INJURY TO THE DEVELOPING LUNG: EXPERIMENTAL AND CLINICAL ASPECTS

Schade aan de zich ontwikkelende long:
experimentele en klinische aspecten

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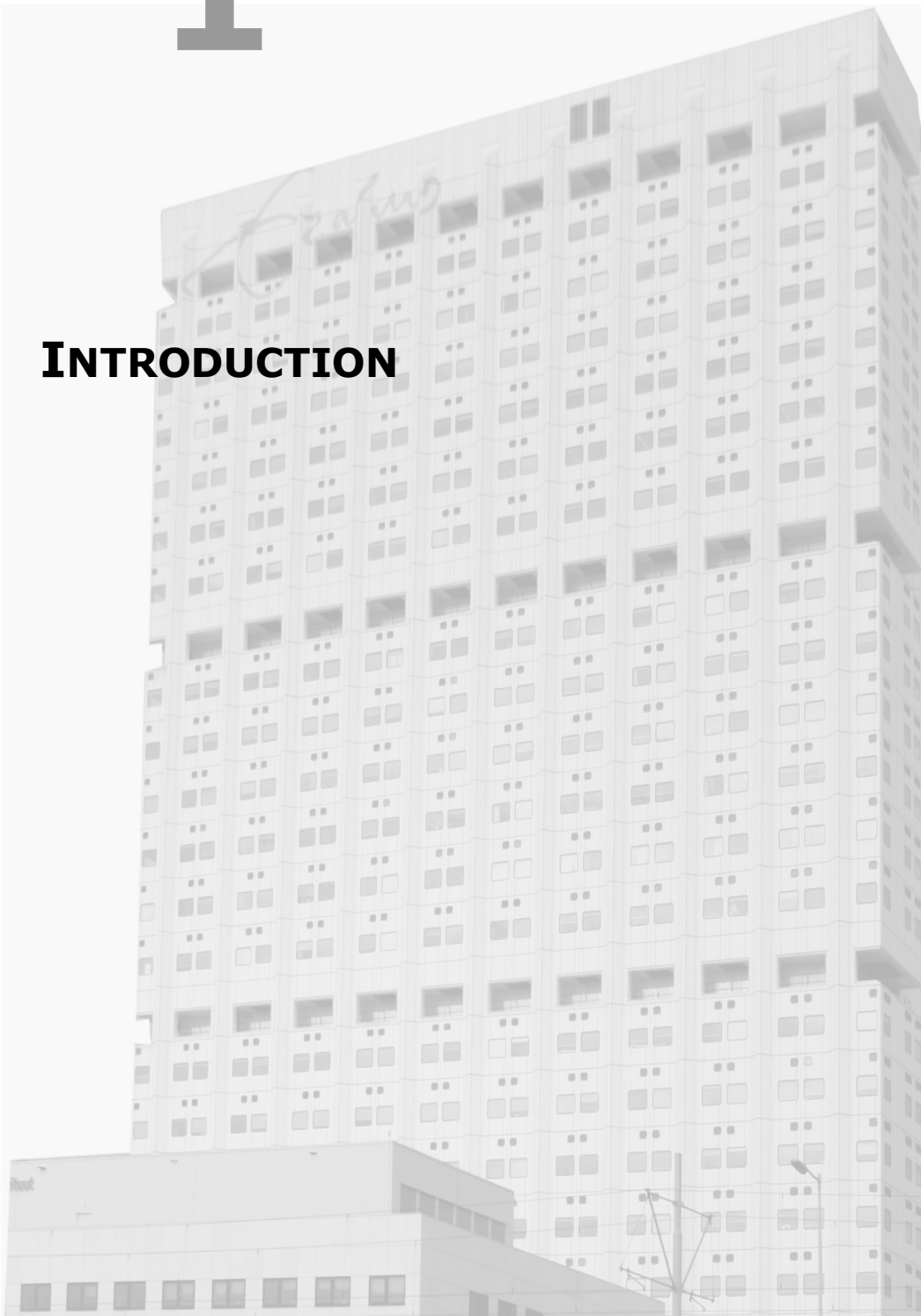
PART I



INTRODUCTION

chapter 1

General introduction and outline of the thesis

Neonatal respiratory insufficiency and failure are common problems for many infants in the intensive care unit, and respiratory therapies in the immediate newborn period typically focus on the lung parenchyma.[1] Because the lung matures relatively late in gestation, immaturity of respiratory function is a significant problem among premature infants. Term infants may also suffer from damage to the normally structured and fully developed lung, such as in case of meconium aspiration syndrome and pneumonia.[2,3] Compromised lung function can also be due to structural abnormalities of the lung, exemplified by congenital diaphragmatic hernia of other major congenital anomalies such as congenital cystic adenomatoid malformation of the lung (CCAM) and congenital lobar emphysema.[4-6] In addition, immaturity of the lung's type II cells, surfactant insufficiency or inactivation, fluid filled parenchyma from retained fluid or infiltrate, and terminal air sacs or canaliculi instead of alveoli result in diminished compliance and need for respiratory support.[7] Mechanical ventilation of a developing lung can lead to chronic lung disease characterized by arrest of alveolar and vascular development.[8] Understanding how alveoli and the underlying capillary network develop and how these mechanisms are disrupted in the above mentioned conditions is crucial to develop efficient and effective therapies for lung disease characterized by alveolar damage.

One such therapy is treatment with surfactant, and this has significantly reduced neonatal mortality for premature infants with lung immaturity and respiratory distress[2] It is also successfully used for near-term infants with severe respiratory distress, along with others therapies such as "gentle ventilation".[9] Surfactant treatment may greatly benefit newborn infants with respiratory compromise secondary to a number of insults, but further prospective evidence of its efficacy in such disorders is needed.

Late Lung development

The lung is composed of branched tubes that conduct air to a vast gas-exchange system. The terminal gas-exchange surface is composed of sacs called alveoli, which are lined by blood capillaries.[10] Development of the lung involves complex mesenchymal-epithelial interactions.[11,12] Based on the formation of key components, three distinct stages of lung development are identified: initial bud formation, dichotomous branching, which produces the air conducting systems, and formation and maturation of the terminal elements to produce the gas-exchange surface.[10] Based on histological features, lung development can be classified into different stages. The embryonic stage is characterized by the formation of the lung bud as a simple budding of the epithelium from the ventral diverticulum of the foregut into the surrounding mesenchyme. Then, the resulting lung bud undergoes rapid dichotomous branching via a complex epithelium-mesenchyme interaction. This is the pseudoglandular stage, characterized by the formation of the conductive airway tree down to the terminal bronchioles.[13,14] Next, the canalicular stage shows expansion of the airway tree and massive organ growth along with differentiation of the air space epithelium cell types. This stage is also marked by massive increase in

the number of capillaries in the primitive mesenchyme.[10] The main gas exchange surface is formed in the next periods, the saccular and alveolar stages. Transformation of the immature saccular lung, with a limited gas exchange area, to a mature lung with a large internal surface area entails thinning of alveolar walls, growth of a capillary network, and extensive subdivision of gas exchange units. This period is marked by interstitial fibroblast proliferation while epithelial cells flatten and decrease in number, resulting in a net thinning of distal airspace walls.[15] Concurrently, the alveolar capillary network becomes more complex. Alveolar septation begins as secondary crests that extend from the primary alveolar walls.[10,16-18] These crests, or septa develop through deposition of new basement membranes, outgrowth of epithelial cells and myofibroblasts at the tips of septa, and elastin deposition.[19,20] Septation is developmentally regulated, occurring primarily from postnatal day 4 through 14 in rats and mice and during the last month of gestation and the first postnatal years in humans. Septum formation seems to involve mechanisms that are different from those implicated in the preceding steps in lung development.[10,21,22]

Septum differentiation

Little is known about the signals for the selection of a specific septation initiation site over others. A number of theories have been put forward to explain the crucial steps. Various stimuli, including genetic factors, oxygen tension, nutrition and hormones, have been shown to modulate distal growth of the lung.[23,24] These stimuli exert their effects via interaction with specific developmentally regulated molecular pathways that have been evolutionarily conserved.

Many investigators agree that the initial deposition of elastin at specific sites in the walls of the developing sacculae marks the onset of septa formation.[25,26] First expressed near airway branching points during the pseudoglandular stage, elastin gene expression peaks during alveolarization when elastin fibers localize to the tips of the alveolar secondary crest, forming rings that surround the alveolar entrances.[26] The elastin fibers deposit in bundles within alveolar walls.[19,20] As elastic fibers are the primary tissue components in which the energy of mechanical stretch is stored and released in the septum, these fibers and the elastin-expressing cells (myofibroblast, fibroblasts) fundamentally determine septal mechanical properties and strain-related signal transduction. Mechanical stresses provide signals for the orientation and differentiation of alveolar myofibroblasts as well as for directional organization of a complex elastic fiber network. Elastin expression in alveolar myofibroblast is upregulated by transforming growth factor-β (TGF-β) and downregulated by fibroblast growth factor (FGF).[27-32] Inhibition of lysyl oxidase-mediated crosslinking, exposure to hyperoxia, dexamethasone, depletion of alveolar myofibroblast, or targeted ablation of the elastin gene all result in decreased alveolar deposition and enlarged emphysema-like alveoli containing fewer septa. Moreover, there is a link between retinoids and lung elastin.[29,33] A subset of lipid-laden alveolar myofibroblasts metabolizes retinoids, which upregulate elastin

expression. Deletion of retinoic acid receptors leads to reduced lung elastin and alveolar numbers.[34-36]

During transition from the saccular to the alveolar stage, the walls of the terminal respiratory units become thinner by apoptosis, and a single layer of capillaries replaces the double-capillary network found in rudimentary alveolar walls.[15] For the septum to achieve its final mature morphology and role, two preconditions are critical, namely the presence of a mature microvasculature and the thinning of the septal mesenchyme, which provides the shortest distance for gas exchange. Thinning of the mesenchymal tissue involves apoptosis.[15] Postnatally, there is a substantial reduction in the number of interstitial myofibroblasts resulting from increased apoptosis during alveolarization.

Vascular development

Vascular development occurs via two processes: (1) vasculogenesis, the development of blood vessels from the differentiation of angioblasts in the mesoderm, and (2) angiogenesis, which is classically described as sprouting of blood vessels from existing vessels, but can also occur by "intussusception", i.e. the formation and growth of a transcapillary tissue pillar that eventually divides an existing capillary segment into two parts.[37-40,41] The bronchial vessels develop with preacinar airways; development is complete by approximately 16 weeks gestation with further growth in size to match lung growth. Bronchial vessels are generally not found in the peripheral acinar region and thus do not normally participate in alveolar gas exchange. Preacinar pulmonary arteries, supplied by the right heart, grow simultaneously with the airways into the intra-acinar region and fuse with the peripherial microvasculature that has arisen from the mesenchyme by vasculogenesis.[42,43] It is not yet fully understood how capillary invasion and alveolar septation interact with each other. The importance of vascular supply to alveolarization was demonstrated by studies using antiangiogenic agents, e.g. inhibitors of the vascular endothelial growth factor (VEGF) receptor.[44,45] In the developing lungs, VEGF isoforms and its receptors have been identified as being important for endothelial survival and proliferation in the alveolar wall. Other than VEGF, growth factors like platelet derived growth factor-B (PDGF-B) and its receptors are crucial for vascular growth and integrity during the alveolar phase by promoting angiogenesis via upregulation of VEGF and basic fibroblast growth factor (FGF).[46] Furthermore, sonic hedgehog (shh), which is known to be expressed in developing lungs, has been shown to act on fibroblasts to induce an array of angiogenic factors like VEGF, angiopoietin-1 and -2 (Ang-1 and Ang-2).[46]

Animal studies suggest that normal pulmonary vascular growth depends on complex signaling between the developing alveolar epithelium and the adjacent pulmonary capillaries. In particular, the mitogenic peptide VEGF is secreted by alveolar epithelial cells and promotes endothelial cell migration and differentiation. Disturbances of these complex signaling systems in humans may result in

disturbance of normal lung growth and development, such as in the case of bronchopulmonary dysplasia.[47,48]

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that most commonly occurs in premature infants who have needed mechanical ventilation and oxygen therapy for acute respiratory distress. It may also occur, however, in premature infants who have had few signs of initial lung disease.[8] The definition and exact clinical criteria of BPD have undergone several changes over the last 30 years. Currently, many neonatologists use the following criterion: oxygen dependency at 36 weeks postconceptional age in children with a history of assisted ventilation and characteristic radiographic abnormalities.[8] Infants at risk for BPD are born at below 28 weeks' gestation, at a stage when the parallel processes of alveolarization of the distal lung saccules and development of the capillary bed are just beginning. Although the disorder is most often associated with premature birth, it is also seen in infants born at term who needed aggressive ventilation for severe acute lung disease.[49] For these children, alternative diagnostic criteria have to be used, which regrettably have not been clearly defined. The best available definition is that of Bancalari *et al* from 1979, which includes assisted ventilation for at least 3 days, continuing respiratory symptoms and continued oxygen dependency at 28 days postnatal age, as well as typical radiographic abnormalities.[50,51] Although surfactant therapy, antenatal steroids, and advances in neonatal intensive care and other treatments have greatly improved the outcomes, the overall incidence of BPD has not changed over the past decade. Nevertheless, the severity of this disorder has been modulated by changes in clinical practice.[52] The overall incidence of BPD is reported to be about 20% of ventilated newborns, with variability between centers.[53] Classically, BPD occurs in the preterm infant lung exposed to hyperoxia and mechanical ventilation, resulting in extensive alveolar fibroproliferation, bronchovascular smooth muscle cell hyperplasia and inhibition of distal lung formation leading to long term pulmonary dysfunction persisting into adolescence and adulthood.

The "new BPD", lacks the severe bronchovascular lesions and interstitial fibrosis, and shows abnormal lung development with simplified acinar structure, poorly formed secundary crests, dysmorphic alveolar capillaries and expression of angiogenic factors and their receptors.[23]

In a primate model of BPD induced by mechanical ventilation of premature baboons, decreased lung capillary density was associated with a reduction in lung VEGF.[48] The lungs of human infants who died with BPD show reduced VEGF expression as well.[54-58]

Inflammation

A number of studies over the past few years have implicated inflammatory mediators in the development of BPD. Epidemiologic studies suggest that either pre- or postnatal exposure to these mediators may predispose to the development of BPD. These mediators may be released in response to infectious (chorioamnionitis, sepsis, pneumonia) and non-infectious (oxygen, mechanical ventilation) stimuli.[59,60] Increasing evidence indicates that BPD results, at least in part, from a persistent imbalance between pro-inflammatory and anti-inflammatory mechanisms which favors pro-inflammatory mechanisms.[61] Neutrophils and macrophages play a crucial role in pulmonary inflammation. Infants at various stages of developing BPD have much higher and persisting numbers of neutrophils and macrophages in their bronchoalveolar lavage fluid compared to infants who have recovered from respiratory distress syndrome (RDS) without developing RDS. The initiation of mechanical ventilation is followed by a neutrophil influx into the airways within minutes. The inflammatory response to injury due to mechanical ventilation may include activation of macrophages that generate early reponse cytokines like Tumor Necrosis Factor-α (TNF-α) and interleukin-1 (IL-1). Increased protein levels and high mRNA expression of pro-inflammatory cytokines and chemokines (TNF-α, IL-1, IL-6, CXCL8) have been detected in airway secretions and bronchoalveolar cells of infants with developing BPD.[62] These cytokines stimulate vascular endothelial cells to express vascular adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), and E-selectin. Blood neutrophils adhere to endothelial cells and transmigrate into the interstial and alveolar compartments. The increased levels and enhanced mRNA expression of pro-inflammatory cytokines present in the airways and pulmonary tissue reflect the inability to regulate inflammation through an adequate expression of the anti-inflammatory cytokines e.g. IL-4, IL-10, IL-12, IL-13 and IL-18.[63,64] Cellular IL-10 mRNA is undetectable in most airway samples of preterm infants with RDS, but is expressed in samples from term infants. Injury to the lung is related to generation of oxidants by phagocyte cells and the release of proteases.[65,66]

Proteases and protease inhibitors

Data from in vitro studies, animal experiments and observations in preterm infants with BPD clearly indicate that an imbalance between proteases and protease inhibitors may contribute to the pathogenesis of BPD.[66-70] Neutrophils and macrophages present at sites of inflammation release various potent proteases that are thought to play essential roles in the destruction of the alveolar-capillary unit and/or the extracellular matrix. An imbalance between elastase and antiproteases has clearly been demonstrated in preterm infants with RDS and BPD. An imbalance between the cystein proteases cathepsin B, H, L and S and their inhibitors has been described in animal models and infants with BPD.

The matrix metalloproteinases (MMPs) process a large array of extracellular and cell surface proteins under normal and pathological conditions. MMPs and their tissue

inhibitors (TIMP) play a key role in epithelial-mesenchymal interactions that are essential for lung development. Infants who develop BPD show an increased amount of MMP 2 and a higher ratio of MMP 9 to TIMP 1 in the first days of life.[67,69] A study of the pathology of BPD using immunohistochemical detection of MMPs and TIMPs from lung sections of preterm infants in various stages of BPD revealed changing concentration of TIMP 1 from acute to chronic stages, which was mostly associated with differentiation of fibroblasts.[67]

The interactions between the surfactant proteins and MMPs and TIMPs in the alveolar space most likely play an important role in the lung maturation process, but more information is needed to identify the exact nature of these interactions in newborns undergoing ventilatory support using different modalities, such as conventional mechanical ventilation, oscillation, and other.[71-73]

Ventilator-induced lung injury

Mechanical ventilation may injure the lungs or exacerbate the pre-existing condition that necessitated mechanical ventilation. Ventilator-induced lung injury (VILI) may be associated with alveolar structural damage, pulmonary edema, inflammation and fibrosis.[74] Recovery from VILI requires clearance of pulmonary edema and alveolar structural repair. The pathogenetic mechanisms for the development of VILI include high airway pressure (barotrauma), large gas volumes (volutrauma), alveolar collapse and re-expansion (atelectrauma) and increased inflammation (biotrauma).[75-77] Oxygen toxicity, barotrauma but also volutrauma and oxygen toxicity induce an infammatory response, which persists in infants who develop BPD. Ventilator- induced lung injury itself is multifactorial.

Volutrauma refers to a more subtle type of lung injury that can occur secondary to pulmonary over-distention induced by mechanical ventilation. Excessive inspiratory lung- stretch can lead to the development of pulmonary edema, diffuse alveolar damage, increased epithelial permeability, and increased microvascular permeability. The term volutrauma indicates that the critical variable for injury is not the airway pressure per se, but rather lung volume or end-inspiratory stretch.[63,64,78]

Atelectrauma refers to the damage that can occur when lungs are allowed to become atelectatic and then are reexpanded. Studies performed during conventional mechanical ventilation of surfactant-depleted lungs with various levels of PEEP support the concept that repeated opening and closing of terminal units causes additional injury. It seems likely that unstable lung units may be damaged by repeated opening and closing tidal ventilation. Keeping end-expiratory lung volumes high enough to prevent atelectasis, and using small tidal volumes to prevent overdistension, improved surfactant efficacy, at least in premature rabbits.[79-81]

Biotrauma, a relatively newly described response to mechanical stress, is characterized by the release of inflammatory mediators from cells within the lung. These mediators can cause further injury to lung tissue and to other organ systems. A number of studies have provided evidence that mechanical ventilation of injured lungs can exacerbate lung injury and lead to an additional inflammatory response.[64] Although subject to some debate, most experimental studies demonstrate that overstretching lung cells can lead to an increase in lung cytokines.[82] Under conditions of increased lung permeability, these cytokines may translocate from the alveolar space to the systemic circulation. Within this context, conventional ventilation in a rabbit model of lung injury produced severe hypoxemia and pathologic evidence of an influx of large numbers of neutrophils into the lung.[83,84] This was in marked contrast to findings in animals that were neutrophil-depleted with nitrogen mustard prior to lung lavage: the absence of neutrophilic infiltration was associated with increased PaO_2.[85-87] These results suggest a key role for neutrophil-released mediators in ventilator-induced lung injury.

High frequency oscillation in a similar lung lavage model significantly decreased the concentrations of inflammatory mediators, including thromboxane B2 and platelet activating factor, when compared to conventional mechanical ventilation.[88] Injurious forms of mechanical ventilation can also lead to higher cytokine concentrations in previously healthy lungs. These higher concentration are associated with an increase in c-fos mRNA, an early response gene. This would seem to suggest that the concept of mechano-transduction (the conversion of cell or receptor deformation into biochemical responses) might be crucial in activating intracellular signal transduction pathways and lead to biotrauma.[89-91] In experiments in which isolated lung cells were submitted to cyclic stretch, increasing cell stretch resulted in an increased release of variuos inflammatory mediators, including TNF-alpha, IL-6, IL-8, and MMP-9.[92-95] Lung macrophages were the main source of these cytokines, which is associated with up-regulation of the nuclear factor kappa B (NF-kB) transcription factor. Cytokines found in the lavage fluid may not stay in the lung but also reach the systemic circulation.[96-99] In an isolated perfused mouse model, ventilation with high end-inspiratory stretch produced higher cytokines levels in the perfusate of the lungs.[97] Another group found that application of zero PEEP with a moderately large tidal volume (16 mL/kg) in an acid aspiration lung injury model in the rat resulted in higher serum TNF-alpha and macrophage inflammatory protein-2 (MIP-2) concentration over a four hour period. When the same tidal volume was used with a higher PEEP level (5 cmH_20), there was no significant increase in serum TNF-alpha. These data suggest a mechanism (release of cytokines and/or inflammatory mediators into the systemic circulation) by which mechanical ventilation might have systemic consequences leading to end-organ failure.[97] Further evidence for this comes from a rabbit acid aspiration model, in which an injurious ventilatory strategy induced epithelial cell apoptosis in the kidney. Using the kidney cells in culture the researchers went on to suggest that apoptosis might have been mediated by a circulating factor, - soluble Fas Ligand (sFasL).[98,100,101]

Finally, they demonstrated a relevance to humans: patients with acute respiratory distress syndrome (ARDS) who were ventilated with a lung protective strategy had lower levels of sFasL than those ventilated using a standard ventilatory approach.[100] There are other mechanisms by which mechanical ventilation could produce systemic effects such as translocation of bacteria or endotoxins from the lungs into the systemic circulation.[102,103]

Pulmonary surfactant

Surfactant replacement therapy has now become the standard therapy for the premature infant with respiratory distress.[104] There is a significant body of evidence for profound functional surfactant deficiency in near-term infants with respiratory failure requiring mechanical ventilation with high mean airway pressure and high FiO_2.[105] This may either represent a primary deficiency such as occurs in hyaline membrane disease (HMD) or be a result of surfactant inhibition or inactivation.

Surfactant composition and function

Pulmonary surfactant is critical for normal lung functioning and survival after birth. Natural surfactant consists of 80% phospholipids, 8% neutral lipids, and 12% proteins, including both surfactant-associated proteins and proteins from plasma and lung tissue. Saturated phosphatidylcholines, most of which is dipalmitoyl phosphatidylcholine, comprise 60% of the phospholipids. Unsaturated phosphatidylcholine species comprise another 25%, and the remaining 15% is phosphatidylglycerol.[106,107] Surfactant is synthesized by type II alveolar cells, stored in lamellar bodies, and eventually secreted into the airspaces as tubular myelin, which then forms a monolayer at the air-fluid interface. Surfactant reduces surface tension and inhibits lung vascular injury and edema.[108]

There are four surfactant-associated proteins: surfactant proteins (SP) A, B, C, and D. Two of these, SP-A and SP-D, are hydrophobic oligomers of trimeric subunits and are members of the collectin superfamily, containing a collagen-like domain as well as a carbohydrate-binding region.[109-112] Found in both pulmonary and extrapulmonary tissue, they are involved in the immune response to microbial challenges. In this process they bind microorganisms, and modulate various leukocyte functions including chemotaxis, cytokine production, and phagocytosis.[113] In vitro studies suggest a role for SP-A in the formation and maintenance of the tubular myelin structure as well as regulation of surfactant metabolism in the alveolus.[109]

The other two, SP- B and SP-C, are hydrophobic surfactant associated proteins that facilitate adsorption and spread of phospholipids to form a monolayer at the air-liquid interface. SP- B is required for proper packaging of surfactant into lamellar bodies and so very essential for good lung functioning.[110]

Surfactant inhibition

There is increasing evidence suggesting that surfactant deficiency may coexist with other lung disorders.[105,111,114-117] An example is meconium aspiration syndrome (MAS), characterized by lung hyperinflation and acute pneumonitis. Abnormal pulmonary function in MAS may be caused in part by disorders of surfactant metabolism.[118] Animal models of meconium aspiration demonstrate inactivation of surfactant with higher surface tension and lower lung volume, compliance, and oxygenation.[118-121] In human infants, concentrations of surfactant inhibitors (e.g. total protein, albumin, membrane-derived phospholipid) were higher in lung lavage fluid of infants with MAS than in controls, but concentrations of surfactant phospholipid and surfactant protein A did not differ.[122] In a small study, there was a trend toward lower surfactant synthesis in neonates with MAS or persistent pulmonary hypertension (PPHN), who required extracorporeal membrane oxygenation (ECMO), compared to control infants who required ventilatory support for non-pulmonary indications.[123] Lower tracheal aspirate concentrations of phosphatidylcholine (PC) and lower incorporation of radiolabeled carbon in PC were seen in infants with MAS or PPHN compared to controls.[106] Cleary et al. found qualitative decreases in surfactant proteins A and B in meconium-installed animals compared with controls. This suggests that exudative lung injury with meconium instillation is associated with lower levels of SP-A and SP-B in the large aggregate fraction of lung surfactant.[124] The authors speculated that less secretion and/or more degradation account for lower levels of SP-B in bronchoalveolar fluid.

Meconium aspiration syndrome

Meconium aspiration syndrome (MAS) is a respiratory disorder caused by inhalation of meconium in amniotic fluid into the tracheobronchial tree. Aspiration can occur before, during, or immediately after delivery. Most cases of severe MAS appear to result from pathologic intrauterine processes, primarily chronic asphyxia and infection. Meconium-stained amniotic fluid (MSAF) is seen in a median of 14 percent of deliveries (range 6 to 25 percent) and is associated with higher risk of respiratory disorders. MAS occurs in around 11 percent of newborns delivered through MSAF. It occurs more frequently in infants who are postmature and/or small for gestational age. The incidence of MAS may be decreasing due to changes in obstetric practice. In a prospective study of 1365 infants ≥ 37 weeks gestational age born through MSAF, the incidence of MAS decreased nearly four-fold over a period of 8 years. This was associated with a significant reduction in births ≥ 41 weeks gestation (42 to 28 percent), as well as increased use of amnioinfusion, diagnosis of non-reassuring fetal heart rate patterns, and cesarean delivery.[125-128]

Normally, fetal breathing activity moves lung fluid out of the trachea. However, as shown in animals, prolonged hypoxia stimulates fetal breathing and gasping, which may then lead to inhalation of amniotic fluid. This process also occurs in humans. Aspirated meconium can interfere with normal breathing in several ways.[129] These include airway obstruction, chemical irritation, infection, and surfactant inactivation

as described above. Nevertheless, most cases of severe MAS are probably related to intrauterine pathologic processes, primarily asphyxia and infection, rather than to the aspiration of meconium itself.[129] Components of meconium, including bile salts, cause inflammation of the lung that is apparent 24 to 48 hours after inhalation. This produces an exudative and inflammatory pneumonitis with epithelial disruption, proteinaceous exudation with alveolar collapse, and cellular necrosis. Stimulation of polymorphonuclear leukocytes (PMN) may play a role in the pathogenesis of pneumonitis. In in vitro studies, MSAF had chemotactic activity for PMNs that was likely mediated through interleukin-8. This activity appears to come from the MSAF and not from the meconium itself. MSAF appears to be a risk factor for bacterial infection of the amniotic cavity and should alert the clinician to higher risk of neonatal morbidity. Although meconium is sterile, the mucopolysaccharide component provides an excellent growth medium for microorganisms, especially Escherichia coli.[130-139]

Hypoxemia results from several causes, including decreased alveolar ventilation related to lung injury and ventilation-perfusion imbalance with continued perfusion of poorly ventilated lung units. Persistent pulmonary hypertension (PPHN) frequently accompanies MAS, with right-to-left shunting caused by increased pulmonary vascular resistance, and resultant hypoxemia.

The management of MAS is largely supportive. The general approach is similar for all infants and begins with identification of risk factors and anticipation of potential illness. Infants at risk for MAS should be monitored closely to ensure adequate oxygenation and ventilation. All ill newborns should be cared for in a neutral thermal environment to minimize oxygen consumption. Handling of the infant should be limited to avoid agitation and exacerbation of PPHN, if present. ECMO may be life-saving in infants who do not respond to mechanical ventilation, surfactant therapy, and/or iNO.[140-142]

Lung Hypoplasia

The true incidence of lung hypoplasia is unknown, but the etiologies include prolonged rupture of membranes, fetal renal dysplasias and obstruction, and fetal neuromuscular diseases. In cases of premature rupture of membranes at 15 - 28 weeks' gestation, the reported incidence of pulmonary hypoplasia ranges from 9 - 28%.[143] Lung hypoplasia also occurs in association with congenital diaphragmatic hernia and congenital cystic lung lesions such as cystic adenomatoid malformation. High-risk congenital diaphragmatic hernia survivors, who were mechanically ventilated, show an incidence of BPD of more than 30%.[144]

Pulmonary hypoplasia or aplasia is part of the spectrum of malformations characterized by incomplete development of lung tissue.[145] The severity of the lesion depends on the timing of the insult in relation to the stage of lung development and the presence of other anatomic anomalies. The hypoplastic lung

consists of a carina, a malformed bronchial stump, and absent or poorly differentiated distal lung tissue. In more than 50% of these cases, coexisting cardiac, gastrointestinal, genitourinary, and skeletal malformations are present, as well as variations in the bronchopulmonary vasculature.[6,146-148] For lung development to proceed normally, physical space in the fetal thorax must be adequate, and amniotic fluid must be brought into the lung by fetal breathing movements, leading to distension of the developing lung.[149] Several factors affect the volume and composition of the fluid in the fetal lung. For example, the volume of liquid in the lung is determined by the net rate at which liquid is secreted across the pulmonary epithelium (4 - 5 mL/kg/h) and by the rate at which it flows from the trachea into the fetal pharynx.[150-153] The pressure in the fetal trachea is normally about 2 mm Hg higher than in the amniotic fluid, thus preventing outflow of fetal lung fluid. Any alteration in the critical volume and pressure relationships of amniotic fluid in the trachea and lung during the canalicular stage of fetal lung development at 15 - 28 weeks' gestation can induce hypoplasia.[149] Lung development is regulated by several transcription factors, such as thyroid transcription factor 1 (TTF-1), member of the hepatocyte nuclear family, and peptide growth factors. Signals from these growth factors are integrated with environmental influences, such as lung fluid volume and hypoxia, to cause cellular proliferation and differentiation.[149,154] A key component of lung development is the branching morphogenesis that results from interaction between the epithelial and mesenchymal components. Growth factors are produced by mesenchymal tissue in the lung and are present in amniotic fluid. A number of growth factors and their receptors, all of which affect fetal lung development, show temporally and spatially regulated expression.[14]

Lung development starts with branching morphogenesis and is completed postnatally with the development of alveoli.[14] Fetal urine is an important component of amniotic fluid during late gestation and contributes to lung growth.[143] During fetal development, the kidney is also a major source of proline. Proline aids in the formation of collagen and mesenchyme in the lung, thus explaining the severe pulmonary hypoplasia in renal agenesis and dysplasia.[155-158] Pathologically, the hypoplastic lung has reduced weight, alveolar number, fewer generations of airways, and hypoplasia of the corresponding pulmonary arteries. Epithelial differentiation is delayed, and is associated with surfactant deficiency.[159-161]

OUTLINE OF THE THESIS

This thesis contains different studies describing clinical and experimental aspects of lung development and therapy of acute lung injury. The findings may lead to better understanding of the processes underlying acute and chronic lung injury and may help develop of new therapeutic and possible preventive strategies.

There are seven parts to this thesis. **Part I** gives an overview of the subject. **Chapter 1** is a general introduction. We describe late lung development and vascular development, as well as the development of BPD and aspects involved in the pathogenesis of this chronic lung disease. **Chapter 2** describes ventilator management of children with congenital lung abnormalities.

In **Part II, Part III**, **Part IV**, and **Part IV**, the main research questions are addressed:

- Which regulatory mechanisms are involved in normal and abnormal late lung development? (**Part II**)
- What are possible risk factors for the development of BPD? (**Part III**)
- What is the role of natural and synthetic surfactant in acute pulmonary disease in childhood? (**Part IV** and **Part V**)

In more detail, **Part II** and **Part III** describe several aspects of BPD. In **chapter 3**, we characterize the role of TGF-β signalling during alveolarization in the normal rodent and human lungs. In **chapter 4**, the effects of hyperoxia on the TGF-β/BMP (bone morphogenetic protein) system in an animal models of BPD are investigated. **Chapter 5** describes how hyperoxia regulates Hypoxia-Inducible Factors-1α and -2α, prolylhydroxylases, vascular endothelial growth factor and its receptors Flt-1 and Flk-1, as well as nitric oxide synthetase-1, -2, and -3, dimethylarginine dimethylaminohydrolase, the cytokines TNF-α, IL-6, and the surfactant proteins SP-B and SP-C. Also, their interrelations are described. In **chapter 6**, we describe pulmonary development and surfactant protein transcription in an animal model of hypoxia-induced intrauterine growth retardation. **Chapter 7** is a study into the risk of BPD in a group of very preterm, small for gestational age infants. **Chapter 8** is a short letter, based on the results of **chapters 6 and 7**, written as a comment on a review article on BPD. **Chapter 9** describes a clinical study into pulmonary inflammation as an effect of prolonged mechanical ventilation. In **chapter 10**, the results of a prospective study into the NO-system in BPD are reported.

Part IV and V describe the surfactant system and its role in therapy of acute pulmonary diseases. **Chapter 11** is a multicenter randomized study into surfactant treatment in severe acute respiratory distress syndrome in children. **Chapter 12** shows the effects of synthetic and natural surfactant on the interaction of TNF-α-activated endothelial cells and neutrophils in vitro. In **chapters 13 and 14**, an animal model of meconium aspiration is used to study the effects of natural bovine and synthetic SP-C surfactant on pulmonary inflammatory processes, lung function and the pulmonary surfactant system. In **chapter 15**, we investigate how positive end-expiratory pressure affects the response to natural and synthetic surfactant in ventilated immature newborn rabbits. **Chapter 16** compares a ventilation strategy (Open Lung Concept) with surfactant replacement therapy in a piglet model of neonatal ARDS.

Part VI discusses some of our results and reviews aspects of disturbances of late lung development in **chapter 17**. **Part VII**, **Chapters 18 and 19** contain the general conclusions of the thesis and describes future research perspectives in this field. In **chapter 20**, the results of this thesis are summarized.

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chapter

2

Ventilator management for congenital abnormalities

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Ventilator Management for Congenital Abnormalities

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CONGENITAL LUNG ABNORMALITIES

Congenital lung anomalies are increasingly discovered on routine prenatal ultrasound or incidentally during postnatal imaging for respiratory insufficiency of unknown origin.[1] Infants born with compromised respiratory status, whether due to immaturity or other conditions such as congenital lung hypoplasia or space-occupying processes including lobar emphysema, often require mechanical ventilation and supplemental oxygen to maintain adequate oxygenation and ventilation. Individually and in combination, oxygen and mechanical ventilation predispose the newborn to ventilator-induced lung injury (VILI).[2] For years, VILI was synonymous with barotrauma/volutrauma.

VENTILATOR-INDUCED LUNG INJURY OF THE DEVELOPING LUNG

Maturation in the human lung continues well after the newborn period and through childhood. Compared with adults, pediatric patients show a spectrum raging from neonatal to infant and adult stages of lung development.[3] Important differences between infant and adult lungs include a different alveolar structure, matrix composition, and angiogenesis, especially in the phase in which new alveoli are formed and septa differentiate (mainly during the first few years of life).[4–6]

The effect of mechanical ventilation on the developing lung cannot be fully understood without considering the effects of intubation and endotracheal suctioning, which are integral components of the ventilation process. Both interventions contribute to injury to the tracheobronchial tree and damage to the ciliated cells of the tracheal epithelium and mucociliary transport system.[7,8] Disruption of the epithelium results in retained secretions and debris, thus setting the stage for inflammation and pulmonary infection.

In addition to intubation and endotracheal suctioning, high concentrations of oxygen also disrupt the epithelial cells and cilia activity. When the tracheal epithelium is exposed to high concentrations of oxygen, the cilia cavity stops.[9] Initiation of mechanical ventilation with high oxygen concentrations causes injury to the lung through various inflammatory mediators.[10] Pulmonary macrophages are activated and release substances that cause an accumulation of polymorphonuclear cells in the alveolar compartment. These leukocytes contain elastases and collagenases that can further damage the lung connective tissue.[11,12] Elastase is known to play an essential role in the pathogenesis of acute and chronic lung disease in the immature lung. Although still controversial, it is generally accepted that increased production of cytokines, chemokines, and other inflammatory mediators is one of the major mechanisms of VILI in the immature and hypoplastic lung.[13–16] Inflammatory cytokines and other inflammatory mediators appear to influence the severity of lung injury and the risk for chronic lung disease (as has been reviewed by several authors) for the premature

lung, the so-called bronchopulmonary dysplasia (BPD).[17,18] The concept of VILI must be regarded much more as a secondary than a primary injury in a structurally immature lung that is fluid filled and hypoplastic. Ongoing exposure to potentially noxious stimuli, such as mechanical forces, infectious agents, or toxic agents, together with the need for endotracheal suctioning, may cause further damage.[19] The individual response and susceptibility to lung injury may vary extensively. Previous lung damage seems to be essential for ventilation to cause an increased systemic inflammatory response in normal humans. However, VILI is not only caused by classic barotraumas (air leaks), but also by more subtle forms of lung injury such as epithelial and endothelial damage leading to fluid and protein leak into airways, alveoli, and interstitium; surfactant disruption and inactivation; and mediator-induced airway and systemic inflammation *(biotrauma)*.[2] The process of inflammation is a vital response to injury whereby blood leukocytes recruit into the alveolar compartment, activate tissue macrophages, and produce a series of inflammatory mediators. The inflammatory process may trigger events that lead to cell regeneration and healing, or progression of the inflammatory response, which often lead to progressive organ dysfunction. Schultz et al. demonstrated an association between prolonged mechanical ventilation and pulmonary inflammation in ventilated infants with an immature lung by inducing proinflammatory cytokines and by failing to stimulate the anti-inflammatory cytokine IL-10.[15]

The injury caused by ventilation has been attributed to two major mechanisms: (1) overdistension of terminal airways *(volutrauma)* and (2) shear stress forces on the epithelial layer of small airways and terminal lung units during closing and cyclic reopening of these units *(atelectrauma)*.[20–22]

Results of a number of animal studies have shown that peak inspiratory pressure (PIP) above 30 cm H_2O can produce injury in normal lungs after relatively short periods of mechanical ventilation. Dreyfuss et al. have shown that ventilation at a PIP of 45 cmH_2O, together with a low positive end-expiratory pressure (PEEP), can produce pulmonary edema and protein leakage in about 20 minutes.[23] This ventilation-induced protein leak results in denaturation of surfactant and loss of surfactant functions[24,25]; this pattern may develop early in postnatal life. Bjorklund et al. have shown that as few as five high-peak airway pressure breaths immediately after birth in the premature lamb model are sufficient to cause diffuse alveolar damage and hyaline membrane formation.[26]

Lung injury secondary to mechanical ventilation is certainly one of the major contributors to mortality in cases of lung hypolasia and is a major risk factor for the bad outcome following acute respiratory failure, particularly in neonates with congenital diaphragmatic hernia (CDH).[27–29] Experimental data have resulted in a trend toward using a lung protective strategy in patients with acute respiratory failure by ventilating with reduced tidal volumes and the introduction of pressure-limited "permissive hypercapnia" ventilation in the treatment of adults and children with acute respiratory failure. This concept of "gentle ventilation" was introduced by Wung et al. and is characterized by preservation of spontaneous breathing, permissive levels of hypercapnia ($PaCO_2$, 60 to 65 mmHg or 9 kPa), and avoidance of high inspiratory airway pressures.[30] Iatrogenic lung injury is avoided where possible. Retrospective series have shown improved survival in infants with persistent pulmonary hypertension of the newborn (PPHN) when hyperventilation was not used.[27] In addition, improved survival and reduced need for extracorporeal membrane oxygenation (ECMO) support was shown when PIP was limited to 30 cmH_2O or less without attempts to correct hypercarbia in infants with lung hypoplasia.[31–33]

CONGENITAL DIAPHRAGMATIC HERNIA

Over the past 20 years, pulmonary hypoplasia and pulmonary hypertension have been recognized as the two cornerstones of the pathophysiology of CDH, while more recently VILI has been recognized as a contributing iatrogenic factor for the development of chronic lung disease.[34,35]

The degree of pulmonary hypoplasia was initially thought to correlate directly with survival, although this may not be true in most infants. With the advent of neonatal mechanical ventilation in the 1960s, many CDH patients with previously fatal respiratory failure were surviving long enough to undergo surgical repair.[36,37]

Pathologic examination of the hypoplastic lungs from patients with CDH revealed a marked reduction in the number of bronchial branchings, but the development of pulmonary acini and alveoli was relatively less affected. The lung injury secondary to mechanical ventilation in these patients was characterized by hyaline membrane formation, parenchymal hemorrhage pneumothorax, and pulmonary interstitial emphysema. Lung injury in patients with CDH was microscopically more evident in the ipsilateral lung. Regarding the relationship between respiratory measurements and lung injury, a clinical study on barotrauma in lung hypoplasia found evidence of pulmonary hemorrhage in lungs that were ventilated with high peak pressure of 50 cmH_2O.[38]

Mechanical ventilation is the initial therapy for infants with respiratory failure due to CDH. In the delivery room, infants with CDH should immediately be intubated to prevent hypoxia-induced pulmonary vasoconstriction.

Because of gastric and abdominal distension and compression of the lung, supply of oxygen by bag-masking must be avoided. To minimize lung injury, the newborn should be ventilated initially with peak pressure below 25 cmH_2O.[32,33] Any delay in obtaining an airway can intensify acidosis and hypoxia, which triggers pulmonary hypertension. For decompression of the abdominal contents in the thorax and thus to help the available lung tissue to expand, early use of a nasogastric tube and continuous suctioning of the stomach are warranted. For optimal mechanical ventilation, blood pressure support by isotonic fluid and inotropic drugs such as dopamine and/or dobutamine should be given to maintain arterial mean blood pressure levels at 50 mmHg and thus to minimize any right to left shunting.

The aim of mechanical ventilation is to administer peak pressure to maintain preductal oxygen saturations above 80% or preductal partial oxygen pressure (Pa_{O_2}) above 60 mmHg. Initiation of conventional ventilator management includes pressure-limited ventilation at rates of 30 to 100 breaths per minute at peak pressures of 20 to 25 cmH_2O. Peak inspiratory pressures that exceed 28 cmH_2O are used only for a short period as a bridge to ECMO. The early institution of high-frequency oscillation (HFO), especially in case of CO_2 retention, should be considered. PEEP should be maintained at physiologic levels (3 to 5 cmH_2O) whenever possible. Hyperventilation, hypocarbia, and alkalosis may decrease ductal shunting and control pulmonary hypertension in CDH, but do so at the expense of increased barotrauma.[39] Permissive hypercapnia, the so-called gentle ventilation approach, is now commonly used in neonates with CDH, with increased survival compared with hyperventilation and alkalization.[27,28,40,41]

HFO is reserved for neonates who continue to have hypoxia and hypercarbia refractory to conventional ventilation. Although the indications for HFO are not clearly defined, there are observational reports of effective P_{CO_2} reduction and increased survival in neonates with CDH.[42–44] In one such study, the use of HFO avoided hyperventilation as well as the need for ECMO. Although there are no randomized controlled studies (RCTs), HFO may have a role in managing neonates, especially in avoiding the need for ECMO.[45] Clinical studies of HFO and mean airway pressure (MAP) in lung injury found that HFO provides better oxygenation and higher MAP without increasing the incidence of barotrauma. On the other hand, low MAP settings do not allow the alveoli to open in the low-compliance lung, which leads to the development of atelectasis and an increase in the amplitude of swing pressure, resulting in excessive expression of cytokines in the airway. At present, no available RCTs show the benefit of HFO as an initial ventilation modality in CDH.

To adjust the ventilator settings, frequent preductal arterial blood gases are important parameters to be determined. Fi_{O_2} should only be weaned after a period of stabilization and should be done very slowly to prevent (recurrent) pulmonary hypertension. Deep analgosedation and sometimes even the use of muscle paralysis may enhance compliance and reduce vasoconstriction, potentially leading to lower ventilator settings. However, the loss of the spontaneous contribution to minute ventilation increases the third space edema and negates the benefits of paralysis and therefore should be avoided.[46,47] In summary, although the optimal mode of ventilation in CDH remains controversial, clinical data suggest that management strategies designed to limit lung distension and barotrauma result in improved survival.[27,28]

Surfactant

Studies of different animals models have shown that the lungs of animals with CDH are surfactant deficient. Surfactant phospholipids and apoprotein SP-A were decreased in nitrofen CDH rats.[48,49] However, their synthetic capacity to produce surfactant was equal to that in controls. The administration of surfactant therapy has been suggested in the treatment of infants with CDH.[44,50] A body of data demonstrates that the lungs of infants with CDH are surfactant deficient.[51,52] Whether a primary surfactant deficiency truly exists or whether secondary inactivation of surfactant is the underlying problem is the subject of ongoing debate. A report from the Congenital Diaphragmatic Hernia Registry did not find that surfactant therapy improved outcome.[49] As a consequence, the routine giving of surfactant to infants with CDH initially is not recommended. Using surfactant in neonates with a gestational age of 34 weeks can be considered in the event that clinical radiologic findings of alveolar atelectasis are suggestive of respiratory distress syndrome. Cogo et al. studied the surfactant phosphatidylcholine (PC) kinetics in CDH patients who did not require ECMO by using stable istopes.[53] Although the amounts of surfactant disaturated surfactant synthesis (DSPC) and SP-A in the tracheal aspirates of CDH patients were reduced, these patients had rates of endogenous surfactant DSPC synthesis comparable to control patients.[54,55] The decreased surfactant PC synthesis in CDH patients who require ECMO could serve as a rationale for the need for ECMO in this particular group and might be a result of lung damage by VILI. Nevertheless, because of several side effects (e.g., bronchus obstruction, hypoxia), surfactant administration should be used with caution in infants with CDH.

Persistent Pulmonary Hypertension of the Newborn in Lung Hypoplasia

In many neonates with respiratory insufficiency, pulmonary hypertension (PPHN) complicates the clinical presentation.[39] PPHN is defined as failure of the

pulmonary circulation to adapt normally to extrauterine life, resulting in unoxygenated blood shunting to the systemic circulation.[56] PPHN remains a significant problem in infants with CDH.[57] Multiple factors, including decreased cross-sectional area of the pulmonary arteries due to lung hypoplasia, increased media thickness of the pulmonary arteries, adventitial thickening, blunted oxygen-induced vasodilatation, and increased endothelin-A receptor expression, are thought to contribute to the pulmonary hypertension seen in CDH.[58] The diagnosis of PPHN should be considered in any infant with CDH and other cases of pulmonary hypertension in which a difference in pre- and postductal saturations exists. The diagnosis of PPHN should be confirmed by cardiac ultrasound.

The management of PPHN in infants with CDH is largely supportive. It is directed toward promoting a progressive decline in the ratio of pulmonary vascular resistance (PVR) to systemic vascular resistance (SVR) to maintain adequate tissue oxygenation until PVR falls. The initial treatment of the newborn with PPHN includes correction of hypothermia, hypoglycemia, hypocalcemia, anemia, hypovolemia, and stress. Although the use of alkalinizing agents is controversial, correction of metabolic acidosis is standard.[39] Because PVR is elevated in PPHN, decreased SVR or poor cardiac output result in decreased mean systemic blood pressure, which can increase right-to-left shunting. Therapy of elevated PVR includes aggressive support of cardiac function and perfusion with volume and inotropic agents to maintain the mean arterial blood pressure at a level that minimizes right-to-left shunting (>50 mmHg).

Inhaled Nitric Oxide

Inhaled nitric oxide (iNO) is a selective pulmonary vasodilator and is widely accepted as the gold standard treatment in PPHN.[59,60] Its use has contributed to reduced rates of ECMO. Nitric oxide (NO) is produced in endothelial cells during conversion of L-argine to L-citrulline by NO-synthetase. NO diffuses from endothelial cells into adjacent smooth muscle cells to cause vasodilatation through activation of soluble guanylate cyclase (sGC) and the production of cyclic guanosine monophosphate (cGMP). cGMP stimulates a cGMP-dependent kinase, causing vasodilation through myosin phosphorylation. To date, results of iNO therapy in patients with CDH have been poor. In CDH, iNO does not appear to be of long-term benefit. In the one trial that specifically addressed this issue, 53 infants with CDH and hypoxemic respiratory failure (gestational age, 34 weeks) were randomly assigned to receive either iNO (20 ppm) or 100% oxygen. Death before 120 days or need for ECMO was not significantly different in iNO or control groups.[61] ECMO use occurred significantly more in the iNO group, although the percentage of infants who died was not different. Although a transient improvement occurred in approximately 50% of the infants treated with iNO, it did not consistently improve oxygenation, nor did it decrease the use of ECMO. With the increasing use of iNO and HFO, the absolute number of non-CDH, noncardiac neonates with hypoxemic respiratory failure requiring ECMO has decreased. Initiation of ECMO has become progressively later, likely because of the use of these rescue therapies, but the overall mortality rate remains unchanged despite this delay. Inhaled therapy with NO might be helpful in stabilizing some patients for transport and initiation of ECMO.

Phosphodiesterase Inhibitors

Approximately 30% of patients with PPHN do not respond to iNO and require alternative treatments. Substances that stimulate the formation of the second messengers cyclic adenosine monophosphate (cAMP) or cGMP have proved useful in the treatment of various forms of precapillary pulmonary hypertension.[62] Milrinone is a bipyridine compound that selectively inhibits phosphodiesterase type 3 (PDE3) and may lead to early and sustained improvements in oxygenation without compromising hemodynamic status. Prospective evaluation of the acute clinical and physiologic effects and long-term outcome of intravenous milrinone therapy as an alternative cardiotrophic agent or as a combination therapy with iNO in neonates with PPHN in lung hyoplasia is required.[63]

Sildenafil (Viagra), a phosphodiesterase type 5 inhibitor (PDE5), is an oral agent that has been shown to selectively reduce pulmonary vascular resistance in both animal models and adult humans and produced vasodilation by increasing cGMP through inhibition of the phosphodiesterase involved in the degradation of cGMP to guanosine monophosphate. PDE5 is a key regulator of NO-induced vasodilation in the postnatal pulmonary arteries. PDE5 inhibitors were shown to be effective in decreasing pulmonary arterial pressure and pulmonary vascular resistance in several neonatal models of acute pulmonary hypertension. One of the recent adult randomized studies in humans shows that oral sildenafil significantly improves exercise tolerance, cardiac index, and quality of life in adult patients with primary pulmonary hypertension.[64–66]

Prostacyclin

Prostacyclin (PGI_2) stimulates membrane-bound adenylate cyclase, increases cAMP, and inhibits pulmonary artery smooth muscle cell proliferation in vitro.

Although the use of systemic infusions of PGI_2 may be limited by the development of systemic hypotension, inhaled PGI_2 has been shown to have vasodilator effects limited to the pulmonary circulation. Reports in children have been encouraging, but to date there have been few reports of inhaled PGI_2 use in neonates with PPHN. The actions of inhaled PGI_2 and iNO appear to be additive in humans and even synergistic in animal studies. Rebound PPHN following withdrawal of iNO has been mitigated by intravenous PGI_2 in children with PPHN following CDH.[67]

PULMONARY HYPOPLASIA DUE TO OTHER CAUSES

Pulmonary hypoplasia is part of the spectrum of anomalies characterized by incomplete development of lung tissue. The severity of the lesion depends on the timing of the insult in relation to the stage of lung development and the presence of other anatomic anomalies. The hypoplastic lung consists of a carina, a malformed bronchial stump, and absent or poorly differentiated distal lung tissue. In more than 50% of these cases, coexisting cardiac, gastrointestinal, genitourinary, and skeletal malformations are present, as well as variations in the bronchopulmonary vasculature. Isolated primary pulmonary hypoplasia is rare but in its milder forms can present a diagnostic dilemma. It usually presents at or shortly after delivery fo a child in varying degrees of respiratory distress who may require high ventilatory pressures and has noncompliant lungs. There may be a genetic component, but more commonly pulmonary hypoplasia is secondary to an underlying abnormality such as restrictive malformation of the chest wall and decreased fetal breathing (fetal neuromuscular disease), decreased fetal lung fluid (prolonged rupture of membrane, fetal renal dysplasias and obstruction), and decreased vascular supply (tetralogy of Fallot, interrupted pulmonary artery).[68] In cases of premature rupture of membranes at 15 to 28 weeks' gestation, the reported incidence of pulmonary hypoplasia ranges from 9% to 28% (13% in most studies).[69] In different studies, mortality rates of 71% to 95% have been reported during the perinatal period in patients with pulmonary hypoplasia. Even after correction of the underlying abnormality (if possible), the pulmonary hypoplasia may be so severe as to be incompatible with life. Postnatal growth of the lung can occur, but in severely affected children, even newer technologies (such as ECMO) have contributed little to an improved outcome. In milder cases (e.g., certain instances of primary pulmonary hypertension or children who have had maturation arrest due to oligohydramnion following premature rupture of membranes), artificial pulmonary support may maintain oxygenation while alveolar growth occurs. Ventilatory strategies have changed in the last decade toward use of permissive hypercapnia and gentle ventilation. Partial liquid ventilation has also been tried in individual cases, but without definite advantages.[70] The concept of a liquid tissue interface is still very promising, and phase I clinical trials have recently begun. A decreased response to NO is believed to occur with pulmonary hypertension associated with hypoplasia. Apart from respiratory insufficiency in premature infants, no appropriate RCTs are available that evaluate HFO versus conventional ventilation.[71]

CONGENITAL CYSTIC LUNG LESION

Lesions such as congenital cystic adenomatoid malformations (CCAMs), sequestrations, bronchogenic cysts, and congenital lobar emphysema may be asymptomatic at birth or at the time of discovery later in life.[72] Some authors advocate simple observation because of the lack of data regarding the incidence of long-term complications.[72] However, there are very few described cases in which CCAM and intralobar sequestration have remained asymptomatic throughout life; complications eventually develop in nearly all patients. The most common complication is pneumonia, which may respond poorly to medical treatment. Other complications include the development of malignancies (carcinomas and pleuropulmonary blastomas), pneumothorax, and hemoptysis or hemothorax. Because lung resection will be required sooner or later for CCAM, intralobar sequestration, and intrapulmonary bronchogenic cysts, our approach is to not wait until complications occur. For patients diagnosed prenatally, we recommend surgery at 3 to 6 months of life at the latest so that compensatory lung growth may occur. At this age the postoperative course is usually smooth, with a low risk of complicating pulmonary hypertension, and long-term follow-up has shown normal respiratory function. Mediastinal bronchogenic cysts also tend to become symptomatic, and elective resection is recommended. On the other hand, asymptomatic congenital lobar emphysema may regress spontaneously, and observation is justified. The management of small noncommunicating extralobar sequestrations is more controversial; it is known that these lesions can remain asymptomatic throughout life, but complications may develop and they are sometimes difficult to differentiate from neuroblastoma.

Congenital cystic lung lesions (especially CCAM and/or pulmonary sequestration) often present as a benign pulmonary mass in infants and children and are traditionally described as a multicystic lung mass resulting from a proliferation of terminal bronchiolar structures with an associated suppression of alveolar growth. Death in utero or at birth in such fetuses and neonates is often due to hydrops and pulmonary hypoplasia.

Hydrops occurs in 45% of fetuses with CCAM and is reported to be associated with combined fetal and postnatal mortality rates of 68% to 89%. The mortality rate is less than 10% when fetal hydrops is not present.[72,73]

The presentation of congenital cystic lung lesions is variable. Many patients with congenital lung cystic lesions are identified by routine prenatal ultrasound examination. Controversy exists as to the management of newborns with asymptomatic congenital lung cyst lesions. In case of isolated cystic lesions with overt mediastinal shift, drainage during fetal life by placement of a pigtail to guarantee flow of fluid may be considered. In the management of infants with congenital cyst lung lesion, the same therapeutic approach as that in patients with hypoplastic lungs with persistent pulmonary hypertension and poor compliance with increased pulmonary vascular resistance has to be considered. Postnatally some patients with CCAM may even need ECMO, either due to respiratory insufficiency or postsurgically after pneumectomy resulting from increased blood flow to the remaining lung.[74,75]

CONGENITAL LOBAR EMPHYSEMA

Congenital lobar emphysema (CLE) is a developmental anomaly of the lower respiratory tract that is characterized by overexpansion of a pulmonary lobe with resultant compression of the remaining ipsilateral lung, and it is a potentially reversible cause of respiratory distress in the neonate.[76] Mediastinal shifting away from the increased-volume lung can also compress the contralateral lung. The abnormality is related to a congenital bronchial narrowing. In these cases weakened or absent bronchial cartilage is present, such that there is inspiratory air entry but collapse of the narrow bronchial lumen during expiration. This bronchial defect results in lobar air trapping. In case of congenital extrinsic compression, such as by a large pulmonary artery, affected cartilage rings are malformed, soft, and collapsible as a result of the long-term in utero extrinsic effect.[77]

Emergency surgical lobectomy was once considered the only treatment for CLE, but appropriate care may be nonsurgical in some infants with only moderate respiratory distress. Prevention of endotracheal intubation should be considered to diminish the risk of progressive hyperinflation. Maintaining ventilatory pressures and volume as low as possible avoids producing ventilator-related hyperexpansion of an affected lobe. Management by a more conservative gentle ventilation technique is often successful. Fewer surgeries result because the affected lobe only occasionally continues to expand after diagnosis and initial treatment. Infants with CLE who are not clinically in respiratory distress and who are able to feed and grow do not necessarily need surgery. Lobar emphysema can occur in hypoalveolar (fewer than the expected number of alveoli) and polyalveolar (more than the expected number of alveoli) forms.

CONGENITAL TRACHEAL OBSTRUCTION

Laryngotracheal stenosis is a congenital or acquired narrowing of the airway that may affect the glottis, subglottis, or trachea. It causes severe symptoms and should be suspected in children less than 1 year of age with either multiple episodes of croup or croup that fails to respond to medical management or requires endotracheal intubation. The trachea and the upper airway, although considered to be relatively rigid conducting airways, do show some changes in caliber during the normal respiratory cycle. There is expansion of the intrathoracic airways along with the expanding lungs, while the extrathoracic airways diminish in caliber due to their intraluminal pressure being lower than atmospheric pressure. The reverse of this process occurs during expiration. If the intrathoracic trachea is soft, the narrowing will accentuate during expiration due to positive intrathoracic pressure. The mechanics of critical tracheal stenosis is such that it would severely compromise delivery of gases beyond the obstruction allowing adequate emptying of the lungs as well. Other obstructive upper airway anomalies (e.g., subglottis stenosis) could be overcome by the use of tracheostomy, but in more distal lesions of the trachea, ventilatory management is more challenging. With conventional ventilator settings in a patient with severe tracheal stenosis, there would be inadequate delivery of gases beyond the site of obstruction, with build-up of proximal pressure resulting in progressive CO_2 retention and inadequate lung expansion. Because of inadequate emptying of the lungs during expiration, air trapping occurs, along with a subsequent decrease in cardiac output. In order to ensure adequate delivery of gases beyond the obstruction, it is necessary to prolong the inspiratory time so that adequate lung expansion can be achieved. In view of the markedly increased resistance, the time constant will also be increased, justifying the need for high inspiratory time. For this, the frequency of breaths would have to be kept at a low value in order to allow adequate time for expiration and at the same time avoid progressive air trapping. For patients undergoing surgical repair of the tracheal lesions, ECMO has also been used.[78–81]

CONGENITAL ALVEOLAR CAPILLARY DYSPLASIA

Congenital alveolar capillary dysplasia (ACD), with or without misalignment of the pulmonary veins, is a rare

cause of PPHN.[82] This malformation represents a failure of capillaries to extend into alveolar tissue of the lung and is an unusual cause of pulmonary hypertension, persistent fetal circulation, and respiratory distress in the newborn. Histology shows increased septal connective tissue and pulmonary veins accompanying small arteries in the centers of the acini, rather than occupying their normal position in the interlobular septa. The number of pulmonary arteries is decreased, and they show increased muscularization. Pulmonary lobules are small, and radial alveolar counts may be decreased. Alveoli are decreased in complexity, their walls contain few capillaries, and there is poor contact of capillaries with alveolar epithelium. The primary defect is poorly understood. ACD causes severe and irreversible PPHN with a uniformly fatal outcome. Although most cases are sporadic, a familial predisposition has been reported, and a number of studies have suggested that ACD be considered in any infant with severe respiratory acidosis and PPHN who fails to improve after the application of routine treatment modalities. As Michalsky et al. reported,[83] the usual presentation is that of a term neonate, appropriate for gestational age, who appears to be normal at the time of delivery. Most infants develop progressive respiratory distress and cyanosis with hypoxia, respiratory acidosis, and hypotension within 48 hours of birth. The rapidly progressive nature of this process results in the need for full ventilatory support soon after the onset of symptoms. Associated anomalies have been noted in approximately 50% of infants with ACD.

Initial chest radiographs are often reported to be unremarkable or to show a mild hazy pattern. Radiographic changes associated with barotraumas may develop later in the course of treatment. Because of its lethal outcome, ACD should be diagnosed as early as possible.

Clinicans should have a high suspicion for ACD in a term infant with good Apgar scores who goes on to experience respiratory deterioration within a few hours of age. The patient may have transient response to NO, minimal response to HFOV, and variable response to prostacyclin. Although ECMO is typically used as rescue therapy, resulting in rapid hemodynamic stabilization, open lung biopsy should be considered before initiation of ECMO to prevent the institution of futile and expensive treatment modalities.[83]

PULMONARY ALVEOLAR PROTEINOSIS AND INTERSTITIAL LUNG DISEASES

A number of genetic and environmental factors have been clearly identified as affecting the severity of neonatal respiratory distress syndromes.[84] Congenital alveolar proteinosis syndromes are characterized by the accumulation of surfactant material in the alveolar space.[85] SP-B deficiency leads to alveolar proteinosis, but it is likely that many other causes may lead to similar pulmonary pathology. Interstitial lung diseases (ILDs) in childhood are a diverse group of conditions primarily involving the alveoli and perialveolar tissues and leading to derangement of gas exchange, restrictive lung physiology, and diffuse infiltrates on radiographs.[86] Childhood ILD is not a disease entity but rather a group of disorders. However, most ILDs share a common pathophysiologic feature: namely, structural remodeling of the distal airspaces, leading to impaired gas exchange. In general, this remodeling has been believed to be the sequela of persistent inflammation; however, more recently the paradigm has shifted away from inflammation to one of tissue injury with aberrant wound healing resulting in collagenous fibrosis.[85] The multiple possible diagnostic entities and lack of RCTs make specific recommendations regarding treatment of childhood ILD impossible. If the process is secondary to an underlying condition, patients should be treated for the underlying disease. The appropriate management depends on the patient's age at presentation, the severity of symptoms, and the anticipated course of the disease. Mechanical ventilation is necessary in children with congenital alvelveolar proteonisis and in some case of interstitial lung diseases. No reports show any benefit from the use of HFO or other unconventional forms of mechanical ventilation.

CONCLUSIONS

VILI is one of the major contributors to mortality and morbidity in patients with congenital lung anomalies. The clinical picture has changed following the application of a lung protective strategy consisting of preservation of spontaneous ventilation, permissive levels of hypercapnia ($PaCO_2$, 60 to 65 mmHg or 9 kPa), and avoidance of high inspiratory airway pressure (25 to 28 cmH_2O), increasingly known as the gentle ventilation concept. More newborns with severe respiratory insufficiency are saved and the amount of pulmonary sequalae (BPD, chronic infections, progressive pulmonary vascular disease) diminished. There is a continuing need for RCTs with enough power to determine the role of HFO compared with conventional ventilation, as new modalities such as liquid ventilation are still experimental at this stage.

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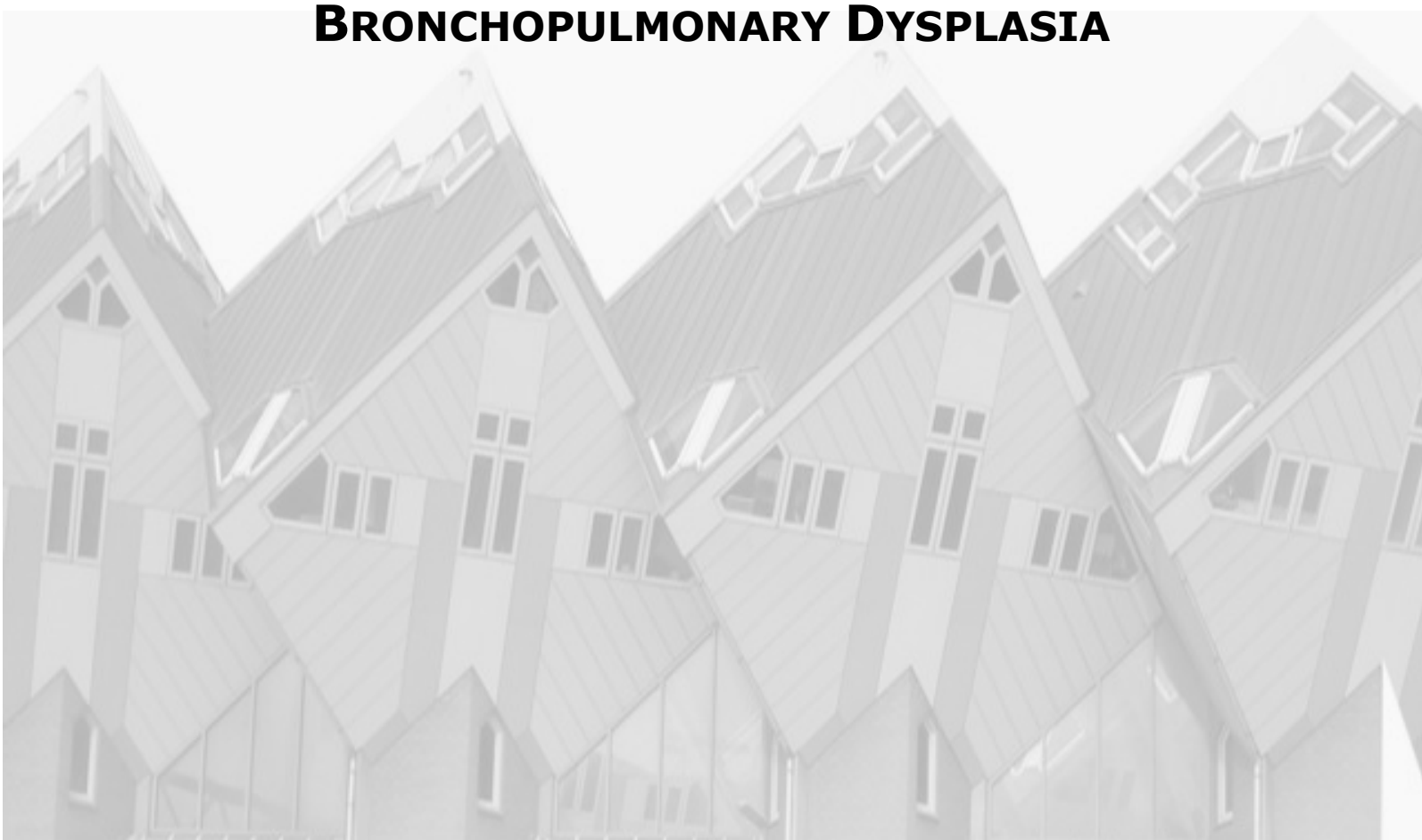
PART II

EXPERIMENTAL ASPECTS OF LUNG DEVELOPMENT AND BRONCHOPULMONARY DYSPLASIA



chapter 3

TGF-β signaling is dynamically regulated during the alveolarization of rodent and human lungs

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TGF-β Signaling Is Dynamically Regulated During the Alveolarization of Rodent and Human Lungs

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Although transforming growth factor-beta (TGF-β) signaling negatively regulates branching morphogenesis in early lung development, few studies to date have addressed the role of this family of growth factors during late lung development. We describe here that the expression, tissue localization, and activity of components of the TGF-β signaling machinery are dynamically regulated during late lung development in the mouse and human. Pronounced changes in the expression and localization of the TGF-β receptors Acvrl1, Tgfbr1, Tgfbr2, Tgfbr3, and endoglin, and the intracellular messengers Smad2, Smad3, Smad4, Smad6, and Smad7 were noted as mouse and human lungs progressed through the canalicular, saccular, and alveolar stages of development. TGF-β signaling, assessed by phosphorylation of Smad2, was detected in the vascular and airway smooth muscle, as well as the alveolar and airway epithelium throughout late lung development. These data suggest that active TGF-β signaling is required for normal late lung development. *Developmental Dynamics 237:259–269, 2008.* © 2007 Wiley-Liss, Inc.

Key words: development; alveolarization; septation; TGF-β, transforming growth factor; mouse; rat; human

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INTRODUCTION

The process of lung development strives to maximize the gas exchange surface area, while minimizing the blood–air barrier (Roth-Kleiner and Post, 2003; Copland and Post, 2004). This is achieved by branching of developing airways in *early lung development* and progressive subdivision of developing airspaces into alveoli in *late lung development* by a process of alveolarization (Roth-Kleiner and Post, 2003; Copland and Post, 2004). Alveolarization begins with branching of distal airway saccules into immature alveoli, in the canalicular phase of lung development, and continues through the saccular phase, into the alveolar phase, where alveolar septae, supported by the extracellular matrix (ECM), divide the terminal respiratory saccules, increasing the number of alveoli. Alveolarization is driven by growth factor-mediated communication between the different cell types of the developing lung (Warburton et al., 2000; Cardoso, 2001; Jankov and Tanswell, 2004). The pleiotropic roles played by the transforming growth factor-beta (TGF-β) family in control-

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ling cell proliferation, transformation and apoptosis, as well as ECM deposition and remodeling, have led to TGF-β being accredited with a key role in lung development (Jankov and Tanswell, 2004; Roth-Kleiner and Post, 2005).

TGF-β signaling is initiated by binding of TGF-β ligands to the type II TGF-β receptor (Tgfbr2, also called TβRII), which then complexes with a type I TGF-β receptor (either Tgfbr1, also called activin-like kinase [ALK]-5; or Acvrl1, also called ALK-1). The type I receptor transmits signals within the cell by means of second-messenger Smad proteins, namely Smad2, Smad3, and Smad4, or by Smad-independent pathways. TGF-β signaling is potentiated by two accessory type III TGF-β receptors: Tgfbr3 (also called betaglycan), and endoglin (CD105, the *eng* gene-product). TGF-β signaling is also regulated by Smad6 and Smad7, inhibitory Smads that antagonize TGF-β signaling. Activated (phosphorylated) Smads are translocated into the nucleus, where they regulate gene transcription and, hence, cell function (Massagué, 1998).

Several studies have implicated TGF-β signaling in early lung development. All three TGF-β isoforms (Pelton et al., 1991) and type I (Zhao et al., 2000b) and type II (Zhao and Young, 1995) TGF-β receptors are expressed in the embryonic rodent lung. Exogenous addition of TGF-β ligands inhibited airway branching in vitro (Sakurai and Nigam, 1997; Liu et al., 2000), and down-regulation either of Tgfbr2 (Zhao et al., 1996), or of Smad2, Smad3, or Smad4 (Zhao et al., 1998), which would block TGF-β signaling, enhanced lung branching in vitro. In line with these findings, overexpression of the inhibitory Smad, Smad7, which antagonizes TGF-β signaling, promoted lung branching in vitro (Zhao et al., 2000a).

TGF-β signaling has received much less attention in the context of late lung development. Adenoviral-mediated transfer of TGF-β1 to the neonatal rat lung (Gauldie et al., 2003) and overexpression of TGF-β1 between postnatal day (P) 7 and P14 in the mouse lung (Vicencio et al., 2004) both disrupted alveolar development, providing indirect evidence that TGF-β was a negative regulator of alveolarization. However, blockade of TGF-β signaling by ablation of Smad3 between days P7 and P28 generated a similar phenotype in mice, indicating that TGF-β can also act as a positive regulator of alveolarization (Chen et al., 2005). Similarly, Smad3 deficiency in mice resulted in progressive airspace enlargement with age, implicating TGF-β in the maintenance of alveolar integrity in the developed lung (Bonniaud et al., 2004). Together, these data suggest that TGF-β plays a finely tuned and key role in the alveolarization process, as well as in the maintenance of alveolar structure. No study to date, however, has assessed the expression of the TGF-β signaling system over the course of late lung development. We describe here that the expression, localization, and activity of components of the TGF-β signaling machinery are dynamically regulated over the course of late lung development in rodents and humans.

RESULTS

Early lung development encompasses the embryonic stage (4–7 weeks after conception in humans, embryonic days [E] 9.5–12 in mice), and continues through the pseudoglandular (5–17 weeks in humans, E12–E16.5 in mice) and canalicular (16–26 weeks in humans, E16.5–E17.5 in mice) stages (Copland and Post, 2004). The alveolarization process begins at the end of the canalicular stage. As late lung development proceeds, distal airways form saccular units in the saccular stage (24–38 weeks in humans, E17.5–P4), and secondary septae then divide these units (*septation*) during the alveolar stage (36 weeks preterm to 36 months postnatal in humans, P4–P28 in mice; Copland and Post, 2004). A progressive decrease in the size of the alveolar air spaces, together with a concomitant increase in the total number of alveoli, occurs during this critical period of late lung development (Copland and Post, 2004).

The progress of late lung development in the mouse was characterized by dramatic, dynamic changes in the abundance of mRNA encoding components of the TGF-β signaling machinery (Fig. 1) as assessed by semiquantitative and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). In particular, the two type I TGF-β receptors exhibited opposite patterns in mRNA abundance, where *acvrl1* mRNA abundance significantly increased over time (compare stages E15 and P21; Fig. 1B,F), whereas *tgfbr1* mRNA abundance significantly decreased over time (compare stages E15 and P21; Fig. 1C,F). A biphasic pattern was observed for *tgfbr3* mRNA abundance (Fig. 1A,F), while *eng* mRNA abundance significantly increased during late lung development (compare stages E15 and P21; Fig. 1F). Due to large variations within each group, changes in the abundance of *tgfbr2* mRNA were not detected (Fig. 1A,D). The abundance of mRNA encoding Smad proteins was also regulated during late lung development in the mouse. The mRNA levels of *smad2*, *smad3*, and *smad4* were significantly decreased by day P28 compared with stage E15 (Fig. 1A,G). In the case of *smad2*, mRNA abundance was significantly reduced as early as day P3 compared with stage E15 (Fig. 1A,G). In contrast, the *smad6* and *smad7* mRNA levels significantly increased during late lung development in the mouse (compare stages E15 and P21; Fig. 1A,G).

Immunoblot analysis of TGF-β receptors and Smad proteins in extracts from mouse lungs at different stages of development (Fig. 2) revealed comparable patterns to those observed in the mRNA profiles (Fig. 1). Expression levels of Acvrl progressively increased while those of Tgfbr1 progressively decreased (compare the expression levels of both molecules at stages E15 and P28 in Fig. 2B). Two splice-isoforms of the *tgfbr2* gene have been described (Hirai and Fijita, 1996; Krishnaveni et al., 2006), which encode high and low molecular mass Tgfbr2 protein isoforms. Both the higher molecular mass Tgfbr2 isoform 1 (also called TβRII-B) and the lower molecular mass Tgfbr2 isoform 2 were detected in mouse lung extracts (Fig. 2A,B). Expression levels of Tgfbr2 also exhibited dynamic regulation during late lung development, peaking at the late saccular stage (Fig. 2A,B). Consistent with the mRNA abundance patterns, all three Smad proteins investigated, Smad2, Smad3, and Smad4, initially exhibited stable expression

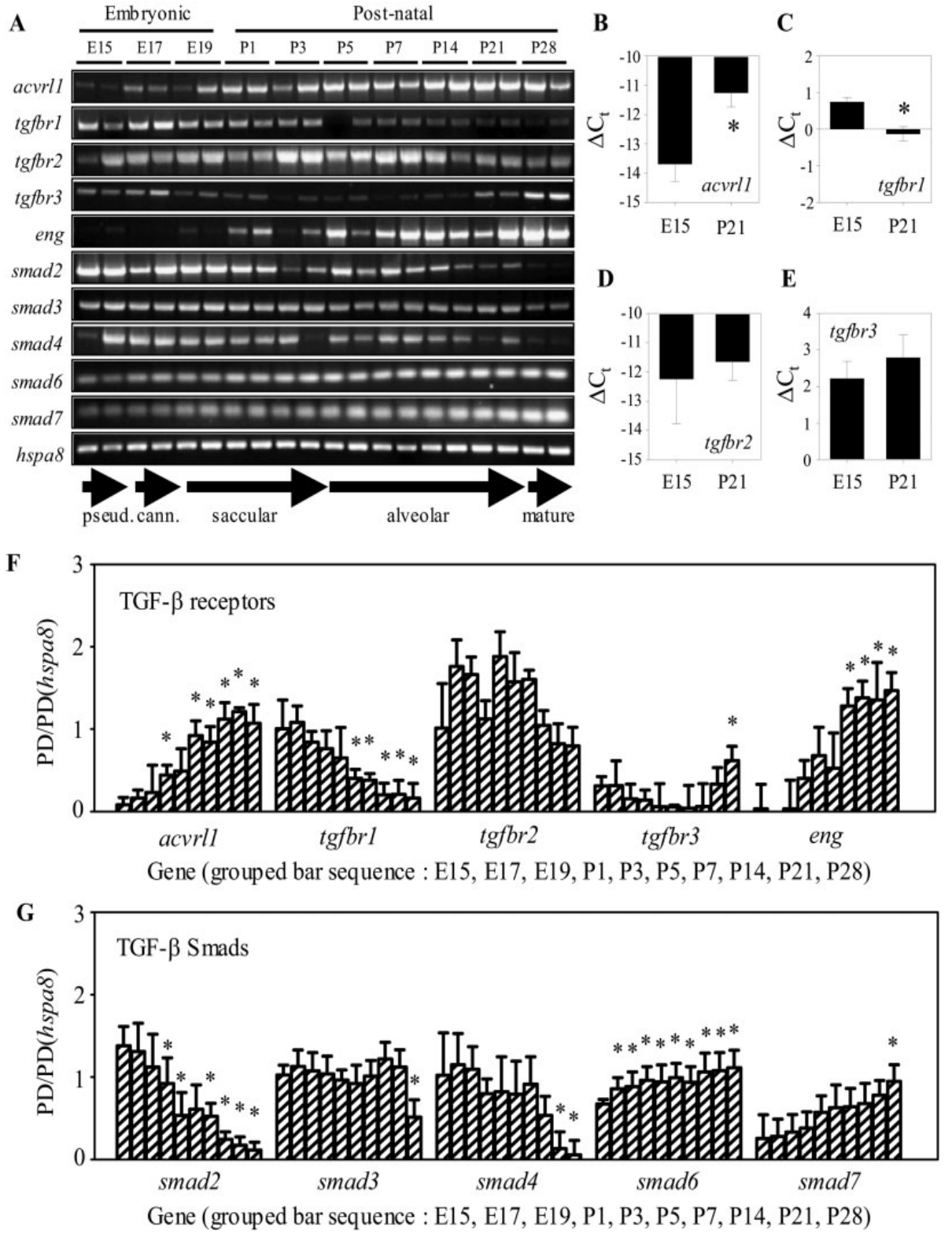

Fig. 1. Expression of the transforming growth factor-beta (TGF-β) signaling machinery during the late lung development in mice. **A:** Changes in the expression of genes, assessed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR), encoding TGF-β signaling machinery during late lung development. Amplicons are illustrated from two different mouse lungs per developmental stage, representative of the patterns observed in six other mouse lungs at the same developmental stage. **B–E:** Changes in mRNA expression were also confirmed by real-time RT-PCR for the classic TGF-β receptors *acvrl1* (B), *tgfbr1* (C), *tgfbr2* (D), and *tgfbr3* (E). **F,G:** Changes in mRNA expression of genes encoding TGF-β receptors (F) and TGF-β Smads (G) assessed by semiquantitative RT-PCR (for which representative examples are illustrated in A) were quantified by densitometric analysis of amplicons from six to eight different mouse lungs per developmental stage. Band intensities (described by pixel density, PD) from samples were normalized for loading using the *hspa8* amplicon from the same sample. Data reflect the mean normalized PD ± SD (n = 6–8). *$P < 0.05$ vs. embryonic day (E) 15. Pseud, pseudoglandular; can, canalicular.

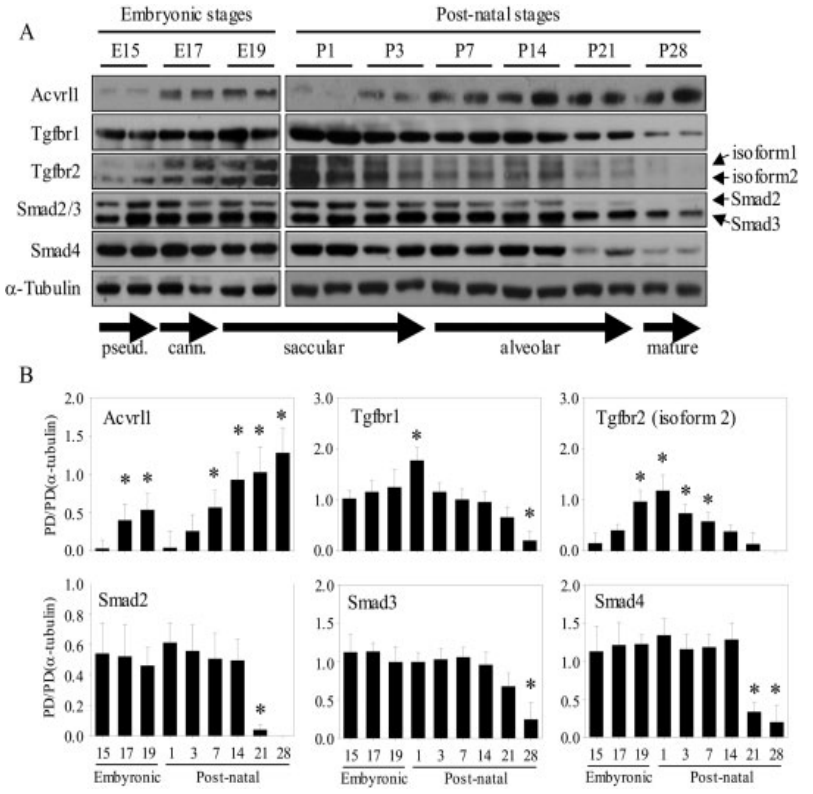

Fig. 2. Changes in protein expression of the transforming growth factor-beta (TGF-β) signaling machinery, assessed by immunoblot, during late lung development in the mouse. **A:** Blots illustrate 10-μg samples from two different mouse lungs per developmental stage, which are representative of the patterns observed in at least six other mouse lungs at the same developmental stage. **B:** Bands derived from six different mouse lungs were quantified by densitometric analysis. Band intensities (described by the pixel density, PD) from samples were normalized for loading using the α-tubulin band from the same sample. In the case of Tgfbr2, the lower (more heavily expressed) isoform 2 was analyzed. Data reflect the mean normalized PD ± SD (n = 8). *$P < 0.05$ vs. embryonic day (E) 15. Pseud, pseudoglandular; can, canalicular.

over the course of late lung development; however, the expression of all three Smads was significantly reduced at day P28 compared with stage E15 (Fig. 2A,B). Taking the RT-PCR and immunoblot data together, these data suggest that Tgfbr1/Tgfbr2/Smad2,3,4-mediated signaling is dampened while Acvrl1/Eng/Smad6/Smad7-mediated signaling is potentiated, as normal late lung development in the mouse proceeds. Interestingly, in a hyperoxia model of bronchopulmonary dysplasia, a disorder characterized by arrested alveolar development, the opposite trend is noted, where Tgfbr2 and Smad4 levels are dramatically up-regulated (Alejandre-Alcázar et al., 2007a).

In addition to changes in total expression levels in the lungs of healthy mice, both TGF-β receptors and Smad proteins exhibited different localization during late lung development. Antibodies directed against Acvrl1 were not suitable for immunohistochemical assessment of Acvrl localization in the mouse (data not shown), however, both Tgfbr1 (Fig. 3A) and Tgfbr2 (Fig. 3C) exhibited staining in the vessel walls of the developing lung at postnatal day P7, and in the case of Tgfbr1, some staining in the interstitium was also observed. In contrast, no staining was observed in the epithelial lining of the airways (insets to Fig. 3A,C). Toward the end of alveolarization (stage P28), staining in vessel walls for Tgfbr1 (Fig. 3B) and Tgfbr2 (Fig. 3D) was lost, although staining was now evident in the epithelial lining of the airways.

Smad protein localization was also different during late lung development (Fig. 3E,F). Staining for Smad3 was evident in the vessel walls, including the endothelium, as well as in the interstitium and in the subepithelial layer of the airways in the lungs of mice at day P7 (Fig. 3E). An abundance of nuclear-localized staining for Smad3 was observed in this tissue, indicating that TGF-β signaling was active. Little or no staining for Smad3 was observed in the in the airway epithelium (Fig. 3E). By day P28, much of the interstitial staining for Smad3 was lost, although staining, including nuclear staining, for Smad3 was still observed in the vessel walls (Fig. 3F). Additionally, an abundance of cytosolic and nuclear staining was now observed in the epithelial lining of the airways (Fig. 3F).

To localize activated TGF-β Smads, mouse lung sections were stained for phospho-Smad2 (Fig. 4A,B). Nuclear-localized phospho-Smad2 was evident in the interstitium, airway epithelium, and in the vessel walls of mouse lungs at day P7 (Fig. 4A). By day P28, the interstitial staining was lost; however, staining was still detected in the airway epithelium and in the vessel walls (Fig. 4B), indicating that TGF-β signaling was broadly active at the start of alveolarization (day P7), but was confined to the epithelial lining of airways toward the end of alveolarization (day P28).

Smad activation was assessed directly by immunoblot for phospho-Smad2 in whole-lung extracts from mice over the course of late lung development (representative blots are illustrated in Fig. 4C; and multiple blots are quantified in Fig. 4E,F). A robust phospho-Smad2 signal was obtained at the early alveolarization stages (days P3 and P7 in Fig. 4C, F), which is consistent with the immunohistochemical data presented in Figure 4A. By day P28, the phospho-Smad2 signal was significantly reduced (day P28 in Fig. 4C,F). However, normalization of the phospho-Smad2 signal for total Smad2 (Fig. 4F) clearly illustrates that, while the abundance of phospho-Smad2 at day P28 is significantly less than that at day P3 (Fig. 4E), the proportion of the total Smad2 pool that is activated (phosphorylated) is significantly increased at day P28 compared with day P7 (Fig. 4F). These data suggest that TGF-β signaling remains active into late lung development and, together with the histochemical data for phospho-Smad2 (Fig. 4A,B), suggest that

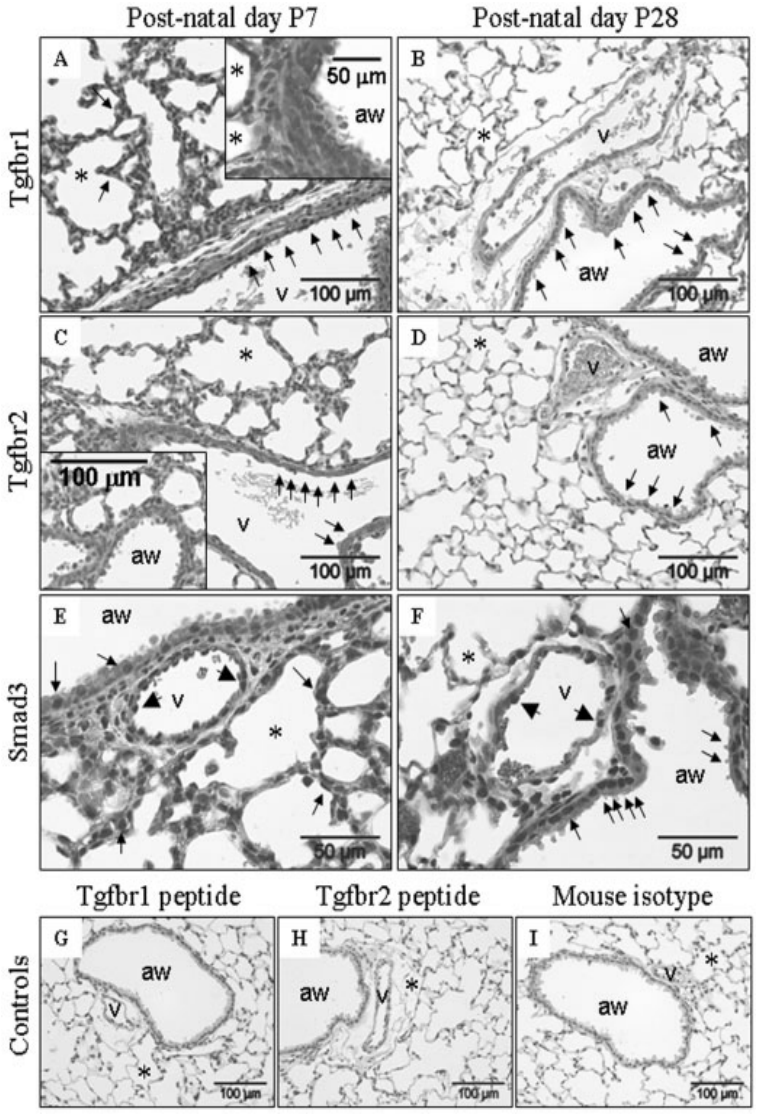

Fig. 3. Changes in localization of components of the transforming growth factor-beta (TGF-β) signaling machinery during late lung development in the mouse. **A–F:** Type I TGF-β receptor (Tgfbr1; A,B), type II TGF-β receptor (Tgfbr2; C,D) and Smad3 (Smad3; E,F), expression was localized in the lungs of mice at postnatal day (P) 7 (A,C,E) and P28 (B,D,F) by immunohistochemistry. Insets in A and C, which are not taken from areas in the larger panels, illustrate airway staining. Images represented for P7 and P28 tissues are derived from the same tissue block; however, the panels are representative of staining patterns observed in three animals per developmental stage. **G–I:** Staining of sections after preadsorption of antibodies with competing peptides for Tgfbr1 (G) and Tgfbr2 (H), or replacing the antibody with a species-matched isotype control, in the case of Smad3 (I). Arrows indicate examples of stained cells. Arrowheads in E and F indicate nuclear-localized Smad3 in the lung vasculature. Asterisk, alveolus; aw, airway; v, vessel.

the bulk of this signaling at day P28 occurs in the airway epithelium.

TGF-β signaling was also assessed indirectly, in whole-lung extracts from mice over the course of late lung development using two TGF-β/Smad2,3 target genes: *ctgf* (encoding connective tissue growth factor) and *serpine1* (encoding plasminogen activator inhibitor-1; Wu et al., 2007; Zakrzewicz et al., 2007). Whereas both genes were expressed throughout late lung development (Fig. 4D), their expression was significantly increased at day P28 compared with stage E15 (Fig. 4G). These data are consistent with the increased proportion of phospho-Smad2 seen at day P28 (Fig. 4F). However, they are not consistent with the increased overall abundance of phospho-Smad2 between days P3 and P14. It may well be that these genes are induced by TGF-β in specific cell types (for example the airway epithelium) where TGF-β/Smad2,3 signaling is active only later in lung development (as suggested by the immunohistochemical analysis for Smad3 and phospho-Smad2 in Figs. 3 and 4). Alternatively, the expression of *ctgf* and *serpine1* may also be positively and negatively regulated by other factors active during lung development, hence, the lack of correlation with the overall abundance of phospho-Smad2 between days P3 and P14. Indeed, *ctgf* can also be induced by, for example, angiotensin II (Iwanciw et al., 2003) and *serpine1* can be induced by mechanical stimulation of the airways (Chu et al., 2006). Because all of these factors do impact late lung development, *ctgf* and *serpine1* expression may not represent ideal indirect readouts of TGF-β/Smad2,3 signaling in this context.

To assess the extent to which these patterns were comparable to human lung development, sections of healthy human lungs in the canalicular, saccular, and alveolar stages of development were screened by immunohistochemical analysis for the expression and localization of components of the TGF-β signaling machinery (Figs. 5, 6). In whole-lung extracts from mice, one of the type I TGF-β receptors, Acvrl1, exhibited a progressive increase in expression as late lung development proceeded, as assessed by RT-PCR (Fig. 1A,B,F) and immunoblot (Fig. 2A,B). A similar trend was observed in human lungs, where progressively more Acvrl1 staining was observed in the canalicular (Fig. 5A,E), saccular (Fig. 5B, F), and the alveolar (Fig. 5C,G) stages. Diffuse staining was observed in the interstitium throughout these stages. Staining was most evident in the bronchial epithelium, and increased in intensity in the vascular smooth muscle (Fig. 5C,G; thick arrows) in the alveolar stage. The anti-Acvrl1 antibody did not stain mouse tissue (data not shown); therefore, comparisons with Acvrl1 localization in rodents could not be made. In the case of the second type I TGF-β receptor, Tgfbr1, no staining was evident in the pseudoglandular stages (not shown); how-

ever, staining of uniform intensity was observed diffusely in the interstitium and was pronounced in the airway epithelium in the canalicular, saccular, and alveolar stages of human development (Fig. 5A–C,E–G). Additionally, pronounced staining was observed in the vascular smooth muscle in the saccular and alveolar stages (Fig. 5B,C,F,G). A similar staining pattern was observed for Tgfbr2 in human lungs (Fig. 6A–C,E–G), where diffuse staining was evident in the interstitium, as well as in the epithelial lining of the developing airways. This staining increased in intensity between the canalicular and alveolar stages, becoming most intense in the alveolar stage (Fig. 6C,G). Staining for Tgfbr2 was also evident in the vessel walls in the saccular (Fig. 6B,F) and alveolar (Fig. 6C,G) stages.

Active TGF-β signaling was demonstrated throughout human lung development by the presence of nuclear-

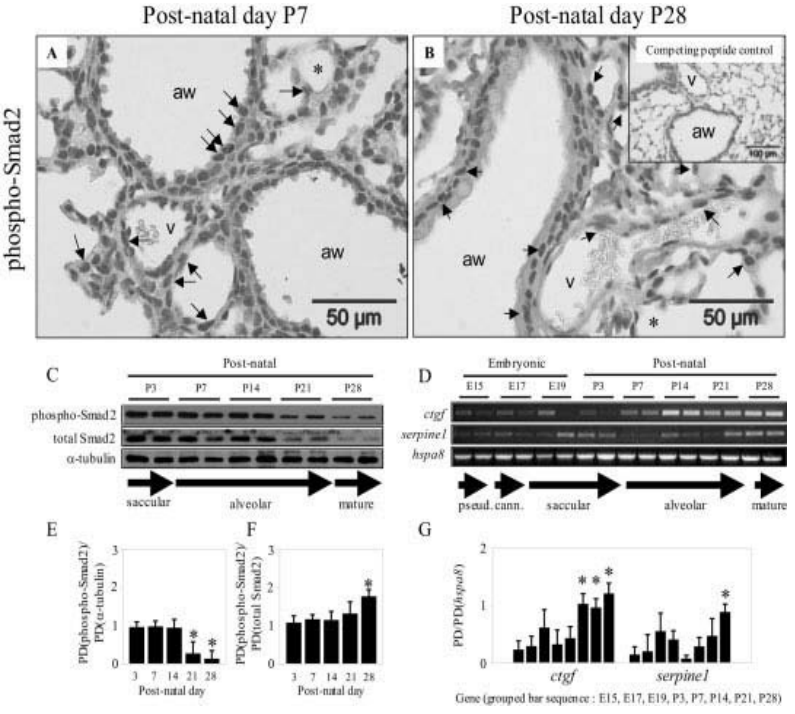

Fig. 4.

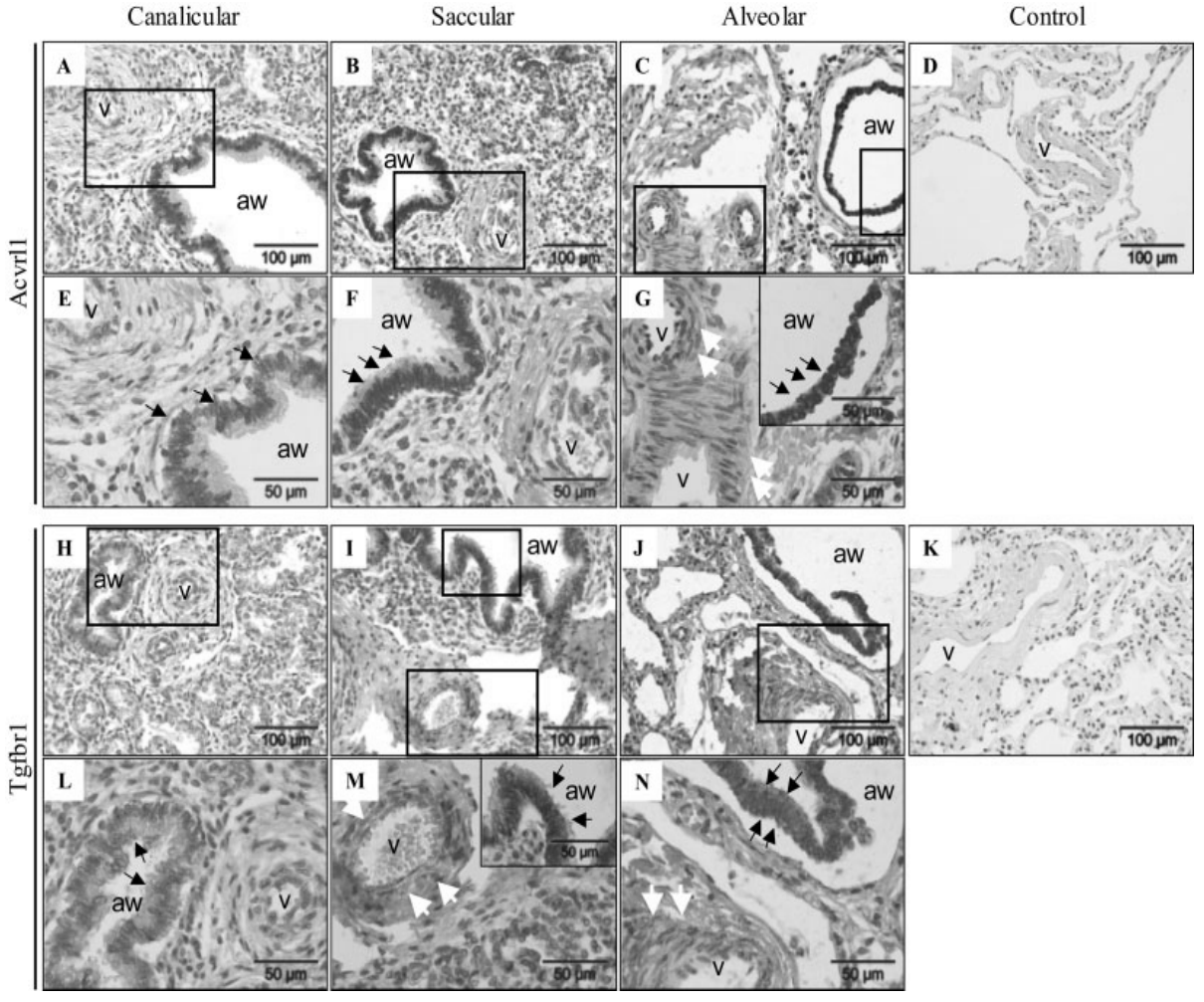

Fig. 5.

localized phospho-Smad2 in the developing lungs, in the canalicular (Fig. 6H,L), saccular (Fig. 6I,M), and alveolar (Fig. 6J,N) stages. Staining was observed in the interstitium, in the vessel walls and was pronounced in the epithelial lining of the developing airways (Fig. 6). Additionally, nuclear phospho-Smad2 staining was also observed in the airway smooth muscle in the alveolar stage of human lung development (Fig. 6J,N). Together, these data indicate that components of the TGF-β signaling machinery are expressed during late lung development in humans and that the expression of various components of this machinery are differentially regulated as late lung development proceeds. Additionally, TGF-β signaling per se was active during human late lung development. The alveolar phase of lung development in humans spans the period from 36 weeks gestation to 2.5 years postnatal. The tissue sections illustrated here are derived from neonatal patients who died preterm in utero, or in the first 3 weeks after delivery. Therefore, all sections illustrated here are in the very early alveolar stage of lung development, probably equivalent to between days P3 and P7 in neonatal mouse lungs. With this in mind, the expression patterns observed for Acvrl1, Tgfbr1, Tgfbr2, and phospho-Smad2 appear to be consistent between mouse and human over the course of late lung development.

DISCUSSION

Our data suggest that TGF-β signaling may be required for normal late lung development in mammals. Some notable patterns in TGF-β receptor expression were observed. Two type I TGF-β receptors exist: Acvrl1 and Tgfbr1. The expression of these two receptors was differently regulated in the mouse, with a progressive increase in Acvrl1 expression and a progressive decrease in Tgfbr1 expression during late lung development. Acvrl is believed to play a role in the endothelium in the maturation phase of angiogenesis (Lamouille et al., 2002), while the more ubiquitously expressed Tgfbr1 mediates ECM deposition and remodeling (Massagué, 1998). The patterns in Acvrl vs. Tgfbr1 expression that we observed over late lung development suggest that endothelial maturation is favored, while ECM production is gradually down-regulated. However, pronounced staining for Acvrl1 was also noted in the vascular smooth muscle and the airway epithelium, for which no function has yet been ascribed. To our knowledge, this is the first report of Acvrl1 expression in either cell type.

What other roles may TGF-β play during late lung development? Dynamic changes in the expression of components of the TGF-β signaling system were observed in several compartments of the developing lung. Diffuse staining in the interstitium was observed throughout late lung development. Type II pneumocytes serve as progenitor cells for the alveolar epithelium and are an important component of the interstitium. The differentiation of type II pneumocytes is regulated, at least partially, by a balance between glucocorticoid and TGF-β signaling (McDevitt et al., 2007). Furthermore, transdifferentiation of type II pneumocytes into type I pneumocytes is an important feature of the alveolarization process (Copland and Post, 2004). TGF-β/Smad2,3,4 signaling plays a key role in this type II to type I pneumocyte transdifferentiation (Bhaskaran et al., 2007), which may explain the active TGF-β signaling observed in the interstitium during the alveolar phase of late lung development, where type II to type I pneumocyte transdifferentiation would occur.

The most pronounced staining for TGF-β signaling components in mouse and human tissue was observed in the airway epithelium. Furthermore, pronounced staining for nuclear-localized Smad3, and nuclear-localized phospho-Smad2 was observed in the airway epithelium as late lung development proceeded. TGF-β is a key growth factor driving terminal squamous differentiation of cultured bronchial epithelial cells in vitro, as well as changes in airway epithelial cell shape, surface area, and adhesion properties (Masui et al., 1986). Those data suggest that TGF-β is

Fig. 4. The transforming growth factor-beta (TGF-β)/Smad signaling axis is active during late lung development in the mouse. **A,B:** Localization of phospho-Smad2 in the lungs of mice at postnatal day (P) 7 (A) and P28 (B). Panels are representative of staining patterns observed in three animals per developmental stage. Arrows indicate examples of nuclear-localized phospho-Smad2. aw, airway; v, vessel. The inset in B illustrates staining of a section after preadsorption of antibodies with a competing peptide for phospho-Smad2/3. **C:** Direct assessment of Smad2 activation during late lung development. Smad2 phosphorylation, total Smad2 levels, and α-tubulin were assessed by immunoblot in protein extracts of lungs from mice during the saccular (P3) and alveolar (P7–P28) stages of late lung development. Blots illustrate 25-μg samples from two different mouse lungs per developmental stage that are representative of the patterns observed in six other mouse lungs at the same developmental stage. **D:** Indirect assessment of TGF-β/Smad signaling by mRNA abundance of the TGF-β/Smad2,3-responsive genes *ctgf* and *serpine1*, determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Amplicons are illustrated from two different mouse lungs per developmental stage, representative of patterns observed in six other mouse lungs at the same developmental stage. **E,F:** Changes in the levels of phospho-Smad2 (normalized for α-tubulin; E) or the proportion of phospho-Smad2 (normalized for total Smad2; F) were quantified by densitometric analysis of immunoblot bands from six mouse lungs per developmental stage. Band intensities (described by the pixel density, PD) for phospho-Smad2 were normalized for α-tubulin or Smad2 using the respective band from the same sample. Data reflect the mean normalized phospho-Smad2 PD ± SD (n = 6). $^*P < 0.05$ vs. E15. **G:** Changes in *ctgf* and *serpine1* mRNA levels were quantified by densitometric analysis of amplicon bands from six mouse lungs per developmental stage. Band intensities (described by the pixel density, PD) from samples were normalized for loading using the *hspa8* amplicon from the same sample. Data reflect the mean PD normalized for the *hspa8* PD ± SD (n = 6). $^*P < 0.05$ vs. embryonic day (E) 15. Pseud, pseudoglandular; can, canalicular.

Fig. 5. Changes in the expression levels and localization of type I transforming growth factor-beta (TGF-β) receptors occur during human late lung development. **A–N:** The type I TGF-β receptors Acvrl1 (A–G) and Tgfbr1 (H–N) were localized in the developing lungs of patients at the canalicular, saccular, and alveolar stages, by immunohistochemistry. The areas illustrated are representative of staining patterns observed in three to five different patients per developmental stage. D and K illustrate staining of sections where the immune antibody was replaced with a species-matched isotype control, in the case of Acvrl1 (D), or after preadsorption of antibodies with a competing peptide, in the case of Tgfbr1 (K). Where high-magnification images (lower row) are derived from the low-magnification image (upper row), the magnified area is demarcated with a solid-lined box. Thin arrows indicate examples of staining in the cells lining the conducting airways and in the interstitium. Thick arrows indicate examples of staining in vessel walls. aw, airway; v, vessel.

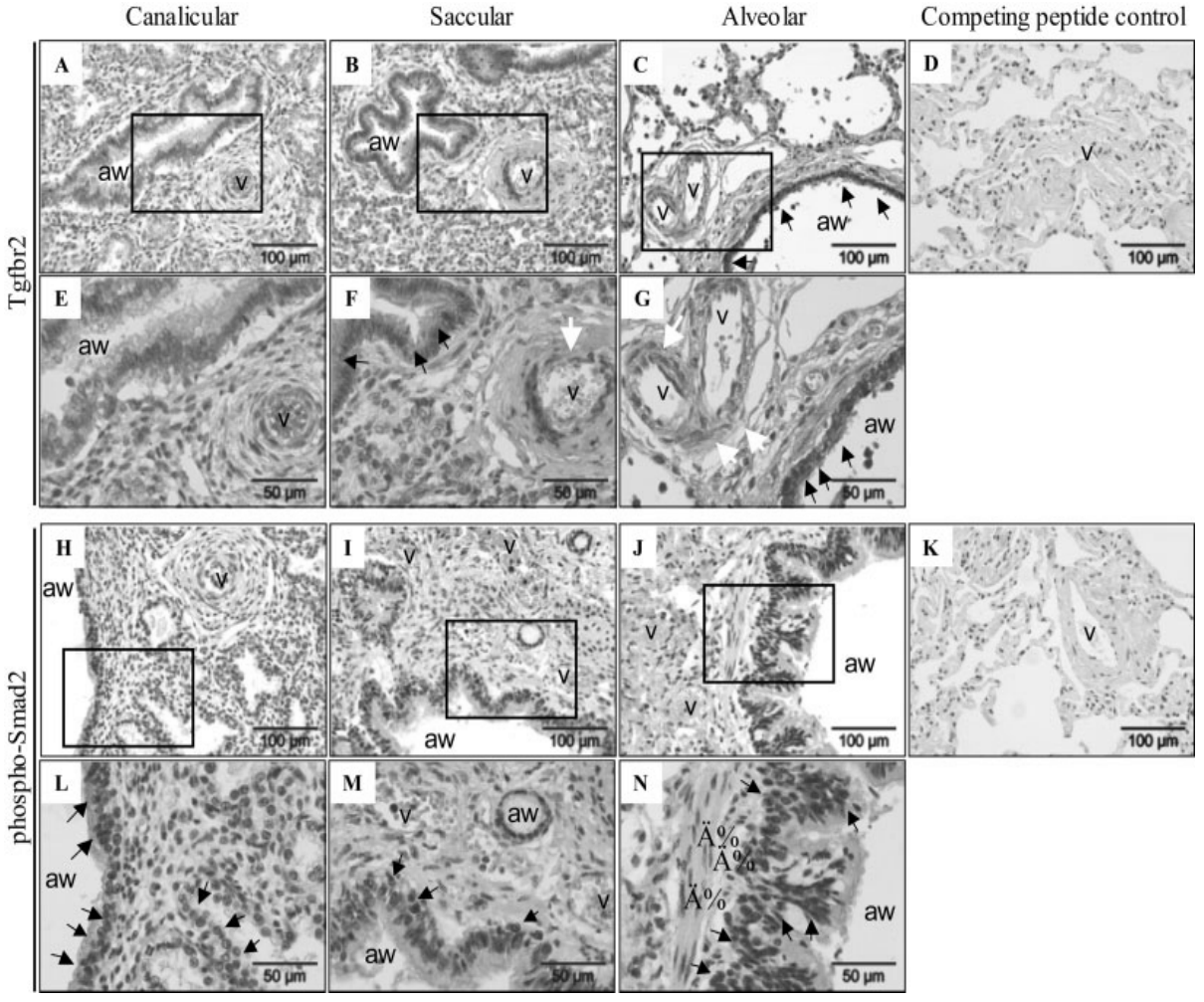

Fig. 6. Changes in the expression levels and localization of the type II transforming growth factor-beta (TGF-β) receptor and phospho-Smad2 during human late lung development. **A–N:** The type II TGF-β receptor Tgfbr2 (A–G) and phospho-Smad2 (H–N) were localized in the developing lungs of patients at the canalicular, saccular and alveolar stages, by immunohistochemistry. D and K illustrate staining of sections where the immune antibody was preadsorbed with competing peptides for Tgfbr2 (D) or phospho-Smad2/3 (K). The areas illustrated are representative of staining patterns observed in three to five different patients per developmental stage. Where high-magnification images (lower row) are derived from the low-magnification image (upper row), the magnified area is demarcated with a solid-lined box. Thin arrows indicate examples of staining in cells lining conducting airways and in the interstitium. Thick arrows indicate examples of staining in vessel walls. aw, airway; v, vessel.

critically involved in airway development, which is consistent with the staining patterns that we observed in the airway epithelium during late lung development. In support of this idea, a significant reduction in the abundance of *tgfb1* (encoding the TGF-β1 ligand) and *tgfbr1* mRNA, as well as a significant reduction in the protein expression of TGF-β1 and TGF-β2 ligands, and Tgfbr1 and Tgfbr2, have been reported in the bronchial epithelium and smooth muscle of a patient with pulmonary acinar aplasia, a severe form of pulmonary hypoplasia (Chen et al., 1999).

Detection of phospho-Smad2 in airway smooth muscle indicated that TGF-β signaling was also active in this tissue. TGF-β exhibits pro-proliferative effects on airway smooth muscle cells (Xie et al., 2007), and active TGF-β signaling in the airway smooth muscle in the developing lung may contribute to the muscularization of the developing airways during late lung development. This idea is supported by studies in asthma, where overactive TGF-β signaling is believed to underlie the airway smooth muscle cell hyperplasia observed in asthmatic patients (Xie et al., 2007).

While TGF-β signaling is required for normal late lung development, this requirement is clearly finely tuned, because too much TGF-β signaling can negatively impact alveolarization. Neonatal rodents chronically exposed to normobaric hypoxia (Vicencio et al., 2002) or hyperoxia (Alejandre-Alcázar et al., 2007a) exhibited arrested alveolarization and developed bronchopulmonary dysplasia. In the case of hyperoxia, this occurred concomitantly with up-regulated Tgfbr2 and Smad4 expression, and increased TGF-β/Smad2,3 signaling in the developing lung (Alejandre-Alcázar et al., 2007a). Dampening of TGF-β signaling in this model by administration of a neutralizing anti–TGF-β antibody largely restored normal alveolar architecture (Nakanishi et al., 2007), validating a pathological role for TGF-β in hyper-

oxia-induced arrest of alveolar development. At the same time, complete blockade of TGF-β signaling by ablation of Smad3 in neonatal mice also arrested alveolarization (Chen et al., 2005). Combining the ideas in those reports with data presented here, it is emerging that, although TGF-β is a negative regulator of airway branching in *early* lung development, we demonstrate here that TGF-β signaling is active in multiple tissue types in the lung during normal *late* lung development. The degree of TGF-β signaling must, however, be precisely controlled, because both up- and down-regulation of TGF-β signaling impairs the alveolarization process.

EXPERIMENTAL PROCEDURES

Animals and Tissue Treatment

The animal ethics authority of the government of the State of Hessen approved all animal procedures. C57BL/6J mice were housed in humidity- and temperature-controlled rooms on a 12:12-hr light–dark cycle and were allowed food and water ad libitum. Mice at stages E15, E17, E19, P1, P3, P5, P7, P14, P21, and P28 were killed by intraperitoneal injection of sodium pentobarbital. Lungs were excised, flushed free of blood with phosphate-buffered saline (PBS), and processed immediately for RNA or protein extraction. For histology, the heart and lungs from mice at days P7 and P28 were excised en bloc, and the lungs were pressure fixed overnight at 20 cm H_2O with 4% (m/v) paraformaldehyde in PBS (20 mM Tris-Cl, 137 mM NaCl, pH 7.6), as described previously (Alejandre-Alcázar et al., 2007a). Paraffin sections (3 μm) were mounted on poly-L-lysine–coated glass slides, dewaxed with xylene (3 × 5 min), and rehydrated in a graduated series of ethanol solutions (100%, 95%, 70% [v/v], and PBS).

Human Tissue

Fetal and neonatal human lung tissue was retrieved from archived autopsy material at the Erasmus University Medical Centre and the University of Giessen Lung Center. Lung samples were normal for their gestational age. All human material was used with the approval of the Human Subjects Review Committees of the Erasmus University Medical Centre and the University of Giessen Lung Center. Autopsy material was derived from fetuses from induced or spontaneous abortions, stillbirth, or neonates that died from nonpulmonary causes within 3 weeks after delivery. None of these infants had infections at the time of death. Tissue was fixed in 4% (m/v) paraformaldehyde in PBS, between 1 and 12 hr after death. Before fixation, tissue was stored at 4°C.

Immunohistochemistry

Hematoxylin staining, and expression of TGF-β receptors, Smads, and smooth muscle actin (SMA) was assessed on 3 μm tissue sections as described previously (Morty et al., 2007), with anti-SMA (1:850; clone 1A4; Sigma, Taufkirchen, Germany); goat anti-Acvrl1 (catalog no. AF370; R&D Systems, Wiesbaden, Germany), rabbit anti-Tgfbr1 (designated R-20, catalog no. SC-399; Santa Cruz, San Fransisco, CA; 1:50; used with blocking peptide SC-399P), rabbit anti-Tgfbr2 (designated C-16, catalog no. SC-220; Santa Cruz, San Fransisco, CA; 1:50; used with blocking peptide SC-220P), rabbit anti-phospho-Smad2 (Ser465/467) (catalog no. 05-953), and mouse anti-Smad3 (clone 2C12; catalog no. MAB10075) were both from Upstate (Charlottesville, VA; both used at 1:50). The anti–phospho-Smad2(Ser465/467) antibody was preadsorbed with a phospho-Smad2/3 competing peptide from Santa Cruz (catalog no. SC-11769P; San Fransisco, CA). For competing peptides, antibodies were preadsorbed for 30 min at a ratio of 1:5 (antibody:peptide; mol:mol) before use. No competing peptides were available for the anti-Acvrl1 and anti-Smad3 antibodies. Therefore, in the case of these two antibodies, species-matched isotype control antibodies (available with the Histostain *Plus* Kit from Zymed, San Fransisco, CA) replaced the primary antibodies. Immune complexes were visualized with a Histostain *Plus* Kit (Zymed, San Fransisco, CA). Although the specificity of all antibodies used for immunohistochemistry has been validated (Zakrzewicz et al., 2007), additional data are included in the relevant panels using antibodies preadsorbed with a competing peptide, or where primary antibodies were replaced with a species-matched isotype control antibody at the same concentration. For mouse and human tissue, all panels illustrated at a particular developmental stage are derived from the same tissue block. The staining patterns depicted are representative for three mice at the same developmental stage, or for between three and five patients at the same developmental stage.

RNA Isolation, Semiquantitative RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was isolated from fresh lung tissue using a Qiagen RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). One microliter of the RT reaction served as a template in a PCR reaction using Platinum *Taq* (Invitrogen, Karlsruhe, Germany). Total RNA was screened by semiquantitative RT-PCR using the following forward (for) and reverse (rev) primers at the stated cycle number: *acvrl1* (29 cycles): 5′-AGG GCC GAT ATG GTG AGG TGT GG-3′ (for), 5′-GCC GGT TAG GGA TGG TGG GTG TC-3′ (rev); *ctgf* (29 cycles): 5′-CCC GCC AAC CGC AAG ATT-3′ (for), 5′;-AGG CGG CTC TGC TTC TCC A-3′; (rev); *eng* (22 cycles): 5′-GAG TCG GCT GTG ATC TAC AGC CTG TGG-3′ (for), 5′-CTG ATG ATC ACC TCA TTG CTG ACC-3′ (rev); *hspa8* (24 cycles): 5′-CAA GCG AAA GCA CAA GAA AGA CAT-3′ (for), 5′-ATA CCA AGC GAA AGA GGA GTG ACA TC-3′ (rev); *serpine1* (26 cycles): 5′-TCA TCA ATG ACT GGG TGG AA-3′ (for), 5′-GCC AGG GTT GCA CTA AAC AT-3′ (rev); *smad2* (24 cycles): 5′-CTC CGG CTG AAC TGT CTC CTA CT-3′, (for), 5′-TTA CAG CCT GGT GGG ATC TTA CA-3′ (rev); *smad3* (24 cycles): 5′-AGA ACG GGC AGG AGG AGA AGT GGT-3′ (for), 5′-GGA TTC GGG GAG AGG TTT GGA GA-3′ (rev); *smad4* (25 cycles): 5′-ACA

GAG AAC ATT GGA TGG AC-3′ (for), 5′-AGT AGC TGG CTG AGC AGT AA-3′ (rev); *smad6* (24 cycles): 5′-GAG CAC CCC CAT CTT CGT CAA-3′ (for), 5′-AAC AGG GGC AGG AGG TGA TG-3′ (rev); *smad7* (25 cycles): 5′;-CCT CCT CCT TAC TCC AGA TA-3′ (for), 5′-ACG CAC CAG TGT GAC CGA TC-3′ (rev); *tgfbr1* (27 cycles): 5′-AGA GCG TTC ATG GTT CCG AGA G-3′ (for), 5′-GGG GCC ATG TAC CTT TTA GTG C-3′ (rev); *tgfbr2* (25 cycles): 5′-GAG AGG GCG AGG GCG AGG AGT AAA GG-3′ (for),. 5′-GTG GTA GGT GAG CTT GGG GT-3′ (rev); and *tgfbr3* (27 cycles): 5′-CCC TGT GTT TGT CCT GAT GAG CGC CTG CC-3′ (for), 5′-CCT ATG TCT AGT ACC ACA GCC ATT C-3′ (rev). Amplicons were separated on a 1% (m/v) agarose gel and visualized by ethidium bromide staining. Amplicons generated with these primer pairs have been validated by sequencing, and the stated cycle numbers lie in the logarithmic phase for each PCR (Alejandre-Alcázar et al., 2007a,b; Zakrzewicz et al., 2007). For quantification of changes in mRNA expression, band intensities from specific samples were normalized for loading using the constitutively expressed *hspa8* band from the same sample. Densitometric analysis of amplicon bands was performed using a GS-800 model calibrated densitometer with Quantity One software (both from Bio-Rad Laboratories, Munich, Germany). Pixel densities were corrected for background staining in the same gel. When the band intensity exceeded the maximum detection limit for the software, that band was excluded from the analyses.

Total RNA was also screened by quantitative real-time RT-PCR exactly as described previously (Alejandre-Alcázar et al., 2007a; Morty et al., 2007; Zakrzewicz et al., 2007) using the following intron-spanning forward (for) and reverse (rev) primers: *acvrl1*: 5′-CAG TCC ACC TGA TTC AGC TTT-3′ (for), 5′-GGC AAC AGG AAT CAA GAT TGT-3′ (rev); *hmbs*: 5′-ATG TCC GGT AAC GGC GGC-3′; (for), 5′-GGT ACA AGG CTT TCA GCA TCG-3′ (rev); *tgfbr1*: 5′-GGG ATT GCC ATA GC-3′ (for), 5′-TCC CTG TTG TGG TGA TGT T-3′ (rev); *tgfbr2*: 5′-CAG AGG GCA CCA CCT TAA AA-3′ (for), 5′-AAT GGT CCT GGC AAT TGT TC-3′ (rev); and *tgfbr3*: 5′-GGG CAT TGC GTT TGC AGC AT-3′ (for), 5′-CTG AGT GCT CCC TAT GCT GT-3′ (rev). Quantitative changes in gene expression were analyzed by comparing the ΔC_t values of the genes of interest (using RNA samples from three different animals per developmental stage, each sample assessed in duplicate), normalized for the ubiquitously expressed pseudogene-free hydroxymethylbilane synthase (*hmbs*) gene.

Protein Isolation and Immunoblotting

Protein extraction from mouse lungs, gel electrophoresis, and immunoblotting were performed as described previously (Alejandre-Alcázar et al., 2007a). Lung protein extracts (10–25 μg) were resolved on 12% polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting. Blots were probed with goat anti-Acvrl1 (catalog no. AF370; R&D Systems, Wiesbaden, Germany; 1:1,000), mouse anti-Tgfbr1 (designated R-20, catalog no. SC-399; Santa Cruz, San Fransisco, CA; 1:1,500 for mouse), mouse anti-Tgfbr2 (designated C-16, catalog no. SC-220; Santa Cruz, San Fransisco, CA;), rabbit anti-phospho-Smad2(Ser465/467) (catalog no. 05-953), and mouse anti-Smad3 (clone 2C12; catalog no. MAB10075) were both from Upstate (Charlottesville, VA; both used at 1:1,000), rabbit anti-Smad4 (catalog no. H-552; Santa Cruz, San Fransisco, CA; 1:1,000), and rabbit anti-Smad2 (catalog no. 51-1300; Zymed, San Fransisco, CA; 1:1,000), while mouse anti–α-tubulin (designated B-7, catalog no. SC-5286; Santa Cruz, San Fransisco, CA; 1:2,500) served as a loading control. Peroxidase-conjugated anti-mouse (1:1,000–1:2,000) and anti-rabbit (1:2,000–1:2,500) secondary antibodies were from R&D Systems (Wiesbaden, Germany). The specificity of all antibodies used for immunoblotting has been validated (Alejandre-Alcázar et al., 2007a). Densitometric analysis of bands was performed as described for PCR amplicons, above.

Statistical Treatment of Data

Data are presented as mean ± SD. Differences between groups were analyzed by analysis of variance and the Student-Newman-Keul post hoc test for multiple comparisons, with a P value < 0.05 regarded as significant.

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chapter 4

Hyperoxia modulates TGF-β/BMP signaling in a mouse model of bronchopulmonary dysplasia

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Hyperoxia modulates TGF-β/BMP signaling in a mouse model of bronchopulmonary dysplasia

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Alejandre-Alcázar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Pérez J, Wygrecka M, Eul B, Köbrich S, Hesse M, Schermuly RT, Seeger W, Eickelberg O, Morty RE. Hyperoxia modulates TGF-β/BMP signaling in a mouse model of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 292: L537–L549, 2007. First published October 27, 2006; doi:10.1152/ajplung.00050.2006.—Prematurely born infants who require oxygen therapy often develop bronchopulmonary dysplasia (BPD), a debilitating disorder characterized by pronounced alveolar hypoplasia. Hyperoxic injury is believed to disrupt critical signaling pathways that direct lung development, causing BPD. We investigated the effects of normobaric hyperoxia on transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling in neonatal C57BL/6J mice exposed to 21% or 85% O_2 between postnatal *days P1* and *P28*. Growth and respiratory compliance were significantly impaired in pups exposed to 85% O_2, and these pups also exhibited a pronounced arrest of alveolarization, accompanied by dysregulated expression and localization of both receptor (ALK-1, ALK-3, ALK-6, and the TGF-β type II receptor) and Smad (Smads 1, 3, and 4) proteins. TGF-β signaling was potentiated, whereas BMP signaling was impaired both in the lungs of pups exposed to 85% O_2 as well as in MLE-12 mouse lung epithelial cells and NIH/3T3 and primary lung fibroblasts cultured in 85% O_2. After exposure to 85% O_2, primary alveolar type II cells were more susceptible to TGF-β-induced apoptosis, whereas primary pulmonary artery smooth muscle cells were unaffected. Exposure of primary lung fibroblasts to 85% O_2 significantly enhanced the TGF-β-stimulated production of the α_1 subunit of type I collagen ($I\alpha_1$), tissue inhibitor of metalloproteinase-1, tropoelastin, and tenascin-C. These data demonstrated that hyperoxia significantly affects TGF-β/BMP signaling in the lung, including processes central to septation and, hence, alveolarization. The amenability of these pathways to genetic and pharmacological manipulation may provide alternative avenues for the management of BPD.

lung development; transforming growth factor-β; bone morphogenetic protein; neonatal chronic lung disease; alveolarization

BRONCHOPULMONARY DYSPLASIA (BPD) was first described by Northway et al. (35) as a disease of preterm infants that received prolonged mechanical ventilation and oxygen supplementation for acute respiratory distress. The impact of oxygen toxicity, barotrauma, volutrauma, and infection on the developing lung resulted in "classic BPD," histopathologically characterized by airway epithelial metaplasia, peribronchial fibrosis, and vascular smooth muscle hypertrophy (36). Advances in surfactant and steroid therapy and ventilation strategies have changed the pathological picture from "classic BPD" to "new BPD," also called chronic lung disease of early infancy (CLD), which is characterized more by alveolar and capillary hypoplasia and less by fibroproliferative airway damage and parenchymal fibrosis (2). Impaired alveolus formation is observed in both forms of BPD and leads to a long-term reduction in total number of alveoli, and hence reduces the surface area available for gas exchange (3, 18, 19).

Whereas the pathogenesis of new BPD is largely unknown (3), hyperoxic injury is thought to disrupt critical signaling pathways that direct lung development, including branching and septation (46, 56). Many signaling pathways critical to these processes have been described (41, 46), notable among them, signaling by the transforming growth factor (TGF)-β superfamily, encompassing the TGF-β and bone morphogenetic protein (BMP) families (17, 41, 53–55).

TGF-β signaling is initiated by binding of TGF-β to the type II TGF-β receptor (TβRII), which subsequently complexes with the type I receptor (ALK-1 or ALK-5). The type I receptor transmits signals within the cell via second-messenger Smad proteins, namely Smads 1–4, or by Smad-independent pathways (27). TGF-β signaling can negatively regulate the branching (17, 41, 54) and septation (15, 52) phases of lung development. In the case of the latter, adenoviral-mediated transfer of TGF-β1 to the neonatal rat lung (15) or overexpression of TGF-β1 between postnatal *days P7* and *P14* in the mouse (52) both induced histological changes analogous to those seen in BPD. Surprisingly, however, Smad3 knockout mice exhibited retarded alveolarization between *days P7* and *P28* (6), suggesting that TGF-β also acts as a positive regulator of septation. This apparent paradox indicates that TGF-β signaling plays a critical and finely tuned role in alveolarization. Consistent with these data, Smad3 deficiency in adult mice caused air space enlargement and centrilobar emphysema in late life (4, 6, 33), suggesting a key role for TGF-β signaling in both the formation of alveoli and the maintenance of alveolar structure.

In contrast to TGF-β, BMP ligands bind their type I receptors ALK-3 (BMPRIa) or ALK-6 (BMPRIb), which activate the type II receptor (BMPRII), thereby initiating a signaling cascade primarily via Smads 1 and 4 (31). BMPs have been accredited with key roles in early lung development, particularly lung branching (9, 17, 53, 54). However, BMP-4 expres-

sion declines prior to the saccular stage, suggesting that BMP-4 is not required for septation (41).

Signaling by the TGF-β/BMP superfamily clearly plays a key role in lung development. It has also been reported that chronic hypoxia can influence TGF-β ligand and receptor expression (51), and that very low birth weight infants receiving oxygen therapy exhibit elevated TGF-β levels in endotracheal aspirates (24). Thus we suspected that oxygen supplementation may influence TGF-β/BMP signaling in the neonatal lung. Such perturbations could underlie the arrested alveolar development associated with BPD. Indeed, the identification of differential gene expression in lungs that develop BPD has been identified as a "research priority" in BPD by a recent National Institute of Child Health and Human Development/ National Heart, Lung, and Blood Institute and Office of Rare Diseases joint workshop (18). Therefore, the object of this study was to investigate the effects of hyperoxia on TGF-β/ BMP superfamily signaling in the lung.

MATERIALS AND METHODS

Chronic exposure to hyperoxia. The government of the State of Hessen approved all animal procedures [Regierungspräsidium Giessen II25.3–19c20–15(1) GI20/10-Nr.22/2000]. Adult and neonatal C57BL/6J mice were housed in humidity- and temperature-controlled rooms on a 12:12-h light-dark cycle and were allowed food and water ad libitum. On *day P1*, pups from four to eight litters (born within 3 h of each other) were pooled and randomized to newly delivered dams. Pups from one half of the combined litters were maintained in 85% (vol/vol) O_2, whereas pups from the other half remained in room air [21% (vol/vol) O_2]. Nursing dams were rotated between hyperoxia and room-air litters every 24 h to prevent oxygen toxicity in the dams. Hyperoxia exposures were done in 90 × 42 × 38-cm Plexiglas chambers, continuously ventilated at a rate of 3.5 l/min. Oxygen levels were monitored with a Miniox II monitor (Catalyst Research, Owing Mills, MD). Dynamic compliance was assessed by the volume-pressure compliance method in anesthetized mice (26), where mice were mechanically ventilated with a tidal volume of 6 ml/kg.

Lung processing and morphometric analysis. Mice were killed by intraperitoneal injection of sodium pentobarbital and were exsanguinated by aortic transection. The heart and lungs were excised *en bloc*, and the lungs were pressure-fixed overnight at 20 cmH_2O with 4% (mass/vol) paraformaldehyde in phosphate-buffered saline (PBS; 20 mM Tris·HCl, 137 mM NaCl, pH 7.6), as described previously (51). Paraffin sections (3 μm) were mounted on poly-L-lysine-coated glass slides, dewaxed with xylene (3 × 5 min), and rehydrated in a graduated series of ethanol solutions [100% (vol/vol), 95% (vol/vol), 70% (vol/vol), and finally PBS]. The mean linear intercept (MLI) and septal thickness were determined on sections stained for smooth muscle actin and counter-stained with hematoxylin and eosin, as described previously (51, 52).

Primary cell isolation and cell culture. Primary mouse alveolar type II (ATII) cells were isolated and passaged exactly as described previously (49). Primary human lung fibroblasts and primary human pulmonary artery smooth muscle cells (PASMC) were isolated from human donor lungs that had been rejected for transplantation, exactly as we have described previously (14, 37). The fibroblast-derived cell line NIH/3T3 and the mouse distal alveolar epithelial cell-derived MLE-12 cell line were maintained and passaged according to the recommendations of the American Type Culture Collection (ATCC, http://www.atcc.org). To investigate the effects of 85% O_2 on the induction of apoptosis, proliferation, or synthesis of extracellular matrix components by TGF-β or BMP, cells were maintained under 21% O_2 or 85% O_2 for 24 h, prior to stimulation with TGF-β1 (2 ng/ml) or BMP-2, BMP-4, or BMP-7 (each at 100 ng/ml) for a further 48 h. Samples were analyzed at 24 and 48 h after application of TGF-β or BMP ligand.

Protein detection by immunoblot. Frozen, unfixed lung tissue was homogenized with a tissue grinder in lysis buffer [20 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100] supplemented with Complete proteinase inhibitor cocktail (Merck Biosciences, Bad Soden, Germany). Lysates from cultured cells were similarly prepared using a cell scraper. Homogenates were clarified by centrifugation (10,000 *g*, 4°C, 10 min). Protein concentration was quantified with a Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Cell extracts (10 μg) resolved on a 10% reducing SDS-PAGE gel were transferred to a nitrocellulose membrane. Blots were probed with the following antibodies: rabbit anti-ALK-3 or anti-ALK-6 (1:2,000; R&D Systems, Wiesbaden, Germany); mouse anti-ALK-5 (1:1,500; Santa Cruz, San Francisco, CA); mouse anti-TβRII (1:1,000; Santa Cruz); rabbit anti-Smad1; rabbit anti-phospho-Smad1 (Ser463/465); mouse anti-phospho-Smad2 (Ser465/467) and mouse anti-Smad3 (both at 1:1,000; Upstate, Charlottesville, VA); rabbit anti-Smad4 (1:1,000; Santa Cruz); and rabbit anti-Smad2 (Zymed, San Francisco, CA); whereas mouse α-tubulin (1:1,000; Santa Cruz) served as a loading control. Peroxidase-conjugated anti-mouse (1:1,000 to 1:2,000) and anti-rabbit (1:2,000 to 1:2,500) secondary antibodies were from R&D Systems. Densitometric analysis of protein bands was performed using a GS-800 model calibrated densitometer with Quantity One software (both from Bio-Rad Laboratories, Munich, Germany). Band intensities from samples were normalized for loading using the α-tubulin band from the same sample. Expression ratios for immunoblot data reflect values normalized for tubulin: [band pixel density (PD) (85% O_2)/tubulin PD (85% O_2)]/[band PD (21% O_2)/tubulin PD (21% O_2)].

Total RNA isolation and semiquantitative and real-time RT-PCR. Total RNA was isolated from unfixed lung tissue or from cultured primary human lung fibroblasts using a Qiagen RNeasy kit (Qiagen, Hilden, Germany), followed by DNase treatment to remove any contaminating genomic DNA. Total RNA was screened for mRNA encoding ALK-1, BMPRII, connective tissue growth factor (CTGF), inhibitor of differentiation (Id) 1, Id2, Id3, and plasminogen activator inhibitor-1 (PAI-1) using the primers indicated in Table 1 and the *hspa8* or *gapdh* genes to demonstrate RNA equivalence in the RT reactions. One microgram of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). One microliter of the RT reaction served as a template in a PCR reaction using Platinum *Taq* (Invitrogen, Karlsruhe, Germany). Amplicons were generated by an initial denaturation (5 min, 96°C), followed by 25 cycles of denaturation (1 min, 96°C), annealing (1 min at the annealing temperature described in Table 1), and primer extension (3 min, 72°C). The PCR reactions were terminated with a 10-min primer extension step at 72°C. Amplicons were separated on a 1% (mass/vol) agarose gel and visualized by ethidium bromide staining. Band intensities from specific samples were normalized for loading using the *hspa8* band from the same sample. Expression ratios for RT-PCR data reflect values normalized for the *hspa8* band: [band PD (85% O_2)/*hspa8* PD (85% O_2)]/[band PD (21% O_2)/*hspa8* PD (21% O_2)].

Quantitative changes in mRNA expression of genes encoding ECM and ECM-remodeling components were assessed by quantitative real-time PCR exactly as described previously (22), using a Sequence Detection System 7700 (Applied Biosystems, Foster City, CA) and the intron-spanning primer pairs indicated in Table 1. The ubiquitously expressed, pseudogene-free hydroxymethylbilane synthase (*hmbg*) gene was used as reference. Cycling conditions were 95°C for 6 min, followed by 45 cycles of 95°C for 20 s, 62°C for 30 s, and 73°C for 30 s. The exclusive amplification of the expected PCR product was confirmed by melting curve analysis and gel electrophoresis. Changes are expressed as: [ligand-induced fold change in cycle threshold (ΔC_t) under 85% O_2]/(ligand-induced fold change in ΔC_t under 21% O_2).

Table 1. *Primers employed for RT-PCR*

Gene	Forward Primer	Reverse Primer	Amplicon Size, bp	Cycle Number	Annealing Temperature, °C
Semiquantitative PCR					
ALK-I	5′-AGGGCCGATATGGTGAGGTGTGG-3′	5′-GCCGGTTAGGGATGGTGGGTGTC-3′	735	29	58
BMPRII	5′-GGGGAAGAAGATAATGCG-3′	5′-GGACATCGAATGCTCAGAGG-3′	601	32	55
CTGF	5′-CCCGCCAACCGCAAGATT-3′	5′-AGGCGGCTCTGCTTCTCCA-3′	449	29	60
gapdh	5′-ACCCAGAAGACTGTGGATGG-3′	5′-TGTGAGGGAGATGCTCAGTG-3′	548	24	60
hspa8	5′-CAAGCGAAAGCACAAGAAAGACAT-3′	5′-ATACCAAGCGAAAGAGGAGTGACATC-3′	474	24	60
Id1	5′-GCCCCAGAACCGCAAAGTGA-3′	5′-AACCCCCTCCCCAAAGTCTCTG-3′	409	25	65
Id2	5′-AAAGCCTTCAGTCCGGTGAG-3′	5′-AGCCACAGAGTACTTTGCTATCAT-3′	400	25	60
Id3	5′-CGCGAGGCCGCGGTAAG-3′	5′-GTTCCGGAGTGAGCTCAGCTGTCT-3′	249	25	65
PAI-1	5′-TCATCAATGACTGGGTGGAA-3′	5′-GCCAGGGTTGCACTAAACAT-3′	508	26	60
Real-time PCR					
Collagen 1	5′-CAAGAGGAAGGCCAAGTCGAG-3′	5′-TTGTCGCAGACGCAGATCC-3′	128	NA	59
Collagen 3	5′-CTGTGAATCATGCCCTACTGGTC-3′	5′-TAGCCTGCGAGTCCTCCTACTG-3′	96	NA	59
Elastin	5′-GGGTTGTGTCACCAGAAGCA-3′	5′-CCCGTAAGTAGGAATGCCTCC-3′	99	NA	59
MMP-1	5′-ACCCCAAAAGCGTGTGACAG-3′	5′-AGAAGGGATTTGTGCGCATG-3′	106	NA	59
MMP-2	5′-CCGCAGTGACGGAAAGATGT-3′	5′-GCCACGAGGAACAGGCTGTA-3′	102	NA	59
hmbg	5′-CCCACGCGAATCACTCTCAT-3′	5′-TGTCTGGTAACGGCAATGCG-3′	70	NA	59
Tenascin-C	5′-CCATCTATGGGGTGATCCGG-3′	5′-TCGGTAGCCATCCAGGAGAG-3′	139	NA	59
TIMP-1	5′-CGCAGCGAGGAGTTTCTCA-3′	5′-AGCTCAGGCTGTTCCAGGG-3′	94	NA	59
TIMP-3	5′-GGACCGACATGCTCTCAAT-3′	5′-CGGTACCAGCTGCAGTAGCC-3′	94	NA	59

BMPRII, BMP type II receptor; CTGF, connective tissue growth factor; Id, inhibitor of differentiation; PAI-1, plasminogen activator inhibitor-1; MMP, matrix metalloproteinase; *hmbg,* hydroxymethylbilane synthase gene; TIMP, tissue inhibitor of metalloproteinase; NA, not applicable.

Immunostaining of lung tissue sections. Expression of TGF-β/BMP receptors and Smad molecules was assessed on 3-μm tissue sections, prepared as described above for lung morphometric analysis. After antigen retrieval (performed in a pressure cooker using 6.5 mM sodium citrate, pH 6.0) and quenching of endogenous peroxidase activity with 3% (vol/vol) H_2O_2 for 20 min, sections were incubated with the relevant primary antibody: mouse anti-SMA (1:450; Sigma, Taufkirchen, Germany), rabbit anti-ALK-3 (1:400; R&D Systems), rabbit anti-ALK-6 (1:350; R&D Systems), rabbit anti-TβRII (H-567, 1:100; Santa Cruz), and rabbit anti-Smad1, anti-Smad3, and anti-Smad4 (all at 1:50; Upstate). Immune complexes were visualized with the relevant peroxidase-coupled secondary antibody, provided in the Histostain *Plus* Kit (Zymed).

Assessment of TGF-β or BMP signaling by luciferase transcriptional reporter assay. Cells (either MLE-12 or NIH/3T3) were seeded in 24-well plates and maintained under 21% O_2 until 70% confluent. Cells were transiently transfected with LipofectAMINE (Invitrogen) according to the manufacturer's recommendations, either with $p(CAGA)_{12}$ (Ref. 11) or with pID120 (Ref. 25), which contain a TGF-β-responsive and a BMP-responsive promoter, respectively, placed immediately upstream of a firefly luciferase gene. Alternatively, cells were transfected with pGL3-basic (containing a promoterless luciferase gene) or pGL3-control (containing a constitutively expressed luciferase gene) both from Promega (Mannheim, Germany) as negative and positive controls, respectively. Cells were incubated for a further 6 h in 21% O_2 and then placed in either 21% O_2 or 85% O_2 for 24 or 48 h, after which cells were stimulated with TGF-β1 (2 ng/ml) or BMP-2 (20 ng/ml) for 12 h. Cells were lysed and processed for determination of firefly luciferase activity exactly as recommended by the manufacturer. To cater for the effects of ligand stimulation and hyperoxia on the baseline transcriptional activity of the cells, values were normalized for the transcriptional activity of the pGL3-control vector.

Assessment of apoptosis and cell-cycle progression by flow cytometry. Identification of apoptotic cells was performed using allophycocyanin-conjugated annexin V (Invitrogen), following the recommendations of the manufacturer, as described previously (45). Necrotic cells were excluded by counter-staining with 2 μg/ml propidium iodide. Cell cycle analysis was performed as described previously (45); briefly, cells were harvested by trypsinization, fixed overnight at 4°C with 75% (vol/vol) ethanol, washed, and incubated in PBS containing 10 μg/ml propidium iodide and 100 μg/ml RNase (Merck Biosciences) for 1 h at 37°C. Data were collected using a FACSCanto flow cytometer and analyzed using a FACSDiva software package (both from BD Biosciences, Heidelberg, Germany). A minimum of 10,000 cells were analyzed per sample. Gates based on forward and side scatter were set to eliminate cellular debris and cell clusters.

Statistical analysis. Values are expressed as means ± SD. Differences between the groups were assessed by one-way ANOVA and a Student-Newman-Keuls test for multiple comparisons, with a P value <0.05 considered significant.

RESULTS

Survival, growth, and respiration of neonatal mice exposed to chronic hyperoxia. For chronic hyperoxia exposure, one group of pups was exposed to 85% O_2 starting at *day P1* and terminating at *day P28*. A second group was exposed to 21% O_2 over the same time period. A 100% survival was recorded for both groups of pups. This high survival contrasts with the poorer survival (60%) observed for FVB/N mice exposed to 85% O_2 between *days P0.5* and *P28* (56), probably reflecting the well-described variability in mouse strain sensitivity to hyperoxia (20).

A marked effect of hyperoxia on growth, as assessed by trends in body mass changes, was observed (Table 2). On *day*

Table 2. *Physiological parameters*

Parameter	21% O_2	85% O_2	P Value
Body mass, g			
day P14	7.2±1.1	6.0±0.9	>0.05
day P28	15.2±0.4	10.2±0.9	<0.01
Dynamic compliance, ml/kPa	5.2±0.3	4.4±0.4	<0.05
Mean linear intercept, μm	20.1±0.6	35.2±3.2	<0.01
Septal thickness, μm	4.4±0.3	5.3±0.2	<0.01

Physiological parameters of mice maintained under 21% O_2 or 85% O_2 assessed at postnatal *day P28* (n = 18 for 21% O_2 and n = 22 for 85% O_2 for all measurements with the exception of dynamic compliance measurements where n = 8 for both groups).

P14, pups exposed to 85% O_2 exhibited a comparable body mass to age-matched pups exposed to 21% O_2 over the same time period (Table 2). However, by *day P28*, pups exposed to 85% O_2 exhibited an average body mass that was 33% lower than that of age-matched pups exposed to 21% O_2 (Table 2).

A marked effect of hyperoxia on dynamic respiratory compliance (C_{dyn}) was also observed, since pups exposed to 85% O_2 between *days P1* and *P28* exhibited a C_{dyn} 25% lower than that observed for age-matched pups exposed to 21% O_2 (Table 2) at *day P28*. Thus, while without effect on survival, exposure to chronic hyperoxia significantly impaired growth and respiratory compliance of neonatal C57BL/6J mice.

Lung morphometry of neonatal mice after exposure to chronic hyperoxia. Alveolar development was impaired in pups exposed to 85% O_2, evident by the enlarged and saccular appearance of air spaces and the reduced number of secondary crests (Fig. 1, *A* and *B*), compared with pups exposed to 21% O_2 (Fig. 1, *C* and *D*). Quantification of these parameters supported our observations. The MLI is inversely proportional to the alveolar surface area. By *day P28*, pups exposed to 85% O_2 exhibited a MLI approximately double that of pups exposed to 21% O_2 (Table 2), indicating a dramatic reduction in alveolar surface area of pups exposed to 85% O_2.

Similarly, mice exposed to 85% O_2 exhibited a significant increase in septal thickness (Table 2), which is typically attributed to the accumulation of extracellular matrix components, namely collagen and elastin fibers, in the interstitium (2). These data indicate that exposure of C57BL/6J neonates to chronic hyperoxia severely impaired alveolarization. This pathology is consistent with morphometric changes observed in FVB/N mice exposed to 85% O_2 (56), as well as in preterm baboon (7) and sheep (1) models of BPD.

Effect of chronic hyperoxia on abundance of TGF-β/BMP signaling molecules in neonatal mouse lungs. Where antibodies were available, lung samples were probed by immunoblotting to investigate whether exposure to 85% O_2 resulted in changes in protein expression between the 85% and 21% O_2 groups. Indeed, pronounced changes were observed for some TGF-β/BMP superfamily receptors (Fig. 2*A*). The abundance of the type I BMP receptors ALK-3 and ALK-6 was reduced (twofold; Fig. 2, *A* and *B*) and increased (fourfold; Fig. 2, *A* and *D*), respectively, particularly at *days P21* and *P28*. The abundance of the type I TGF-β receptor ALK-5 was reduced (fourfold) in the 85% O_2 group (Fig. 2, *A* and *C*), whereas abundance of TβRII, particularly the short isoform (42), was increased (fourfold; Fig. 2, *A* and *E*).

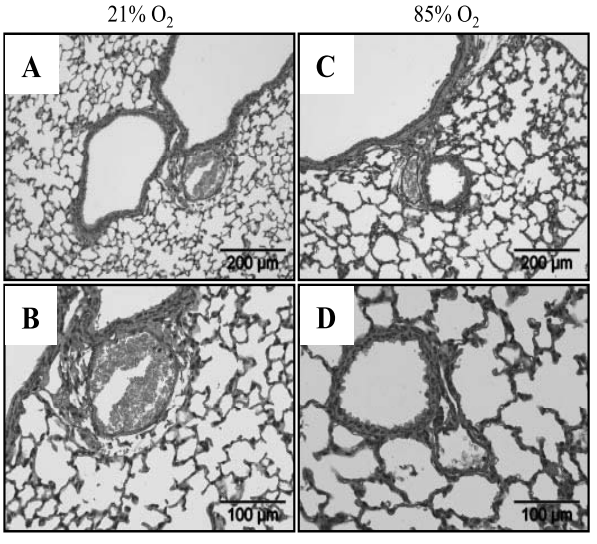

Fig. 1. Chronic hyperoxia impairs alveolarization in neonatal mice. Architectural changes to lung structure were evident in hematoxylin- and eosin-stained lung sections from mice at postnatal *day P28*, after exposure to 21% O_2 (*A–B*) or 85% O_2 (*C–D*).

Temporal changes in the abundance of intracellular signaling components of the TGF-β/BMP system were also observed (Fig. 3*A*). The abundance of Smad1, the key transducer of BMP signals, was increased in the 85% O_2 group. This effect peaked at *day P14* (Fig. 3, *A* and *B*) and remained evident at *day P21*. In contrast, the abundance of Smad3, a transducer of TGF-β signals, was reduced between *days P7* and *P28* (Fig. 3, *A* and *C*). Protein levels of the co-Smad, Smad4, which transduces both TGF-β and BMP signals, were dramatically (sixfold) increased at *days P21* and *P28* (Fig. 3, *A* and *D*). Thus chronic hyperoxia markedly changed the abundance of TGF-β/BMP superfamily proteins in the lungs of neonatal mice.

Protein expression of ALK-1 and BMPRII could not be evaluated by immunoblot, since there are no commercial antibodies available against ALK-1 and a commercial antibody against BMPRII did not work in our hands (data not shown). Therefore, gene expression in the lungs of neonatal mice exposed to 85% or 21% O_2 between *days P1* and *P28* was assessed by semiquantitative RT-PCR (Fig. 2*F*). Levels of mRNA encoding ALK-1 were upregulated in the 85% O_2 group, evident at *days P7*, *P21*, and *P28* (Fig. 2, *F* and *G*). In contrast, with the exception of *day P7*, levels of BMPRII mRNA were unchanged under hyperoxic conditions (Fig. 2, *F* and *H*). Thus, in addition to the changes in TGF-β/BMP superfamily expression observed by immunoblot (Figs. 2*A* and 3), chronic hyperoxia can also alter ALK-1 gene expression in the lungs of neonatal mice. It is important to note, however, that in the hyperoxia-injured lung, mRNA levels do not necessarily correlate with protein abundance. Indeed, for some proteins, such as surfactant protein D, mRNA levels and protein abundance are inversely correlated (57). Therefore, these RT-PCR data should be interpreted with caution.

Effect of chronic hyperoxia on localization of TGF-β/BMP superfamily proteins in neonatal mice. In pups exposed to both 85% O_2 and 21% O_2, ALK-3 staining was evident in both the airway epithelium and septae (Fig. 4*A*). The ALK-6 staining, which was evident in the septae as well as in the airway epithelium of pups exposed to 21% O_2, was more intense in lungs of pups exposed to 85% O_2 (Fig. 4*A*). Similarly, whereas little or no staining for TβRII was evident in lungs exposed to 21% O_2, staining (particularly in the airway subepithelial layer) was evident after exposure to 85% O_2 (Fig. 4*A*). These trends are consistent with a fourfold elevation in ALK-6 (Fig. 2, *A* and *D*) and TβRII (Fig. 2, *A* and *E*) protein abundance in hypoxia-exposed lungs assessed by immunoblot.

Protein abundance of Smad1 in the lungs of pups at *day P28* was similar in the 21% and 85% O_2 groups (although it was significantly elevated at *days P14* and *P21*; Fig. 3, *A* and *B*). Consistent with these data, Smad1 staining, evident in the endothelial layer, as well as in the airway epithelium, was of a similar intensity in lung sections from pups exposed to 21% and 85% O_2 (Fig. 4*B*). In contrast, strong Smad3 staining was

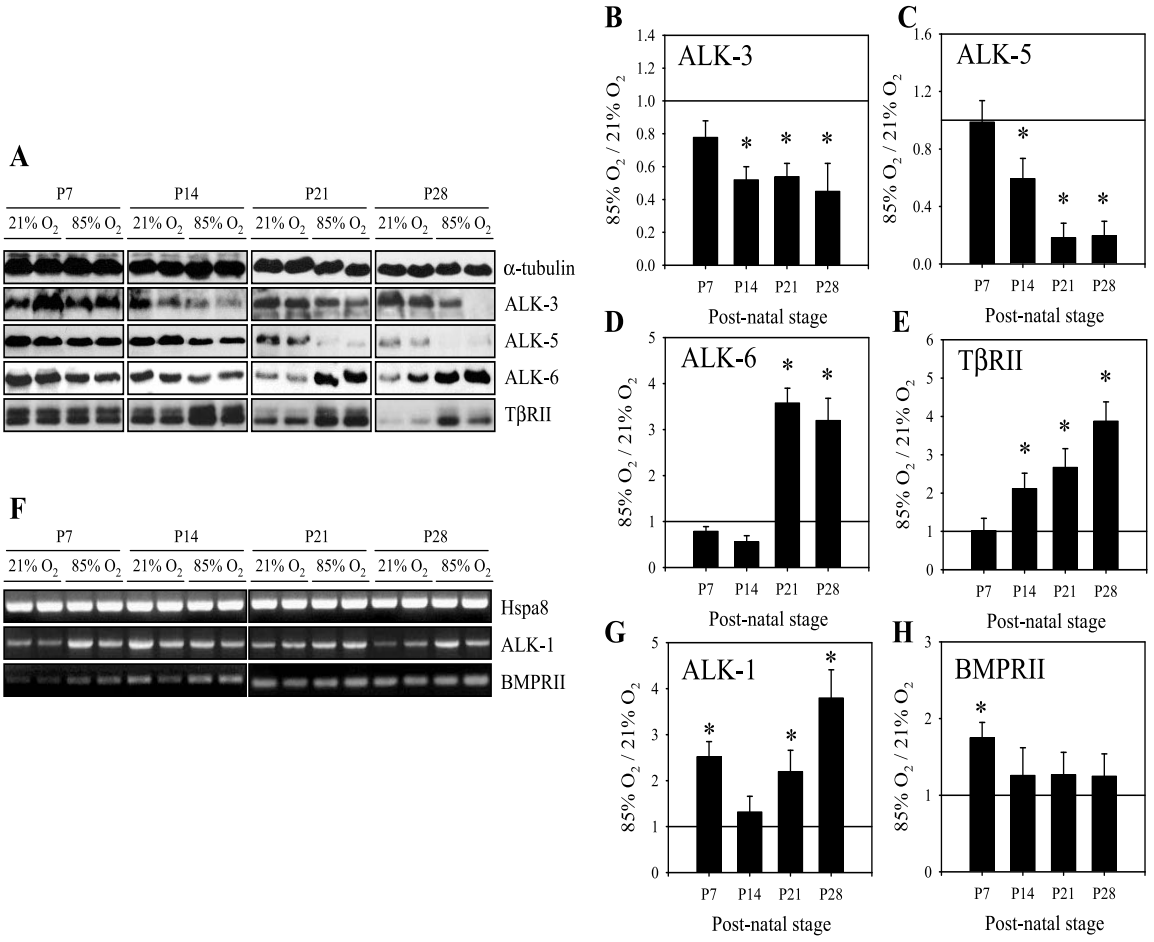

Fig. 2. Chronic hyperoxia alters expression of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) superfamily receptors in neonatal mice. *A*: representative immunoblots illustrating expression of the TGF-β/BMP superfamily receptors ALK-3, ALK-5, ALK-6, and type II TGF-β receptor (TβRII) in lungs extracted at *days P7*, *P14*, *P21*, and *P28* from neonatal mice exposed to 21% O_2 or 85% O_2 from *day P1*. The α-tubulin served as a loading control. Immunoblot data were quantified for ALK-3 (*B*), ALK-5 (*C*), ALK-6 (*D*), and TβRII (*E*); $n = 3–6$ for each bar. $^*P < 0.05$. Changes in expression of ALK-1 and BMP type II receptor (BMPRII) were assessed at the mRNA level because appropriate antibodies were not available. Total lung RNA was extracted, and RT-PCR analysis was performed as described in MATERIALS AND METHODS, to detect genes encoding ALK-5 and BMPRII. A representative ethidium bromide-stained agarose gel is illustrated in *F*, showing PCR amplicons obtained from RNA extracted at *days P7*, *P14*, *P21*, and *P28* from neonatal mice exposed to 21% O_2 or 85% O_2 from *day P1*. The *hspa8* gene was used to demonstrate RNA equivalence in the RT reactions. Quantification of RT-PCR data is illustrated for ALK-1 (*G*) and BMPRII (*H*); $n = 4–6$ for each bar. $^*P < 0.05$.

evident in the airway epithelium and the septae in the lungs of pups exposed to 21% O_2 (Fig. 4*B*), although this staining was less intense in the lungs of 85% O_2-exposed pups (Fig. 4*B*), reflecting the twofold decrease in Smad3 protein abundance evident in immunoblots (Fig. 3, *A* and *C*). A similar correlation was observed in the case of Smad4, which exhibits a dramatic sixfold increase in protein abundance in the lungs of 85% O_2-exposed pups (Fig. 3, *A* and *D*). Smad4 staining was localized primarily to the endothelium in the lungs of pups exposed to 21% O_2 (Fig. 4*B*). After exposure to 85% O_2, staining was more intense and was now also evident in the airway epithelium and the alveolar septae (Fig. 4*B*). Together, these data indicate that chronic exposure changes both the protein abundance and localization of components of the TGF-β/BMP superfamily.

Effect of chronic hyperoxia on TGF-β/BMP signaling in neonatal mouse lungs. To address whether the dramatic alterations in the expression of TGF-β and BMP signaling molecules we observe actually translate to altered TGF-β and BMP signaling in the neonatal mouse lung in response to high oxygen concentrations, we assessed the expression of two TGF-β responsive genes, PAI-1 and CTGF, as well as three BMP-responsive genes, encoding Id1, Id2, and Id3. The PAI-1 mRNA expression was consistently upregulated in lungs of pups exposed to 85% O_2, in which CTGF mRNA levels were also prominently upregulated, particularly at *days P14* and *P21* (Fig. 5*A*). Whereas no quantitative statement could be made regarding Id1 mRNA levels, the mRNA levels of Id2 and Id3 were decreased, notably at *days P14* and *P21*, in lungs of pups exposed to 85% O_2 (Fig. 5*A*). These data suggested that TGF-β signaling was enhanced, whereas BMP signaling was dampened in lungs of pups exposed to 85% O_2. In further support of this idea, we assessed the degree of phosphorylation of Smad1 and Smad2, which are specific transducers of BMP and TGF-β signals, respectively. Indeed, at *day P14*, exposure to 85% O_2 promoted both a significant decrease in Smad1 phosphorylation and a significant increase in Smad2 phosphorylation (Fig. 5, *B* and *C*). By *day P28*, Smad1 phosphorylation was restored

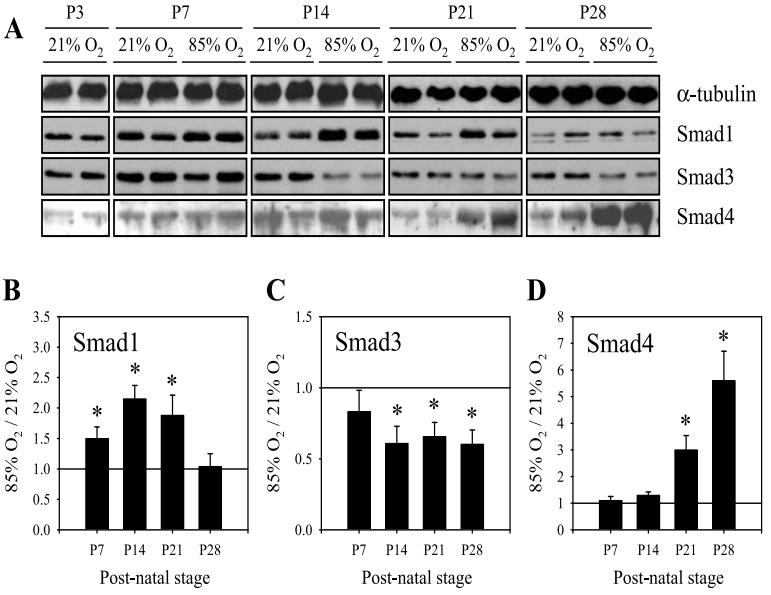

Fig. 3. Chronic hyperoxia alters expression of Smad proteins in neonatal mice. *A*: representative immunoblots illustrating the expression of BMP-specific Smad1, TGF-β-specific Smad3, and the co-Smad, Smad4, in lungs extracted at *days P7*, *P14*, *P21*, and *P28*, from neonatal mice exposed to 21% O_2 or 85% O_2 from *day P1*. The α-tubulin served as a loading control. Immunoblot data were quantified for Smad1 (*B*), Smad3 (*C*), and Smad4 (*D*); $n = 4–6$ for each bar. $^*P < 0.05$.

to levels observed; however, Smad2 phosphorylation remained elevated in the 85% O_2 group (Fig. 5, *B* and *C*). Together, these data indicate that TGF-β signaling is most likely enhanced, whereas BMP signaling is decreased, in lungs from pups exposed to 85% O_2.

Effect of hyperoxia on TGF-β/BMP signaling in epithelial and fibroblast cell-lines. Since the impaired TGF-β/BMP signaling we observed in the lungs of pups may have been attributable to the undernourishment (clearly evident by reduced body mass) of pups exposed to 85% O_2, we sought to validate our observations in vitro in cell culture. Therefore, TGF-β and BMP signaling was assessed in NIH/3T3 (fibroblast) and MLE-12 (epithelial) cells, using a luciferase-based transcriptional reporter assay, employing the TGF-β-responsive reporter $p(CAGA)_{12}$ or the BMP-responsive reporter pId120. Baseline transcriptional activity of the cells was al-

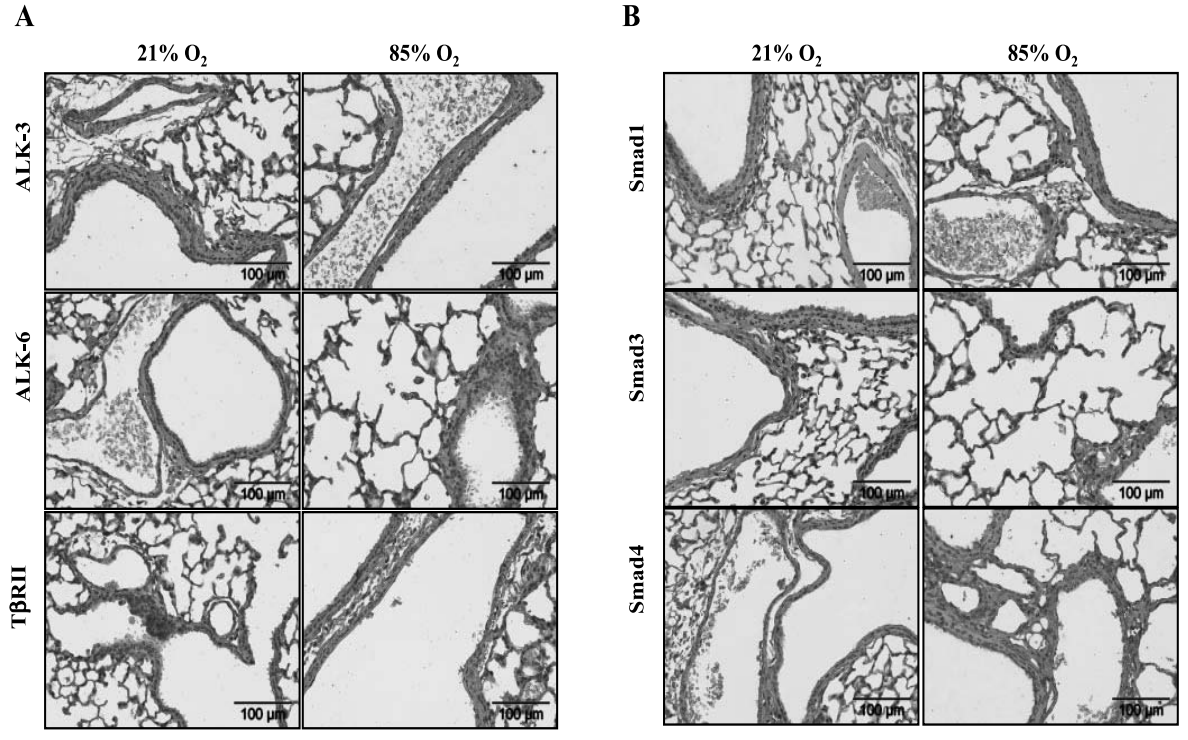

Fig. 4. Immunohistochemical localization of TGF-β/BMP superfamily receptor and Smad proteins in the lungs of neonatal mice chronically exposed to hyperoxia. *A*: representative fields illustrating the expression patterns of TGF-β/BMP system receptors ALK-3, ALK-6, and TβRII in lungs extracted on *day P28* from neonatal mice exposed to 21% O_2 (*left* column) or 85% O_2 (*right* column) from *day P1*. *B*: representative fields illustrate the expression patterns of BMP-specific Smad1, TGF-β-specific Smad3, and the co-Smad, Smad4, in lungs extracted on *day P28* from neonatal mice exposed to 21% O_2 (*left* column) or 85% O_2 (*right* column) from *day P1*.

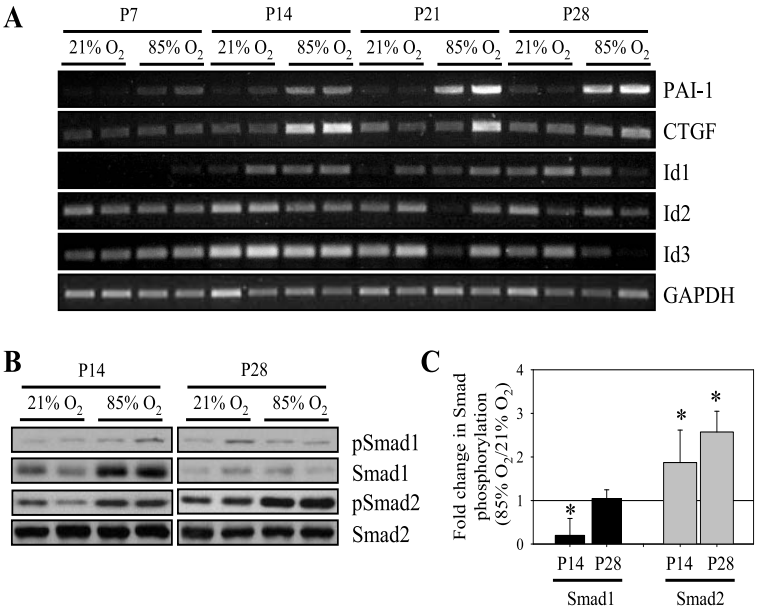

Fig. 5. Induction of TGF-β/BMP signaling in the lungs of neonatal mice chronically exposed to hyperoxia. *A*: expression of mRNA of the TGF-β-inducible genes encoding plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF), and the BMP-inducible genes encoding inhibitor of differentiation 1 (Id1), Id2, and Id3, in lungs extracted at days *P7*, *P14*, *P21*, and *P28* from neonatal mice exposed to 21% O_2 or 85% O_2 from *day P1*. The mRNA expression was assessed by semiquantitative RT-PCR. The *gapdh* gene was used to demonstrate RNA equivalence in the RT reactions. Baseline phosphorylation of Smad proteins was also assessed in lungs extracted at *days P14* and *P28* from neonatal mice exposed to 21% O_2 or 85% O_2 from *day P1* (*B*). Data were quantified by densitometry, by comparing the ratio of phospho-Smad (pSmad) to total Smad in hyperoxic vs. normoxic lungs (*C*). The fold change in Smad phosphorylation was calculated by [pSmad/total Smad (85% O_2)]/[pSmad/total Smad (21% O_2)]; $n = 3–4$ for each bar. $^*P < 0.05$.

tered by hyperoxia (~11% reduced) and TGF-β1 (reduced by as much as 17%), although not by BMP-2, as assessed by changes in the expression of the constitutively active pGL3-control construct (data not shown). Therefore, induction of transcription was normalized for pGL3-control transcriptional activity. Exposure of both MLE-12 and NIH/3T3 cells to 85% O_2 for 24 or 48 h dramatically (3- to 4-fold) increased their sensitivity to TGF-β1 stimulation (Fig. 6, *A* and *B*). In contrast, MLE-12 cells were poorly responsive to BMP-2 stimulation, and MLE-12 responsiveness to BMP-2 was not affected by elevated oxygen concentrations (Fig. 6*C*). NIH/3T3 cells were responsive to BMP-2 stimulation, and after 48 h of exposure to 85% O_2, the responsiveness of NIH/3T3 cells to BMP-2 was significantly decreased (Fig. 6*D*). These data nicely support our contention that hyperoxia has opposing effects on TGF-β and BMP signaling, where TGF-β signaling is enhanced and BMP signaling is dampened.

Effect of hyperoxia on TGF-β- and BMP-induced apoptosis of ATII and PASMC. The hyperoxia-induced apoptosis of ATII cells is believed to be an important factor in oxygen toxicity

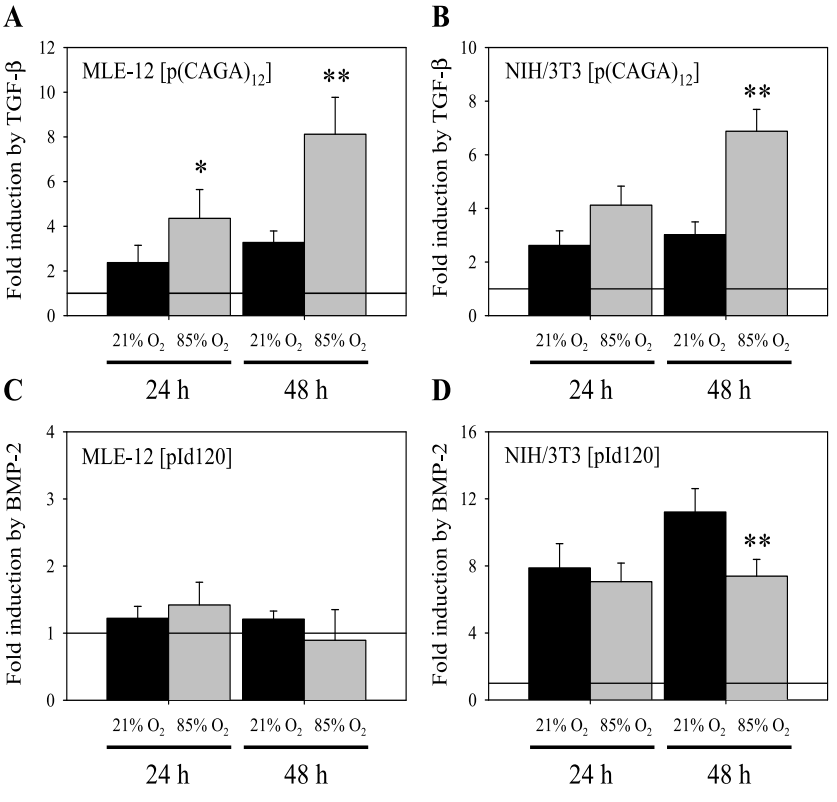

Fig. 6. Effect of hyperoxia on TGF-β and BMP signaling assessed by a luciferase reporter assay. Mouse lung epithelial-12 (MLE-12) (*A* and *C*) or NIH/3T3 (*B* and *D*) cells were transfected with the TGF-β-responsive reporter $p(CAGA)_{12}$ (*A* and *B*) or the BMP-responsive reporter pID120 (*C* and *D*) and maintained in 21% O_2 (black bars) or 85% O_2 (gray bars) for 24 or 48 h prior to stimulation with TGF-β (2 ng/ml) or BMP-2 (20 ng/ml) for 12 h. Ligand-inducible luciferase activity was normalized for luciferase activity of the constitutively active pGL3-control vector to account for effects of hyperoxia or TGF-β/BMP ligands on baseline transcriptional activity. Determinations were made for 3 separate experiments, each in quadruplicate. $^*P < 0.05$; $^{**}P < 0.01$.

and the development of BPD (10). Since TGF-β (27) and BMP ligands (31) have both been ascribed with proapoptotic activity, we sought to determine whether exposure of ATII cells and PASMC altered their sensitivity to TGF-β- or BMP-induced apoptosis. Primary ATII cells and primary PASMC were exposed to 21% O_2 or 85% O_2 for 24 h prior to stimulation with TGF-β1 or BMP-2, or addition of vehicle alone in the case of control experiments, after which cells were assessed for apoptosis after an additional 24 or 48 h. No significant differences in the percentage of apoptotic cells were observed between control and BMP-2-stimulated groups. However, both 24 and 48 h after TGF-β1 stimulation, the percentage of apoptotic cells in the 85% O_2 group was significantly increased compared with the 21% O_2 group (Fig. 7, *A* and *C*). In contrast, neither TGF-β1 nor BMP-2 induced apoptosis in PASMC, irrespective of the oxygen concentration. These data indicated that exposure of ATII cells to 85% O_2 for as little as 24 h could abnormally increase the sensitivity of ATII cells to TGF-β-induced apoptosis.

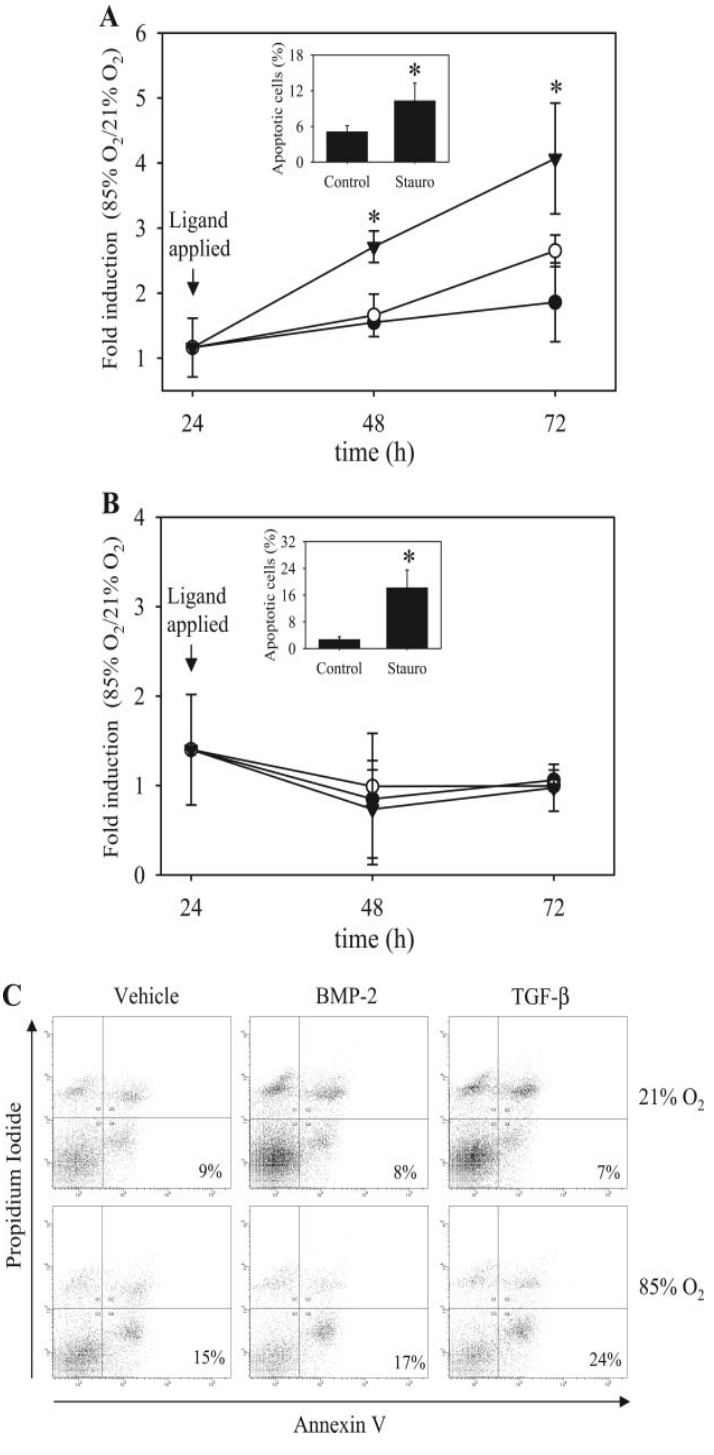

Fig. 7. Effect of hyperoxia on TGF-β- and BMP-induced apoptosis of primary alveolar type II (ATII) cells and primary pulmonary artery smooth muscle cells (PASMC). Primary ATII cells (*A*) and primary PASMC (*B*) were maintained under 21% O_2 or 85% O_2 for 24 h prior to stimulation with vehicle [0.8% (mass/vol) NaCl] alone (●), TGF-β (2 ng/ml; ▼), or BMP-2 (100 ng/ml; ○). Cells were examined for induction of apoptosis by flow cytometry 24 and 48 h after ligand stimulation. Data represent the mean fold change in the percentage of apoptotic cells in the 85% O_2 group vs. the percentage of apoptotic cells in the 21% O_2 group at the time points indicated ± SD ($n = 3$). *$P < 0.05$. Staurosporine (Stauro; 100 nM, 24 h) was employed as a positive control for induction of apoptosis in both cells types in 21% O_2 (*insets*). *C*: original flow cytometry scattergrams from a 72-h time point of an arbitrarily selected representative experiment with ATII cells.

Effect of hyperoxia on TGF-β- and BMP-induced proliferation of ATII and PASMC. Since both TGF-β and BMP ligands can have pro- and antiproliferation activity in variety of cell types (27, 31), we assessed the effects of TGF-β1 and BMP-2 on the proliferation of ATII cells and PASMC after exposure to 85% O_2. Primary ATII cells and primary PASMC were exposed to 21% O_2 or 85% O_2 for 24 h prior to stimulation with TGF-β1 or BMP-2, or addition of vehicle alone in the case of control experiments, after which cells were assessed for apoptosis after an additional 48 h (Fig. 8). No significant changes in cell proliferation were observed, for ATII cells or for PASMC, for either ligand.

Effect of hyperoxia on TGF-β- and BMP-induced extracellular matrix proteins by lung fibroblasts. The production of ECM and ECM-remodeling enzymes (and their inhibitors) by interstitial fibroblasts is a key step in the alveolarization process, and expression of many of these proteins may be stimulated by TGF-β. Exposure of primary lung fibroblasts to 85% O_2 increased the basal expression of both Smad4 and TβRII and increased the basal phosphorylation of Smad2, as assessed by immunoblot (Fig. 9*A*). These data are consistent with our observations made in whole lung extracts from pups chronically exposed to 85% O_2.

Exposure of fibroblasts to 85% O_2 also significantly altered the basal mRNA levels of several important ECM or ECM-remodeling proteins: mRNA levels for the α_1 chains of collagens I and III as well as tropoelastin and tenascin-C were reduced, whereas tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA levels were increased, and matrix metalloproteinase (MMP)-1, MMP-2, and TIMP-3 mRNA levels were unchanged (Fig. 9*B*).

We then assessed whether exposure to 85% O_2 could alter the induction of these genes by TGF-β1 (2 ng/ml) or BMP-2, BMP-4, or BMP-7 (all at 100 ng/ml). The α_1 chains of collagens I and III, and MMP-1, MMP-2, and TIMP-3 were not TGF-β-inducible in 21% O_2, which is consistent with other reports (21) illustrating that none of these genes are normally TGF-β inducible in fibroblasts, in contrast, for example, to collagens IV, VIII, and XV. However, levels of mRNA encoding TIMP-1 (Fig. 9*G*), tropoelastin (Fig. 9*I*), and tenascin-C (Fig. 9*J*) were significantly increased in the presence of TGF-β in 21% O_2. Of particular interest to our study, the ability of TGF-β to increase mRNA levels of TIMP-1 and tropoelastin was significantly enhanced after 24 h of exposure to 85% O_2 (Fig. 9, *G* and *J*). Of further interest, collagen I mRNA levels were increased by TGF-β in 85% O_2 but not 21% O_2 (Fig. 9*C*). Levels of tenascin-C mRNA were also increased by TGF-β in 85% O_2 (Fig. 9*J*); however, this increase was not significantly different with respect to that observed in 21% O_2. With the exception of a small (but significant) decrease in collagen I mRNA levels in response to BMP-7 (Fig. 9*C*), none of the genes we investigated were regulated by BMP (Fig. 9, *C–J*). In sum, our data indicate that exposure of primary lung fibroblasts to 85% O_2 significantly increases the capacity of TGF-β to upregulate levels of mRNA encoding extracellular matrix components.

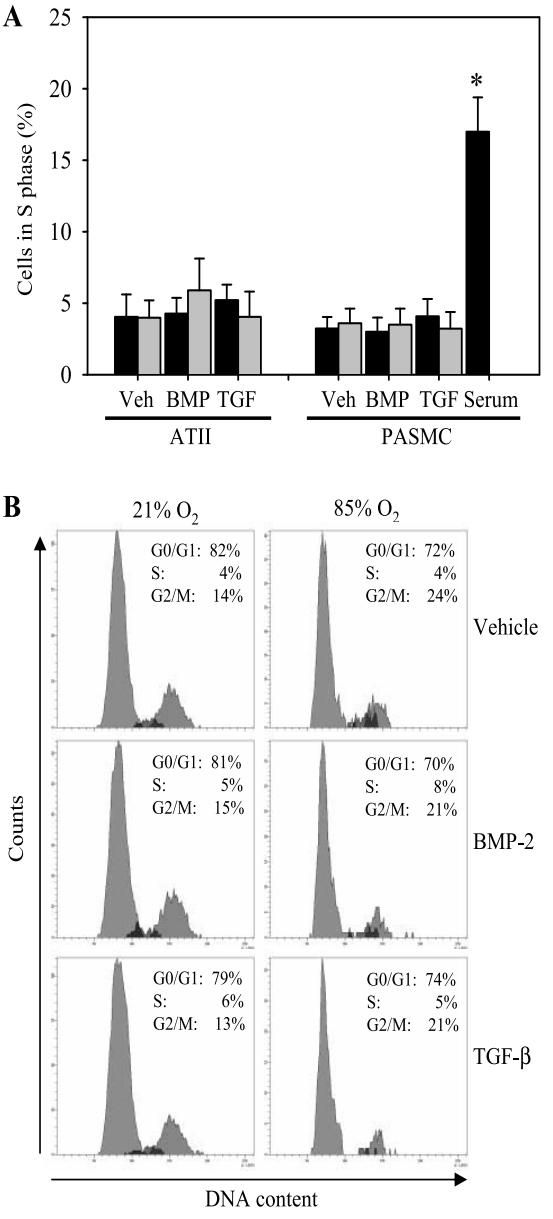

Fig. 8. Effect of hyperoxia on TGF-β- and BMP-induced proliferation of primary ATII cells and primary PASMC. *A*: primary ATII cells and primary PASMC were maintained under 21% O_2 (black bars) or 85% O_2 (gray bars) for 24 h prior to stimulation with vehicle [Veh; 0.8% (mass/vol) NaCl] alone, TGF-β (2 ng/ml), or BMP-2 (100 ng/ml). Cell-cycle analysis of the cell populations was undertaken by flow cytometry 48 h after ligand stimulation. The proportion of cells in S phase was taken as a measure of cell proliferation. Data represent the mean fold change in the percentage of cells in S phase in the 85% O_2 group vs. the percentage of cells in S phase in the 21% O_2 group ± SD ($n = 3$). *$P < 0.05$. In the case of PASMC, analyses were made in the presence of 0.5% (vol/vol) serum, whereas 5% (vol/vol) serum was employed as a positive control for the induction of proliferation in 21% O_2. *B*: original flow cytometry histograms from an arbitrarily selected representative experiment with ATII cells.

DISCUSSION

Alveolarization begins with branching of distal airway saccules into immature alveoli, in the canalicular phase of lung development, beginning on embryonic *day E16* in mice, and continues through the saccular phase and into the alveolar phase, occurring between *days P5* and *P21* (9). During this time, ATII cells proliferate and differentiate into type I cells. Alveolar septae divide the terminal respiratory saccules, increasing the number of alveoli (9). The septae are supported by

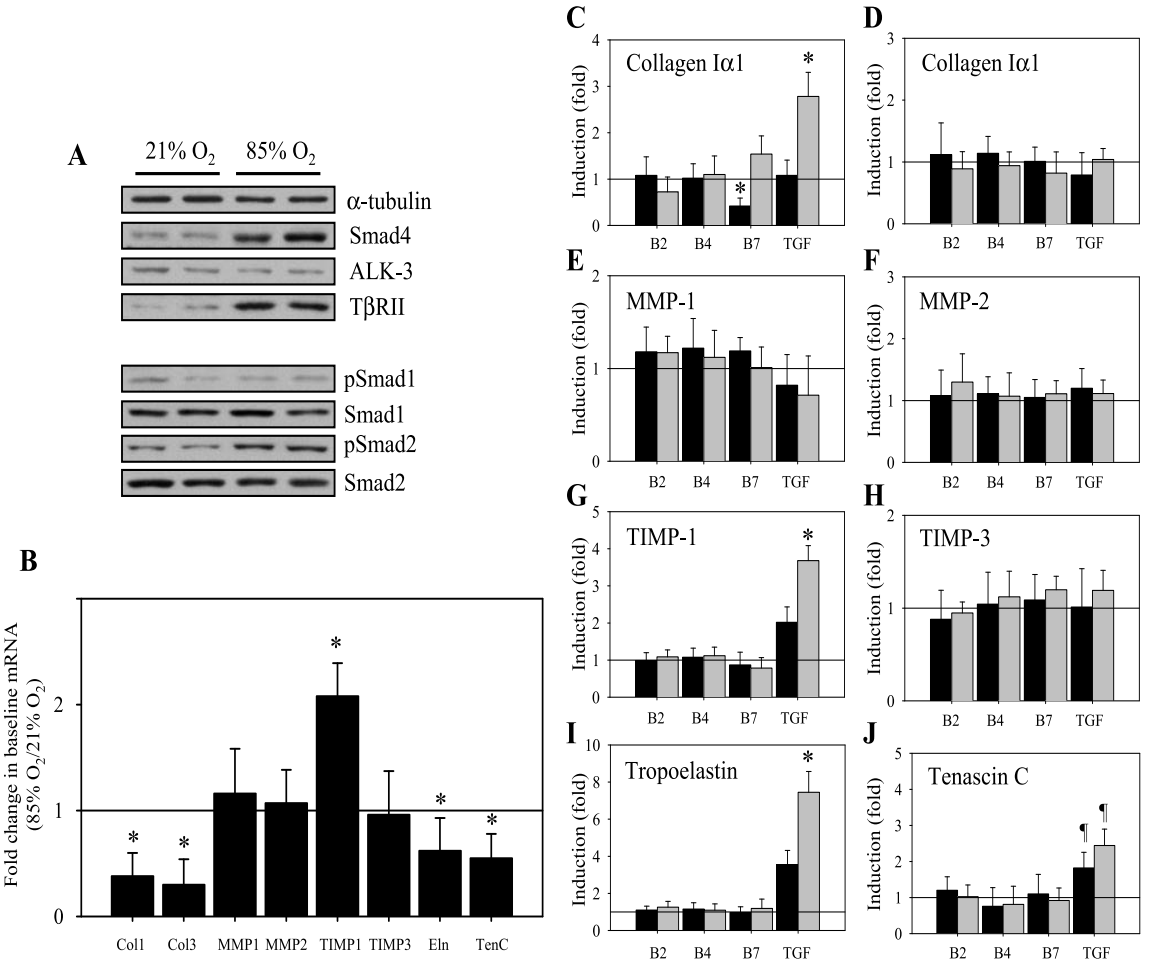

Fig. 9. Effect of hyperoxia on TGF-β- and BMP-induced expression of extracellular matrix components by primary lung fibroblasts. *A*: basal expression of components of the TGF-β/BMP signaling machinery in primary lung fibroblasts cultured for 48 h in 21% O_2 and 85% O_2. *B*: fold change induced by 85% O_2 after 72 h in the basal mRNA levels of genes encoding collagen Iα_1-chain (Col1), collagen III α_1-chain (Col3), matrix metalloproteinase-1 (MMP-1), MMP-2, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-3, tropoelastin (Eln), and tenascin-C (TenC) in primary lung fibroblasts are illustrated. Data represent the mean fold change in cycle threshold (ΔC_t) assessed by real-time RT-PCR in fibroblasts cultured in 85% O_2 for 72 h vs. fibroblasts cultured in 21% O_2 for 72 h ± SD ($n = 3$). *$P < 0.05$. *C*: the induction of these 8 genes by TGF-β1 (2 ng/ml) or BMP-2 (B2), BMP-4 (B4), or BMP-7 (B7) (all at 100 ng/ml), in fibroblasts cultured in 21% O_2 (black bars) or 85% O_2 (gray bars) for 24 h prior to a 48-h stimulation, are illustrated in *C–J*. Fold changes were calculated by: $[(\Delta C_{tHYX,stim}/\Delta C_{tHYX,unstim})/(\Delta C_{tNOX,stim}/\Delta C_{tNOX,unstim})]$, where HYX denotes hyperoxia (85% O_2); NOX, normoxia (21% O_2); stim, ligand-stimulated; and unstim, unstimulated. Data represent the mean fold change ± SD ($n = 3$). *$P < 0.05$, 21% O_2 vs. 85% O_2 groups; ¶$P < 0.05$, induced vs. noninduced groups at the same O_2 concentration.

the ECM, composed of a collagen scaffold, to which glycoproteins (like tenascin) and fibrinous proteins (like elastin) are interwoven (41). The ECM is deposited by fibroblasts and is continuously remodeled by matrix remodeling proteases (41). Epithelial growth occurs concomitantly with that of lung capillaries (46). This alveolarization process is mediated by paracrine, autocrine, and juxtacrine communication between the epithelium and endothelium, and their associated fibroblasts, undertaken by signaling molecules including TGF-β and BMP (17).

Injury to the developing lung, like oxygen toxicity, may disrupt critical signaling pathways that regulate lung development, leading to developmental arrest. In the case of premature infants, who usually present during the canalicular stage, a pronounced arrest of alveolarization is observed culminating in a pathology typical of BPD (2). In this study, we investigated whether exposure of neonatal mice to hyperoxia during the critical alveolarization period had any effect on the expression and function of the TGF-β/BMP system, a key signaling pathway in lung development. Elevated levels of TGF-β ligands have been detected in neonates with BPD undergoing oxygen therapy (24) and in animal models of BPD (5). Furthermore, exposure of ATII cells to 95% O_2 promotes association of Smad2/3/4 complexes with DNA (34), indicating that hyperoxia upregulated TGF-β signaling. However, no study to date has addressed the effect of hyperoxia on the expression of nonligand components of the TGF-β/BMP signaling pathways. The results that we present in the current study indicate that temporal changes in the expression and localization of key signaling components of the TGF-β/BMP system do occur under hyperoxic conditions. Our data clearly indicate that hyperoxia can "tweak" TGF-β/BMP signaling, and that TGF-β-mediated effects are generally potentiated by hyperoxia, whereas BMP signaling is generally dampened.

How could dysregulated TGF-β/BMP signaling arrest alveolarization? TGF-β has potent antiproliferative properties on epithelial cells (43, 58) and some types of smooth muscle cells

(45). The proliferation and differentiation of ATII cells are key steps in the alveolarization process. It is known that TGF-β can arrest proliferation of ATII cells (43) and prevent keratinocyte growth factor-stimulated ATII cell proliferation (58). We demonstrated in our study that after exposure to 85% O_2 for as little as 24 h, primary mouse ATII cells are significantly more sensitive to the proapoptotic effects of TGF-β, although primary smooth muscle cells derived from the pulmonary artery remained unaffected. Considering these data, it may well be that enhanced TGF-β signaling in the alveolar epithelium after exposure to hyperoxia promotes apoptosis (and thus prevents proliferation and differentiation) of ATII cells, thereby contributing to the hypoplasia associated with BPD (2).

We report in our study that neither TGF-β nor BMP-2 promoted the proliferation of ATII cells or PASMC. Although BMPs are accredited with pro-proliferative properties (31), it is noteworthy that we observe a downregulation of ALK-3 and an upregulation of ALK-6 at the protein levels in pups exposed to hyperoxia, since these two receptors have opposing functions: ALK-3 promotes proliferation, whereas ALK-6 is antiproliferative, promoting mitotic arrest and apoptosis (38). These data would suggest that hyperoxia promotes a shift away from ALK-3-mediated proliferation, in favor of ALK-6-mediated growth arrest. Indeed, we did observe increased ATII cell apoptosis in the presence of BMP-2, although the extent was not statistically significant with respect to vehicle controls.

In the context of cell proliferation, the abnormally enhanced TGF-β signaling during exposure to hyperoxia together with the elevated ALK-1 levels we have observed are noteworthy, since ALK-1 is primarily expressed in the endothelium, and ALK-1 activity inhibits proliferation of endothelial cells by inducing G_0/G_1 arrest (23). These two phenomena could, in part, underlie the capillary hypoplasia observed in animal models of BPD (8). However, since we have not focused on endothelial cells in this study, this idea remains entirely speculative.

In addition to a direct effect on cell proliferation and apoptosis, dysregulation of TGF-β signaling would also impact ECM deposition and remodeling, another key step in the alveolarization process (41). TGF-β regulates the secretion of some matrix-metabolizing enzymes: the MMPs and their cognate inhibitors, TIMPs. Throughout the canalicular, saccular, and alveolar phases of normal lung development, MMP-1, MMP-2, MMP-9, and TIMP-2 are strongly expressed in humans (29) and mice (44), and during normal development, MMP-9 expression peaks during alveolarization (5), indicating the importance of matrix remodeling in this process. In our study, we have illustrated that TGF-β stimulation dramatically elevates levels of mRNA encoding TIMP-1 in fibroblasts cultured in 85% O_2, compared with fibroblasts cultured in 21% O_2, whereas mRNA levels of MMP-1 and MMP-2 were either unaffected or decreased. These data are consistent with reports that TIMP-1 protein expression is increased in rat (16) and mouse (5) models of hyperoxia-induced arrest of alveolarization, although no changes in MMP-2 levels were observed. These data are also consistent with reports of elevated TIMP-1 levels in BPD (12).

TGF-β regulates secretion of some components of the ECM, including collagens, elastin, and tenascin-C (41), and their deposition plays a key role in alveolarization. TGF-β can stimulate collagen secretion by primary lung fibroblasts (13) and upregulates expression of collagen (27), elastin (30), and tenascin and fibronectin (59) in lung tissue. In our study, we have illustrated that when fibroblasts are cultured in 85% O_2, levels of mRNA encoding collagen $I\alpha_1$, tropoelastin, and tenascin-C in the primary lung fibroblasts are abnormally increased in response to TGF-β stimulation. Taken together, our data collectively suggest that abnormal upregulation of the TGF-β system in the lung upon exposure to hyperoxia would swing the balance in favor of interstitial ECM deposition and would prevent turnover or breakdown of ECM components. This is consistent with the increased thickening of alveolar septa observed in this and other studies (1, 7), as well as the detection of increased amounts of ECM components in the interstitium in BPD (35, 48) and in several animal models of BPD (1, 7, 39).

In sum, we illustrate in our study that hyperoxia dysregulates both the expression of components of the TGF-β/BMP signaling machinery and TGF-β and BMP signaling per se. We have further demonstrated that this dysregulated signaling has at least two functional consequences relevant to hyperoxia-induced arrest of alveolarization and BPD: *1*) hyperoxia increases the sensitivity of ATII cells to TGF-β-induced apoptosis, and *2*) hyperoxia modulates the expression of ECM and ECM-remodeling components induced by TGF-β in fibroblasts.

The ability of all-*trans* retinoic acid (RA) to rescue failed septation (28) and attenuate oxygen-induced inhibition of lung septation (50) lends credence to our hypothesis. RA is a TGF-β antagonist, since RA prevented TGF-β-stimulated ECM production by lung fibroblasts (40), downregulates TGF-β receptor expression (32), and reverses hyperoxia-induced cell-cycle arrest (34). It may well be that RA exerts its protective effects by downregulating TGF-β signaling, which we illustrate in our study is abnormally upregulated by hyperoxia.

BPD is a significant complication of premature birth, affecting up to 10,000 newborns annually, and has long-term respiratory consequences that reach beyond childhood (47). Intensive research is currently encouraged to delineate signaling pathways that underlie the alveolar and capillary hypoplasia that are the hallmarks of "new BPD" (18). Such research has recently paid off, with the discovery that lung-specific vascular endothelial growth factor gene transfer restored alveolar development in a hyperoxia model of BPD (47). The data we present in the current study implicate a second growth factor signaling pathway, that of the TGF-β/BMP superfamily, in the pathogenesis of BPD. It should be emphasized that our data do not unequivocally demonstrate a causal effect between dysregulated TGF-β signaling and BPD. However, the elevated TGF-β signaling observed in the lungs of neonates exposed to hyperoxia, together with the increased sensitivity of lung fibroblasts and epithelial cells to TGF-β after hyperoxic exposure, are consistent with the increased epithelial cell apoptosis and elevated ECM deposition observed in BPD. Given that the TGF-β/BMP pathways are also amenable to pharmacological and genetic manipulation in the lung, they may provide alternative avenues for the management of this debilitating disorder.

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chapter 5

Hyperoxia-induced injury in late lung development: time-dependent expression of inflammatory and oxygen-regulated genes and their interactions in mice

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Submitted

Abstract

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of early infancy caused by oxidative damage and/or mechanical ventilation that is characterized by impaired pulmonary and vascular development. Angiogenic factors, inflammatory cytokines and the nitric oxide (NO) system have been implicated in the pathogenesis of BPD. All of these factors play important and interrelated roles in normal late lung development. However, little is known about their interactions in BPD. Using a rodent model of hyperoxia-induced BPD, we demonstrated a pronounced dysregulation of expression of a number of these factors during the period of alveolarization and vascularization. Significantly altered expression at different postnatal ages was shown for a selected number of oxygen-regulated factors (up-regulation of hypoxia-inducible factor (HIF)-1α and down-regulation of HIF-2α), angiogenic factors (downregulation of vascular endothelial growth factor (VEGF) and its receptor Flt-1), and components of the NO biosynthetic pathway (up-regulation of nitric oxide synthase (NOS)-2, downregulation of NOS-3 and dimethylarginine dimethylaminohydrolase (DDAH)). Expression of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 was up-regulated, and surfactant proteins (SP)-B and -C were down-regulated. The altered expression of these molecules and their interactions may contribute to our knowledge of the pathogenesis of BPD and identify potential therapeutic targets. Also, it may help define the optimal time point for intervention.

INTRODUCTION

Postnatal lung growth and development are dependent upon angiogenesis and alveolarization, two integrated processes which continue over the first two years of *post natal* life in humans, and three to four weeks *post natal* in mice.[1,2] Preterm birth interrupts normal pulmonary alveolar and vascular development, and leads to long-term abnormalities in pulmonary growth and function. Prematurely born neonates often require oxygen therapy and/or mechanical ventilation due to respiratory insufficiency and hypoxemia. However, both oxygen therapy and mechanical ventilation contribute to lung injury through the release of toxic free radicals, inflammatory cytokines, and by disrupting the production of critical growth factors and other signalling molecules in the lung.[3]

Given that embryonic lung development occurs in a relatively hypoxic environment, HIF, which mediate cellular responses to oxygen levels, have been accredited with key roles in lung development.[4,5] This idea was confirmed by the observation that activation of HIF through inhibition of prolyl hydroxylases restored normal lung development. The function of HIF has been tightly coupled to NOS activity. NOS is responsible for NO production, and is abundant in epithelial and endothelial cells during fetal development. Indeed, abrogation of NO signalling causes alveolar simplification and impaired pulmonary vascular growth. Other factors also impact NO synthesis, and hence, HIF function, in the developing lung.[6] For example, TNF-α is reported to decrease the expression of endothelial NOS (eNOS, NOS-3), but increases expression of inducible NOS (iNOS, NOS-2) in cultured endothelial cells.[7]

Hypoxia-inducible factors also form an important link between NOS activity and that of angiogenic growth factors, such as VEGF and its receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1). The VEGF family of growth factors is believed to have a key role in late lung development with NO expression up-regulating VEGF by stabilizing HIF-1α mRNA and protein.[8,9] Thus, HIF, NOS, and VEGF signalling networks appear to be intimately coupled in driving alveolar and pulmonary vascular development.[10]

Various attempts to prevent hyperoxic lung injury have revealed that restoration of any one of the HIF, NOS or VEGF systems can partially ameliorate the deleterious effects of hyperoxia on late lung development.[11] However, no intervention to date has been able to fully abrogate the development of BPD. Therefore, we have compared the expression of essential components of all three systems (HIF, NOS and VEGF), as well as two pro-inflammatory cytokines (TNF-α and IL-6), as an effort to understand how dysregulated expression of individual components may perturb inter-pathway cross-talk in hyperoxia-induced arrest of alveolar development in mice.

MATERIAL AND METHODS

Animals

Mice (C57BL/6) were housed in humidity- and temperature-controlled facilities in 12 h light-dark cycle and were allowed food and water ad libitum. On the first day after birth (P1), pups and nursing dams were either maintained under constant hyperoxic conditions (85% O_2) or kept in room air (normoxia; 21% O_2) starting at postnatal day 1 (P1) up to P7, P14, P21 or P28. Oxygen levels were monitored using a Miniox II monitor (Catalyst Research, Owing Mills, MD). Dams were exchanged between hyperoxic and normoxic litters every 24 h, to prevent an effect of oxygen in the nursing dams.

Histomorphometry

Mice were sacrificed at day P7, P14, P21 or P28 by intraperitoneal injection of pentobarbital. Lungs were excised, fixed overnight using 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Sections (3 µm) were mounted on glass slides, deparaffinized in xylene and rehydrated in a series of ethanol dilutions. Mean linear intercept and septal thickness were determined on sections stained for smooth muscle actin and counter-stained using hematoxylin and eosin as has been described previously.

mRNA expression

Real-time PCR was pereformed with a TaqMan® ABI PRISM 7700 SDS system (Applied Biosystems, Foster City, CA). Primers and probes were designed using the Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.). Primers were obtained from Roth (Karlsruhe, Germany) and MWG (Ebersberg, Germany); probes were synthesized by Eurogentec (Seraing, Belgium). Sequences are given in table 1.

Pulmonary tissue from five animals per treatment group (hyperoxia and normoxia at P7, P14, P21 and P28) was snap frozen in liquid nitrogen immediately after sacrificing the animals. Lung lobes were homogenized in liquid nitrogen. Total RNA isolation was performed by using the guanidinium-thiocyanate-phenol chloroform method (Trizol, Invitrogen, Karlsruhe, Germany). Reverse transcription was performed using the SuperScript II First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Karlsruhe, Germany).

Real-time analysis was performed as previously described.[12] Briefly, a cycler programme of: 2 min at 50° C and 10 min at 95° C followed by 45 cycles of 15 s at 95° C and 1 min at 60° C. For relative quantification, we applied the ΔΔCT method[13] where mRNA expression of a target gene was normalized for expression of an internal control gene, in this case β-actin.

Immunohistochemistry

Serial sections (3 µm) of paraffin-embedded lung tissue were prepared as described for histomorphometry. Staining was performed according to standard protocols (Histostain® Kit, Zymed Laboratories, San Francisco, CA, U.S.A.). Briefly, deparaffinized and rehydrated tissue sections were blocked with normal serum (goat or rabbit, depending on the species of primary antibody). Antigen retrieval was performed in a pressure cooker using sodium citrate (6.5 mM, pH 6.0). Sections were incubated with primary antibodies directed against NOS-2 and -3, DDAH-2, VEGF, VEGFR1 and VEGFR2, (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:200. Biotinylated secondary antibodies were employed as part of the immunodetection kits (Histostain® Kit; or Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) in the case of DDAH-2). Immune reactions were detected after binding of horse radish peroxidise (HRP)-labelled streptavidin to the secondary antibody and incubation with a HRP-substrate/chromogen mixture (Zymed Laboratories). Counter-staining was performed using Mayer's hematoxylin (Zymed Laboratories).

Statistics

Data are given as mean and standard deviation. The mRNA expression was analyzed using the Mann-Whitney U test and GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, U.S.A.). Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Growth and lung development in mouse pups exposed to chronic hyperoxia

The exposure to chronic hyperoxia ($FiO_2 = 0.85$) was initiated on postnatal day P1 and was terminated on day P7, P14, P21 or P28. Each group contained time-matched normoxic ($FiO_2 = 0.21$) controls. Although the survival rate was 100% in all treatment groups, chronic hyperoxia significantly compromised growth after 14 days, resulting in an average body mass that was a third lower than that of age-matched controls, which is consistent with our findings in this model.[14] Alveolar development was impaired in neonatal mice when exposed to chronic hyperoxia. By day P28, air spaces were dramatically enlarged, suggesting a reduction in alveolar surface area (Figures 1-3).

Proinflammatory cytokines

Expression of TNF-α and IL-6 were determined to explore acute effects in pulmonary tissue due to hyperoxic exposure (Table 2 and Figure 4). Both, TNF-α and IL-6 mRNA expression was up-regulated, starting at day P7 ($P < 0.01$ and $P < 0.02$, respectively; Table 2). Differences remained significant only for IL-6 by day P14 ($P < 0.05$) and day P21 ($P < 0.01$).

Table 1 Sequences of primer and probes used for real time PCR analysis in lung tissue

Gene	Abreviation	Oligonucleotide sequence of primer (FP, forward primer; RP, reverse primer, P; probe)
ß-actin		FP: 5′-AGGTCATCACTATTGGCAACGA-3′ RP: 5′-CAACGTCACACTTCATGATGGA-3′ P: 5′(FAM)-AGCCTTCCTTCTTGGGTATGGAATCCTGT-(TAMRA)3′
Tumor necrosis factor – alpha	TNF-α	FP: 5′-GGCCTCCCTCTCATCAGTTCTAT-3′ RP: 5′-ACGTGGGCTACAGGCTTGTC-3′ P: 5′(FAM)-CCAGACCCTCACACTCAGATCATCTTCTCAA-(TAMRA)3′
Interleukin – 6	IL-6	FP: 5′-TCAATTCCAGAAACCGCTATGAA-3′ RP: 5′-CACCAGCATCAGTCCCAAGAA-3′ P: 5′(FAM)-CCTCTCTGCAAGAGACTTCCATCCAGTTGC-(TAMRA)3′
Neuonal nitric oxide synthase	nNOS, NOS-1	FP : 5′-TCGCTGTGTGGGCAGGAT-3′ RP : 5′-GCGTACTTGACGTGGTTACAGATG-3′ P: 5′(FAM)-TCCAAGCTGCAGGTGTTCGATGCC-(TAMRA)3′
Inducible nitric oxide synthase	iNOS, NOS-2	FP : 5′-GGACCTGGCCACCTTGTTC-3′ RP : 5′-TGCTCCTCTTCCAAGGTGCTT-3′ P: 5′(FAM)-CCTTCAACACCAAGGTTGTCTGCATGG-(TAMRA)3′
Endothelial nitric oxide synthase	eNOS, NOS-3	FP : 5′-GCAGCGGTGGAAATTAATGTG-3′ RP : 5′-GCGTGGTGGTCCACTATGG-3′ P: 5′(FAM)-TGCACAGTTACCAGCTGGCCAAAGTGA-(TAMRA)3′
Dimethylarginine dimethylaminohydrolase 1	DDAH-1	FP : 5′-CCTTTCCAAAAGAACAAATCAACGA-3′ RP : 5′-TGGGTCCGGCCATGCT-3′ P: 5′-(FAM)-TGCTGAAATCTTGGCTGATACTTTTAAGGACTACG-(TAMRA)-3′
Dimethylarginine dimethylaminohydrolase 2	DDAH-2	FP : 5′-CCTGGAGCCCAGCACGTA-3′ RP : 5′-CACAATTCGGAGTCCCAAGTC-3′ P: 5′-(FAM)-CCTGAGGTTGATGGAGTGCGTAAAGCC-(TAMRA)-3′
Hypoxia-Inducible Factor 1 alpha	HIF-1α	FP: 5′-CAGATTGAAGTGCACCCTAACAAG-3′ RP: 5′-GGTTGGTTACTGTTGGTATCATAGACAT-3′ P: 5′(FAM)-TGCAGTGAAGCACCTTCCACGTTGCT-(TAMRA)3′

Hypoxia-Inducible Factor 2 alpha	HIF-2α	FP: 5′-CCAACAGAGGCCGGACTGT-3′ RP: 5′-GGCAGTTGTTGTAGACTCTCACTTG-3′ P: 5′(FAM)-CCACCTGGAAGGTCCTGCACTGCA-(TAMRA)3′
Prolyl Hydroxylase Domain containing protein 1	PHD-1, EGLN2	FP: 5′-TCTGGTCTGACCGACGGAA-3′ RP: 5′-GCCCGGTTCCTTGGCATC-3′ P: 5′(FAM)-CCACCAGGTACGCCAT-(TAMRA)3′
Prolyl Hydroxylase Domain containing protein 2	PHD-2, EGLN1	FP: 5′-TCTAAATAAAGACTGGGACGACCA-3′ RP: 5′-TTTATCCTGTAGTTGCCCAGCTT-3′ P: 5′(FAM)-CGCCACTGCAGCGG-(TAMRA)3′
Prolyl Hydroxylase Domain containing protein 3	PHD-3, EGLN3	FP: 5′-ATGGCCGCTGTATCACCTG-3′ RP: 5′-AATGGGCTCCACGTCTGCT-3′ P: 5′(FAM)-GACGCCAAGTTACACG-(TAMRA)3′
Prolyl Hydroxylase Domain containing protein 4	PHD-4	FP: 5′-CCATCGGCCTGCTCATG-3′ RP: 5′-TTTATCCTGTAGTTGCCCAGCTT-3′ P: 5′(FAM)-AGCATGGACGACCTGATCCGCC-(TAMRA)3′
Vascular endothelial growth factor	VEGF	FP: 5′-GCAGGCTGCTGTAACGATGA-3′ RP: 5′-TCATTCTCTCTATGTGCTGGCTTT-3′ P:5′(FAM)- ACTACCATGCAGATCATGCGGATC-(TAMRA)3′
Vascular endothelial growth factor – receptor 1	VEGF-R1, Flt-1	FP: 5′-CCGAGCCACCAACCAGAA -3′ RP: 5′-CGTGATCAGCTCCAGGTTTGA-3′ P: 5′(FAM)-CCTCACCGTGCAAGGAACCTCAGACA -(TAMRA)3′
Vascular endothelial growth factor – receptor 2	VEGF-R2, Flk-1	FP: 5′-TCTGCTCAAGATAAGAAGACCAAGAA-3′ RP: 5′-TCTCCAGATTTCCGGTGATCA-3′ P: 5′(FAM)-AGCTCATCATCCTGAAGCGCATGGCA -(TAMRA)3′
Surfactant associated protein B	SP-B	FP: 5′-ATGGCCAAGTCGCACCTACT-3′ RP: 5′-CCAGAATTGAGGGCCTTGTG-3′ P: 5′-(FAM)-CCCAGGTGCAGCTATCACGTCGG-(TAMRA)-3′
Surfactant associated protein C	SP-C	FP: 5′-CCACTGGCATCGTTGTGTATG-3′ RP: 5′-GTAGGTTCCTGGAGCTGGCTTA-3′ P: 5′-(FAM)-CTACCAGCGGCTCCTGACGGCC-(TAMRA)-3′

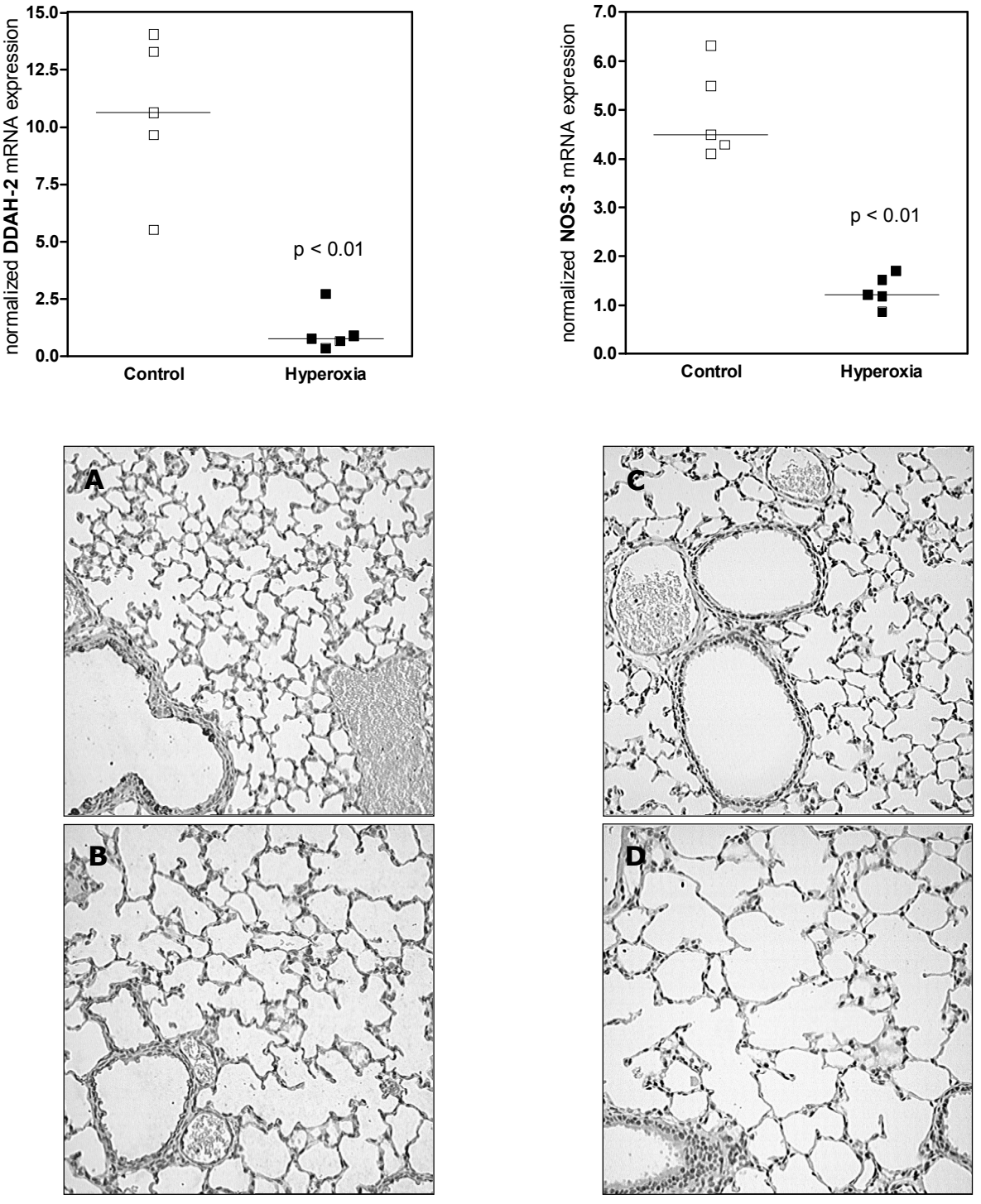

Figure 1 *DDAH-2 and NOS-3 expression in lung sections from mouse pups after chronic hyperoxia until day P14 compared to control animals maintained under normoxic conditions. The upper panel illustrates mRNA expression assessed by real time RT-PCR as a dot-plot, each dot representing the median mRNA expression for a single animal (n = 5, per group). Gene expression was significantly down-regulated in hyperoxic animals compared to controls. The lower panel illustrates immunohistochemical staining for DDAH-2 (A, control; B, hyperoxia) and NOS-3 (C, control; D, hyperoxia) in tissue sections (magnification 20×). In lung sections from hyperoxic mice air spaces were enlarged (as is evident in panels B and D, compared with panels A and C). The DDAH-2 protein expression (red-brownish staining) is evident in lung sections from control animals (A), although not in lung sections from hyperoxic pups (B). Faint differences in NOS-3 protein expression are also visible.*

Expression of enzymes in the NO system

To investigate the induction of NOS genes in lung tissue, mRNA expression of NOS-1 (nNOS), NOS-2 (iNOS) and NOS-3 (eNOS) was assessed by real-time RT-PCR. Expression of NOS-1 mRNA was very low and did not change with time *post partum* (Table 2). Expression of NOS-2, however, was up-regulated in mouse pups exposed to hyperoxia for more than 21 days; this difference was significant for the groups at P21 ($P < 0.01$; Table 2). In contrast, NOS-3 was down-regulated in the first 14 days after exposure to hyperoxia compared to control animals ($P < 0.01$ at day P14), but not thereafter (Table 2).

The decrease in NOS-3 mRNA expression was observed along with a down-regulation of DDAH-2 in hyperoxic pups at day P14 and day P21 ($P < 0.01$ and $P < 0.05$, respectively; Table 2). Expression of DDAH-1 remained at a similar level in both treatment groups over the time-course of the study.

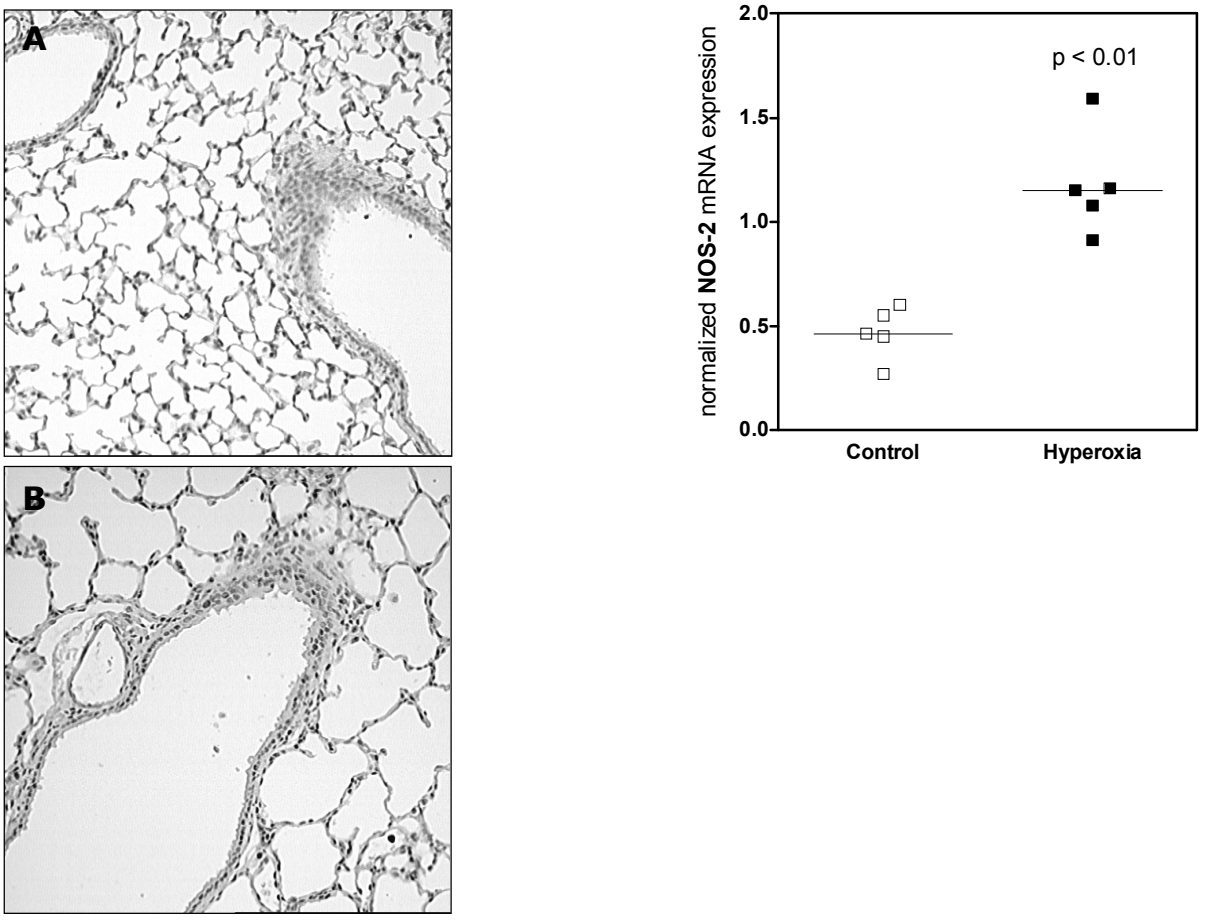

Figure 2 *Expression of NOS-2 (inducible NOS) in mouse lungs at day P21. NOS-2 expression was up-regulated in pulmonary tissue from hyperoxic animals compared to controls. The upper panel illustrates mRNA expression assessed by real time RT-PCR as a dot-plot, each dot representing the median mRNA expression for a single animal (n = 5, per group). The lower panel illustrates immunohistochemical staining for NOS-2 in tissue sections of (A) a control animal and (B) an animal maintained under hyperoxic conditions (magnification 20×), which confirm the increased NOS-2 expression at the protein level (red-brownish staining). Again, dramatically enlarged air spaces can be seen in lung tissue from animals maintained under hyperoxic conditions (panel B), compared with lung sections from animals maintained under normoxic conditions (panel A).*

Table 2 The mRNA expression in mouse lungs after exposure to chronic hyperoxia compared to controls (normoxia). Values given are mean ± SD (95% CI of mean) after real time RT-PCR as $2^{-\Delta\Delta ct}$ normalized for the β-actin as house-keeping gene

Parameter		Control	Hyperoxia	
TNF-α	P7	1.29 ± 0.19 (1.05 - 1.52)	12.79 ± 22.13 (0.00 - 40.27)	$P < 0.01$
	P14	2.24 ± 1.08 (0.89 - 3.58)	2.94 ± 2.48 (0.00 - 6.02)	ns
	P21	1.11 ± 0.18 (0.88 - 1.34)	1.99 ± 0.34 (1.57 - 2.42)	ns
	P28	2.25 ± 1.52 (0.36 - 4.13)	3.60 ± 0.76 (2.65 - 4.55)	ns
IL-6	P7	1.12 ± 0.46 (0.54 - 1.69)	13.92 ± 22.60 (0.00 - 41.97)	$P < 0.02$
	P14	7.25 ± 5.14 (0.87 - 13.64)	20.33 ± 8.67 (9.57 - 31.09)	$P < 0.05$
	P21	1.12 ± 0.54 (0.45 - 1.79)	22.37 ± 12.50 (6.86 - 37.89)	$P < 0.01$
	P28	6.16 ± 6.29 (0.00 - 13.97)	40.44 ± 48.00 (0.00 - 100.00)	ns
NOS-1	P7	0.10 ± 0.05 (0.03 - 0.16)	0.10 ± 0.05 (0.04 - 0.16)	ns
	P14	0.35 ± 0.55 (0.00 - 1.03)	0.08 ± 0.09 (0.00 - 0.20)	ns
	P21	0.08 ± 0.03 (0.04 - 0.11)	0.07 ± 0.04 (0.03 - 0.12)	ns
	P28	0.11 ± 0.10 (0.00 - 0.23)	0.10 ± 0.10 (0.00 - 0.18)	ns
NOS-2	P7	0.50 ± 0.11 (0.36 - 0.64)	0.58 ± 0.14 (0.41 - 0.75)	ns
	P14	1.16 ± 0.36 (0.72 - 1.60)	1.08 ± 0.16 (0.88 - 1.29)	ns
	P21	0.47 ± 0.13 (0.31 - 0.62)	1.18 ± 0.25 (0.87 - 1.49)	$P < 0.01$
	P28	0.62 ± 0.19 (0.39 - 0.85)	0.90 ± 0.20 (0.66 - 1.15)	ns
NOS-3	P7	1.62 ± 0.58 (0.91 - 2.34)	0.99 ± 0.39 (0.50 - 1.47)	ns
	P14	4.94 ± 0.94 (3.77 - 6.11)	1.30 ± 0.32 (0.90 - 1.69)	$P < 0.01$
	P21	1.43 ± 0.40 (0.94 - 1.93)	1,29 ± 0.51 (0.65 - 1.93)	ns
	P28	1.43 ± 0.35 (1.00 - 1.85)	1.77 ± 0.53 (1.11 - 2.43)	ns
DDAH-1	P7	1.91 ± 0.68 (1.07 - 2.75)	2.59 ± 1.62 (0.57 - 4.61)	ns
	P14	2.08 ± 1.00 (0.83 - 3.32)	1.43 ± 0.39 (0.95 - 1.92)	ns
	P21	0.94 ± 0.27 (0.61 - 1.28)	2.31 ± 1.53 (0.41 - 4.20)	ns
	P28	1.45 ± 0.48 (0.85 - 2.05)	2.57 ± 0.96 (1.38 - 3.77)	ns
DDAH-2	P7	2.31 ± 1.53 (0.41 - 4.20)	0.65 ± 0.30 (0.28 - 1.01)	ns
	P14	10.63 ± 3.38 (6.43 -14.82)	1.07 ± 0.93 (0.00 - 2.22)	$P < 0.01$
	P21	0.51 ± 0.12 (0.37 - 0.66)	0.25 ± 0.10 (0.13 - 0.38)	$P < 0.02$
	P28	0.72 ± 0.63 (0.00 - 1.51)	0.45 ± 0.21 (0.19 - 0.71)	ns
HIF-1α	P7	0.74 ± 0.12 (0.59 - 0.89)	0.91 ± 0.25 (0.61 - 1.22)	ns
	P14	0.95 ± 0.53 (0.30 - 1.61)	1.00 ± 0.12 (0.86 - 1.15)	ns
	P21	0.51 ± 0.06 (0.44 - 0.59)	1.20 ± 0.14 (1.03 - 1.37)	$P < 0.01$
	P28	0.63 ± 0.16 (0.43 - 0.84)	1.27 ± 0.25 (0.95 - 1.58)	$P < 0.01$
HIF-2α	P7	3.68 ± 0.71 (2.80 - 4.57)	3.90 ± 0.35 (3.47 - 4.33)	ns
	P14	5.87 ± 4.35 (0.46 -11.28)	3.57 ± 0.44 (3.02 - 4.12)	ns
	P21	8.25 ± 1.09 (6.90 - 9.60)	3.51 ± 0.88 (2.41 - 4.61)	$P < 0.01$
	P28	6.98 ± 3.32 (2.85 - 11.10)	5.89 ± 3.82 (1.15 - 10.64)	ns
VEGF	P7	1.20 ± 0.34 (0.77 - 1.62)	1.12 ± 0.27 (0.78 - 1.46)	ns
	P14	5.23 ± 1.60 (3.24 - 7.21)	1.38 ± 0.31 (0.99 - 1.76)	$P < 0.01$
	P21	1.85 ± 0.42 (1.33 - 2.37)	0.92 ± 0.15 (0.73 - 1.11)	$P < 0.01$
	P28	1.82 ± 0.88 (0.73 - 2.19)	1.76 ± 1.27 (0.19 - 3.34)	ns

Parameter		Control	Hyperoxia	
VEGFR1	P7	1.79 ± 0.45 (1.23 - 2.35)	1.66 ± 0.25 (1.35 - 1.97)	ns
	P14	3.40 ± 0.67 (2.56 - 4.23)	2.04 ± 0.43 (1.51 - 2.58)	$P < 0.05$
	P21	3.62 ± 0.79 (2.63 - 4.61)	2.31 ± 0.59 (1.57 - 3.05)	$P < 0.05$
	P28	3.38 ± 1.34 (1.73 - 5.04)	2.87 ± 1.61 (0.87 - 4.87)	ns
VEGFR2	P7	1.38 ± 0.37 (0.92 - 1.84)	1.59 ± 0.48 (1.00 - 2.19)	ns
	P14	2.48 ± 0.75 (1.54 - 3.41)	1.93 ± 0.36 (1.48 - 2.38)	ns
	P21	1.97 ± 0.28 (1.63 - 2.32)	1.92 ± 0.46 (1.35 - 2.48)	ns
	P28	1.89 ± 0.57 (1.18 - 2.59)	1.84 ± 0.32 (1.44 - 2.24)	ns
PHD-1	P7	1.75 ± 1.06 (0.43 - 3.06)	2.05 ± 1.80 (0.00 - 4.29)	ns
	P14	2.77 ± 0.91 (0.40 - 5.15)	1.40 ± 0.63 (0.62 - 2.17)	ns
	P21	1.13 ± 0.25 (0.82 - 1.44)	0.85 ± 0.07 (0.76 - 0.95)	ns
	P28	1.25 ± 0.70 (0.39 - 2.12)	1.37 ± 0.48 (0.78 - 1.96)	ns
PHD-3	P7	0.44 ± 0.28 (0.09 - 0.79)	0.36 ± 0.25 (0.06 - 0.67)	ns
	P14	0.23 ± 0.22 (0.00 - 0.51)	0.11 ± 0.06 (0.04 - 0.19)	ns
	P21	0.04 ± 0.02 (0.02 - 0.06)	0.04 ± 0.01 (0.02 - 0.06)	ns
	P28	0.06 ± 0.04 (0.01 - 0.11)	0.06 ± 0.01 (0.05 - 0.07)	ns
PHD4	P7	1.29 ± 0.57 (0.57 - 2.00)	1.13 ± 0.40 (0.64 - 1.62)	ns
	P14	3.82 ± 2.63 (0.02 - 6.54)	0.98 ± 0.71 (0.09 - 1.86)	ns
	P21	0.95 ± 0.21 (0.69 - 1.20)	0.49 ± 0.08 (0.39 - 0.58)	$P < 0.01$
	P28	1.17 ± 0.92 (0.02 - 2.31)	1.10 ± 0.67 (0.26 - 1.93)	ns
SP-B	P7	1.41 ± 0.28 (1.06 - 1.76)	1.78 ± 0.25 (1.48 - 2.09)	ns
	P14	2.55 ± 1.07 (1.22 - 3.88)	1.09 ± 0.22 (0.81 - 1.36)	$P < 0.02$
	P21	1.97 ± 0.38 (1.49 - 2.45)	1.36 ± 0.24 (1.06 - 1.66)	P < 0.02
	P28	2.07 ± 0.49 (1.46 - 2.68)	2.27 ± 0.33 (1.86 - 2.68)	ns
SP-C	P7	2.85 ± 0.73 (1.94 - 3.75)	2.21 ± 0.72 (1.32 - 3.11)	ns
	P14	4.45 ± 2.76 (1.02 - 7.88)	0.86 ± 0.24 (0.56 - 1.15)	$P < 0.01$
	P21	1.59 ± 0.39 (1.11 - 2.08)	0.60 ± 0.05 (0.53 - 0.66)	$P < 0.01$
	P28	1.42 ± 0.66 (0.59 - 2.24)	1.09 ± 0.44 (0.54 - 1.63)	ns

Angiogenic factors

The expression of HIF, prolyl hydroxylase domain-containing enzymes (PHD) that regulate HIF stability, as well as that of VEGF and its receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1) were determined, since dysregulated expression of these factors may be responsible for impaired lung development. Expression of HIF-1α was up-regulated in hyperoxic animals at day P21 and day P28 ($P < 0.01$), while HIF-2α expression, was significantly down-regulated at day P21 compared to normoxic animals (Table 2, $P < 0.01$). The mRNA expression of VEGF was decreased in mouse pups exposed to hyperoxia for up to three weeks ($P < 0.01$), while VEGFR1 mRNA expression was reduced ($P < 0.05$) in hyperoxic mice at day P14 and day P21. The expression of VEGFR2 remained unchanged in both groups (Table 2).

Expression of PHD mRNA levels decreased within the study period of four weeks; however, PHD-2 mRNA was not detected. No significant differences were observed

between mRNA expression in control animals and those maintained under hyperoxic conditions, with the single exception of PHD-4 expression being significantly higher in control animals compared to hyperoxic mouse pups at day P21 (Table 2).

Expression of surfactant associated proteins SP-B and SP-C

The mRNA expression of surfactant associated proteins SP-B and SP-C, employed as a measure of lung development and function of surfactant-producing type II cells, was decreased under chronic hyperoxia reaching significance at day P14 and day P21 ($P < 0.05$ for SP-B and $P < 0.01$ for SP-C; Table 2).

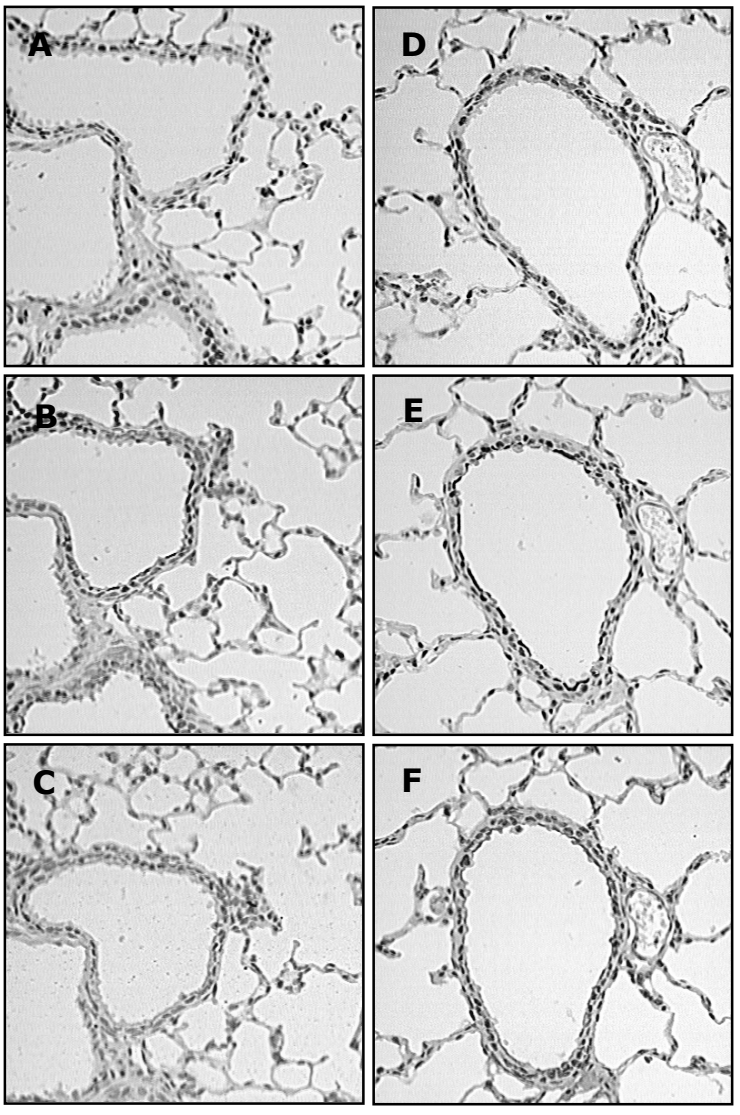

Figure 3 *Serial immunhistological staining for VEGF (A, D), VEGFR1 (B, E) and VEGFR2 (C, F) in normoxic (left panel, A-C) and hyperoxic (right panel, D-F) lung tissue at day P21 (magnification 40×). Decreased expression of VEGF in hyperoxic mouse lungs (D) compared to normoxic controls (A) confirmed the mRNA data, VEGFR1 expression (B vs. E) tended to be lower at the protein level whereas VEGFR2 protein expression appeared to be higher in hyperoxic lung tissue (F) compared to controls (C), but mRNA expression for VEGFR2 was similar in both groups (Table 1).*

Protein expression

Comparing levels of protein expression with that of gene expression, we semi-quantitatively inspected all tissue sections. At day P14, immunhistological staining confirmed the reduced expression of DDAH-2 and NOS-3 found on mRNA level in hyperoxic lung tissue although differences for NOS-3 were weak with regards to immunostaining (Figure 1). At day P21, gene expression for NOS-2, the inducible form of NOS was up-regulated not only as mRNA, but also on the protein level as is shown in figure 2. At the same time, the expression of VEGF and its receptor Flt-1 was reduced in pulmonary tissue from mouse pups kept under hyperoxic conditions compared to normoxic controls (Table 2); however, on the protein level, only VEGF staining revealed corresponding differences; tissue slices treated with Flt-1 antibodies were found to be similar in hyperoxic versus normoxic animals. VEGF receptor Flk-1 seemed to be slightly increased after chronic hyperoxia (Figure 3), although no significant differences were observed on the mRNA level (Table 2).

DISCUSSION

Bronchopulmonary dysplasia develops in the early *post-natal* phase of late lung development, as a result of hyperoxic injury and/or long term mechanical ventilation and is characterized by impaired alveolar and vascular growth as well as various degrees of inflammatory tissue damage.[15] Several signalling pathways, including those induced or regulated by the NOS, HIF and VEGF systems, have been implicated in the development of BPD.[6] These signalling pathways also interact with one another. The aim of this study therefore was, to compare the expression of essential components of all three systems, as an effort to understand how dysregulated expression of individual components may perturb inter-pathway cross-talk in hyperoxia-induced arrest of alveolar development in mice.

In our study, we have employed a model of chronic hyperoxia (FiO_2 = 0.85) in mouse pups for 28 days, which we have previously described in detail[14] and in which lung development was impaired with significantly enlarged air spaces concomitant with a reduction of alveolar surface area. This morphological picture is also found in human BPD, and other animal models of BPD.[16-20]

An early effect of exposure to a hyperoxic environment is the triggering of an inflammatory cascade in the lung.[21] In humans, the presence of some cytokines, for example, IL-6, in tracheal aspirates at birth is an independent risk factor for the development of BPD in preterm infants.[22] In our study, we observed an initial up-regulation of TNF-α expression in the first week of hyperoxic exposure, and IL-6 mRNA expression was increased several-fold throughout the study period (Figure 4). Interestingly, TNF-α not only triggers leukocyte recruitment, but also affects other factors, such as endothelial NOS expression by decreasing NOS-3 promoter activity.[23]

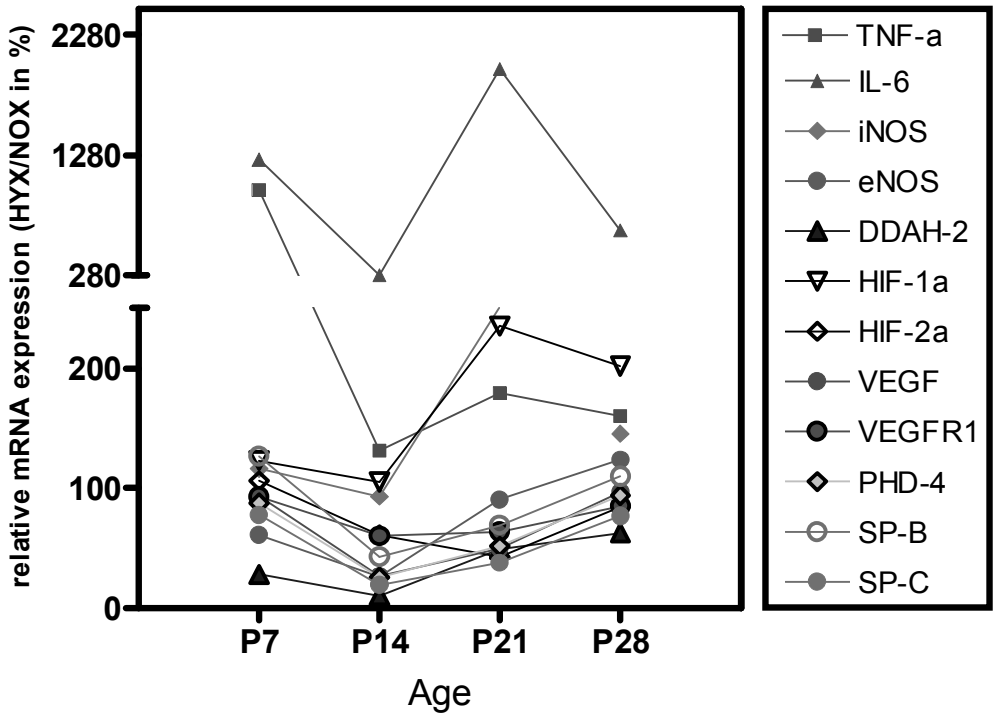

Figure 4 *mRNA expression levels of selected molecules, over the period day P7 to day P28. Only molecules that significantly changed in expression levels (Table 1) are included. The relative mRNA expression was calculated as group means htperoxia (HYX)/normoxia (NOX) over time (P7, P14, P21, P28). Values are presented as a percentage of the normoxia values, and error bars have been omitted for clarity.*

Indeed, we observed that NOS-3 mRNA expression was significantly down-regulated at day P14. Thus, these changes in NOS-3 expression may in part be mediated by an increased expression of pro-inflammatory cytokines.[24] In addition to the decreased NOS-3 expression that we observed, NOS-3 activity may be further reduced indirectly by the down-regulated expression of DDAH-2 we reported here. Nitric oxide synthase is inhibited by endogenous arginine metabolites such as asymmetric dimethylarginine which is degraded by DDAH known to be differentially regulated during lung development.[25] The reduced expression of DDAH might lead to enhanced levels of asymmetric dimethylarginine (ADMA), an inhibitor of NOS-3 activity which then most likely affects HIF function. Of particular interest in our study is the interaction between NOS-3, HIF-2α and VEGF, since HIF stabilization with chemical inhibitors of PHD increases VEGF expression, and hence, lung vascularisation and alveolarization.[9] The reduced NOS-3 activity we report here lowers HIF-2α expression levels, and this in turn, would decrease levels of angiogenic factors such as VEGF.[26]

The altered expression of angiogenic factors plays an important role in the development of BPD resulting in microvascular damage, diminished epithelial cell proliferation, and arrest of alveolarization.[18] Hosford and Olson[18] speculated that hyperoxia-induced changes in VEGF may be an important element in the etiology of BPD. In support of this, Maniscalco et al.[27] has shown that neonatal rabbits exposed

to 100% O_2 for nine days exhibited decreased VEGF mRNA levels and decreased alveolar epithelial cell VEGF expression. Bhatt et al.[28] reported that preterm human neonates with RDS or BPD were deficient in VEGF and Flt-1, both on mRNA and protein level. We demonstrated here that VEGF and its receptor Flt-1 were both down-regulated in our hyperoxia model of BPD, and we propose that reduced NOS-3 activity may, at least in part, mediate this effect. In addition to this impact on vascular development, VEGF also affects surfactant production, i.e. the expression of SP-B. Consequently, VEGF down-regulation would lead to a reduced expression of SP.[5] Since SP-B and SP-C are critically important for surface tension properties in the alveolar compartment, they play a crucial role in directing late lung development.

Returning to the NOS family of enzymes, in contrast to the down-regulated expression of NOS-3, we observed up-regulated expression of NOS-2 after 21 days of hyperoxic exposure. Interestingly, NOS-2 is known to down-regulate SP expression, and these elevated NOS-2 levels would most likely further reduce SP production, which would be additive to the deleterious effects of dysregulated VEGF signalling on surfactant production.[29]

In summary, this paper describes a number of factors involved in the pathogenesis of BPD. Although their roles in lung development and lung injury were described previously, we are the first to report changes in their expression over time, especially during alveolarization and vascularisation. It is during this time, that a disturbance of lung development leads to BPD. Our results show differences in expression of oxygen-regulated factors, angiogenic factors and components of the NO biosynthetic pathway, as well as of pro-inflammatory cytokines and SP over time. In addition, we present a model incorporating all these factors (Figure 5).

In conclusion, hyperoxia affects a variety of factors that impact lung development, including an inflammatory response and disruption of endothelial and epithelial cell function. Therefore, a single preventative measure or therapeutic treatment targeting only one of these factors at a specific point in time may exhibit beneficial effects on that particular parameter, but may not improve the overall clinical outcome. Thus, it is likely that a combination of interventions, such as the application of an inhibitor of NOS-2 as has recently been suggested by Yuba et al.[30] together with high doses of antioxidants for neonates who require hyperoxic treatment, may be more appropriate. Along these lines, the application of NO or the administration of retinoic acid, which has been found to reduce ADMA levels and increase NO synthesis *in vitro* needs to be further investigated. In addition, the changes in expression of these factors were found to be time-dependent.[31] Therefore, in clinical practice, it is not only important to know which pathway(s) to therapeutically target, but also to define the optimal time point for such an intervention.

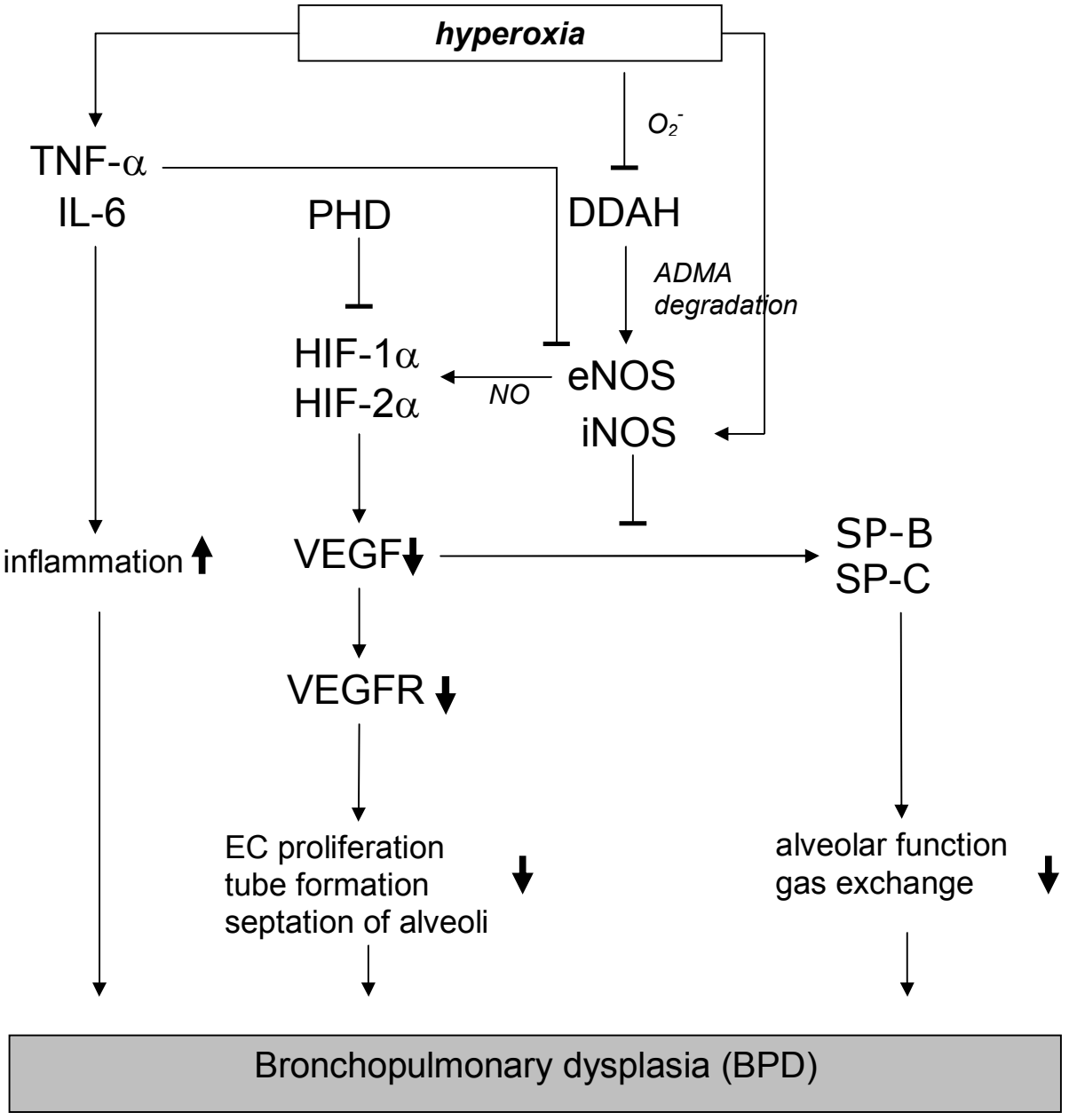

Figure 5 *Hypothesis for the interaction of effects induced by chronic hyperoxia on pulmonary gene expression regulating vascular and alveolar development.*

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chapter

6

Hypoxia-induced intrauterine growth retardation: Effects on pulmonary development and surfactant protein transcription

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Hypoxia-Induced Intrauterine Growth Retardation: Effects on Pulmonary Development and Surfactant Protein Transcription

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Key Words

Intrauterine hypoxia · Intrauterine growth retardation · Lung development · Surfactant proteins

Abstract

Background and Objectives: Preterm infants with intrauterine growth retardation (IUGR) reveal an increased risk for the development of acute and chronic pulmonary disorders, i.e. bronchopulmonary dysplasia (BPD). In order to investigate the effect of IUGR on pulmonary development, an easily reproducible animal model for fetal growth restriction has been established using hypoxia as a sole intervention in the last third of pregnancy. ***Methods:*** Date-mated mice were randomly assigned to either being kept at a fraction of inspired oxygen (FiO_2) of 0.10 (hypoxic group) starting at day 14 or under normoxic conditions until day 17.5 of gestation (control group). Variables of somatic growth were assessed and standardized histomorphometric analyses of pulmonary tissue were performed. Expression of surfactant proteins (SP)-A, -B, -C and -D was determined by quantitative rt-PCR as biochemical indicators for lung development and maturation. ***Results:*** Fetuses were delivered preterm at 0.87 of gestation. Those grown under hypoxic conditions revealed significantly lower birth weights (median: 0.69 vs. 0.97 g in controls; $p < 0.001$), body lengths (median: 17.5 vs. 20.2 mm in controls; $p < 0.001$) and fronto-occipital diameters (median: 9.4 vs. 10.1 mm in controls; $p < 0.001$) compared to controls. Histomorphometric analyses were found to be without significant differences between both groups. On the transcriptional level, however, mRNA expression of SP-A, -B and -C but not SP-D could be shown to be significantly reduced in hypoxic fetuses compared to normoxic controls. ***Conclusions:*** In conclusion, hypoxic conditions from day 14 to 17.5 led to IUGR in preterm mice and to significant alterations of the developing surfactant system. We speculate restricted development of SP gene expression to be a causal factor for the increased risk of acute and chronic pulmonary disorders in preterm infants with IUGR.

Introduction

Research on intrauterine growth restriction (IUGR) has gained increasing interest secondary to its life-lasting consequences [1]. Although the so-called 'Barker hypothesis' is still controversially discussed [2], the main interpretations of long-term consequences of IUGR are widely accepted including an increased risk for the development of the metabolic syndrome and cardiovascular diseases [3].

A specific risk group for devastating consequences of IUGR is represented by preterm infants below 30 weeks of gestational age not only suffering from immaturity but also from direct consequences of IUGR including an increased risk of neonatal mortality and morbidity. With regard to long-term consequences, the clinical outcome in these patients is mainly determined by the increased risk for the development of chronic lung diseases, i.e. bronchopulmonary dysplasia compared to age-matched controls [4, 5].

The 'classical' hypothesis during the 1980s, suggesting preterm infants to be protected from pulmonary complications in case of IUGR [6] reflects neonatal characteristics of infants studied at that time, i.e. mean gestational age <30 weeks. Currently, even more immature infants are representing the high-risk group for perinatal mortality and severe neonatal morbidity including pulmonary complications like the development of acute and especially chronic lung disease (bronchopulmonary dysplasia, BPD). Thus, IUGR has been described to be associated with a higher prevalence of the respiratory distress syndrome (RDS) in preterm infants, and furthermore, pulmonary function later in life has been found to be compromised by fetal growth restriction [7, 8].

In previous studies a complex model was used inducing IUGR by umbilico-placental embolization and thus studying the effects of several variables such as hypoxemia, nutrient deficiency and stress-related hormonal counter-regulation [7, 9, 10].

The aim of the present study therefore was to establish an easily reproducible animal model to investigate the consequences of IUGR on lung development. The focus was placed on the induction of fetal growth restriction by hypoxia still mimicking pathophysiological conditions relevant for human neonates.

Materials and Methods

Animals

Date-mated pregnant mice (C57BL/6, Charles River, Sulzfeld, Germany) were kept under constant conditions with respect to fluid and nutritional supply until day 14 (embryonic day 14; E14). Thereafter, they were randomly assigned to either being kept at a fraction of inspired oxygen (FiO_2) of 0.10 (hypoxic group; n = 5) or under normoxic conditions until day 17.5 of gestation (FiO_2 0.21; control group; n = 5). Normobaric hypoxia was created by means of a ventilated chamber. FiO_2 was held constant by an autoregulatory control unit (model 4010, O_2 controller; Labotect, Göttingen, Germany). Excess humidity in the recirculating system was prevented by condensation in a cooling unit. Carbon dioxide was continuously removed by soda lime. Cages were opened once a day for cleaning as well as for food and water supply. At day 17.5 of gestation, pregnant dams were anesthetized using ketamine/xylamine (40 and 4 μg/g body weight, respectively). Preterm delivery of mouse fetuses was carried out by Cesarean section (normal duration of gestation for mice: 19–21 days).

The study was approved by the Institution for Animal Protection and carried out in compliance with the guide for care and use of laboratory animals published in the National Institutes of Health [NIH publication 85-92, revised 1985].

Auxological Measurements

We obtained auxological data from preterm mice with respect to birth weight, body length and fronto-occipital length by means of a precision scale (Mettler-Toledo, Wiesbaden, Germany) and a digital caliper (Mitutoyo, Neuss, Germany), respectively. For further evaluation of lung development, lungs were excised and kept at –80°C either for molecular analyses and paraffinated for histological investigation.

Lung Histology

Lungs were removed before the first breath filled with fetal lung fluid to standardize histologic evaluation. Tissue slices from paraffin-embedded fetal lungs were obtained from controls (n = 15) and hypoxic animals (n = 14), stained with hematoxylin/eosin, and evaluated by a pathologist (M.E.). Both fetal lungs and the heart were found in the paraffin sections. Random fields from each ventral and dorsal part of the left and right lung were studied. An adequate number of 5 fields were evaluated referring to the limited size of the organ.

Septal thickness was measured after photodocumentation of the lung tissue using Axiovision 3.0 for AxioCam MRc (Zeiss, Germany). Alveolar septa were measured at their thinnest point and averaged over ten measurements per lung. No tangential truncated septa were chosen for measurements.

Analysis of Surfactant Protein mRNA Expression

Messenger RNA expression of surfactant protein (SP)- A, -B, -C and -D was measured in tissue of the whole lung using the real-time RT-PCR technique (TaqMan®). Real-time PCR has been proven to be a sensitive and precise tool for the quantification of mRNA and thus for gene expression studies [11]. Primers and probes for SP-A, SP-B, SP-C, SP-D, β-actin, 18S and porphobilinogen deaminase (PBGD; table 1) were designed using the software Primer Express (Applied Biosystems, Foster City, Calif., USA) following a fixed set of recommendations to allow the application of standardized cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles for 15 s at 95°C and 1 min at 60°C). Control PCRs showed no signal for genomic DNA, proving mRNA specificity. Primers were purchased from Roth (Roth, Karlsruhe, Germany), probes from Applied Biosystems. Lung tissue homogenization was performed in liquid nitrogen and total RNA was then extracted using the acid guanidinium-thiocyanate-phenolchloroform method (Trizol, Invitrogen GmbH, Karlsruhe, Germany). Total RNA isolation, random primed reverse transcription and real-time PCR was performed following a standardized protocol as described previously [12]. Primer and TaqMan probe sequences are depicted below. Relative quantification was performed using the ΔΔCt method, which results in a ratio of target gene expression and the expression of a housekeeping or reference gene. As the validity of this method depends on the constant expression of the housekeeping

Table 1. Primer and TaqMan® probes for quantitative RT-PCR

β-Actin (A)
Forward primer (FP): 5′-AGGTCATCACTATTGGCAACGA-3′
Reverse primer (RP): 5′-CAACGTCACACTTCATGATGGA-3′
Probe: 5′(FAM)-AGCCTTCCTTCTTGGGTATGGAATCCTGT-(TAMRA)3′
18S (S)
FP: 5′-TGCGAATGGCTCATTAAATCAG-3′
RP: 5′-TAGCTCTAGAATCACCACAGTTATCCA-3′
Probe: 5′(FAM)-ATGGTTCCTTTGGTCGCTCGCTCCT-(TAMRA)3′
Porphobilinogen deaminase (PBGD)
FP: 5′-GCGGAGTCATGTCCGGTAAC-3′
RP: 5′-ACTCGAATCACCCTCATCTTTGA-3′
Probe: 5′(FAM)-CCACAACCGCGGAAGAAAACGG-(TAMRA)3′
Surfactant protein A (SP-A)
FP: 5′-AGTTGACTGACTGCCCATTGGT-3′
RP: 5′-ACTCTACGAGATCAAACATCAGATTCTG-3′
Probe: 5′(FAM)-TGCAAGCTGAGGACTCCCATTGTTTGC-(TAMRA)3′
Surfactant protein B (SP-B)
FP: 5′-ATGGCCAAGTCGCACCTACT-3′
RP: 5′-CCAGAATTGAGGGCCTTGTG-3′
Probe: 5′(FAM)-CCCAGGTGCAGCTATCACGTCGG-(TAMRA)3′
Surfactant protein C (SP-C)
FP: 5′-CCACTGGCATCGTTGTGTATG-3′
RP: 5′-GTAGGTTCCTGGAGCTGGCTTA-3′
Probe: 5′(FAM)-CTACCAGCGGCTCCTGACGGCC-(TAMRA)3′
Surfactant protein D (SP-D)
FP: 5′-GGCCTCCATCTGCTGCCT-3′
RP: 5′-GGAGACAGAGGAATCAAAGGTGAA-3′
Probe: 5′(FAM)-AGCAGCACTGTCTGGAAGCCCGC-(TAMRA)3′

gene throughout the set of samples studied, we analyzed gene expression ratios of housekeeper β-actin, 18S or PBGD and the total cDNA content of each sample, that was measured using a method based on the fluorescent stain OliGreen. As β-actin showed both, no differences between different experimental groups and the lowest variation in all samples, it was chosen as reference gene for further analysis.

Statistics

Data are given as mean ± SD or median and range, respectively. Auxological, histological and molecular biological variables were analyzed by Mann-Whitney U tests using SPSS for Windows version 6.1.3. Differences at $p < 0.05$ were considered to be statistically significant.

Results

Macroscopically, animals from the hypoxic group (n = 31) were smaller and less developed compared to controls (n = 31; fig. 1). Auxologic measurements revealed significantly lower birth weights (median: 0.69 vs. 0.97 g in controls; $p < 0.001$), body lengths (median: 17.5 vs. 20.2 mm in controls; $p < 0.001$) and fronto-occipital diameter (median: 9.4 vs. 10.1 mm in controls; $p < 0.001$) in these fetuses (fig. 2).

Using histomorphometry to investigate lung development, there was no significant difference with regard to number of saccular air spaces (60 ± 7 vs. 61 ± 6 per magnification field in hypoxic and control animals, respectively) and septal thickness (12.1 ± 1.3 vs. 11.5 ± 1.8 μm in hypoxic and control animals, respectively) in lung tissue from both groups.

On the transcriptional level, however, functional biochemical variables such as the expression of SP were found to be different between the groups. Thus, pulmonary SP-A mRNA expression was found to be significantly reduced in hypoxic fetuses compared to controls ($p < 0.001$) whereas SP-D mRNA expression was not significantly altered (fig. 3).

With regard to the hydrophobic SP, SP-B and SP-C, mRNA expression was significantly lower ($p < 0.05$ and $p < 0.01$, respectively) in experimentally induced IUGR compared to normoxic fetuses (fig. 4).

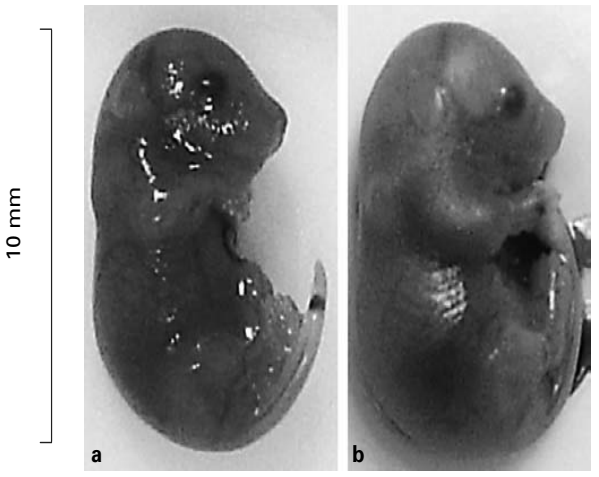

Fig. 1. Photographs of fetuses immediately after delivery. Animal after hypoxia (**a**), compared to a normoxic control (**b**).

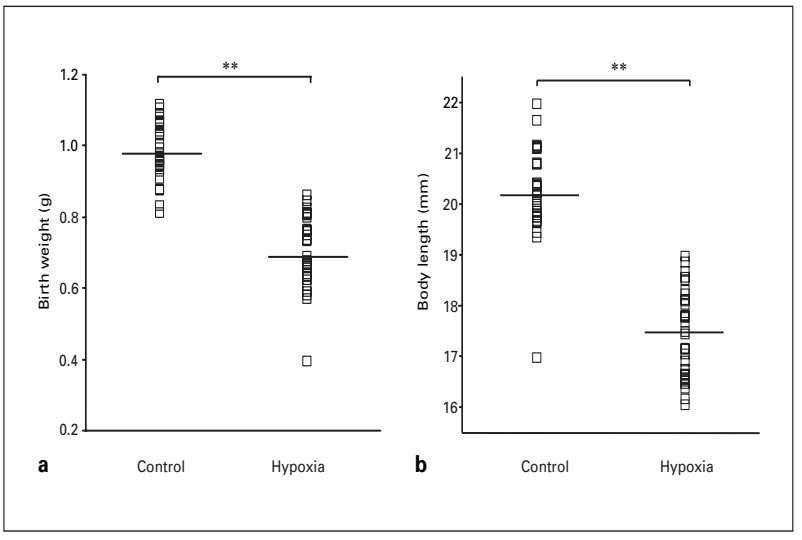

Fig. 2. Growth parameters regarding (**a**) birth weight (in g) and (**b**) body length (in mm) of fetuses kept under hypoxic conditions during pregnancy (FiO_2: 0.1) compared to normoxic controls. Each square indicates one fetus. Medians are given. ** $p < 0.001$.

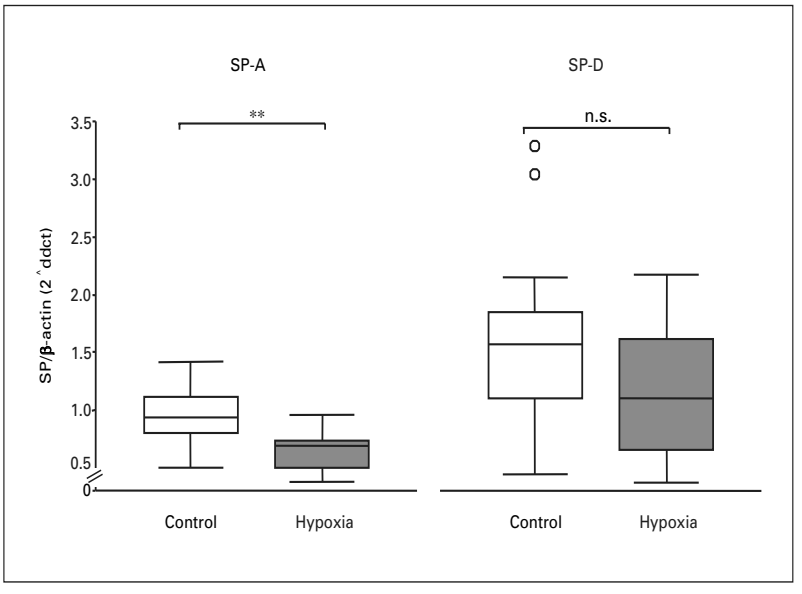

Fig. 3. Pulmonary SP-A and SP-D mRNA expression. SP mRNA expression was normalized to β-actin. Median, 95% CI, range and outlier (○) are given. ** $p < 0.001$; n.s. = not significant.

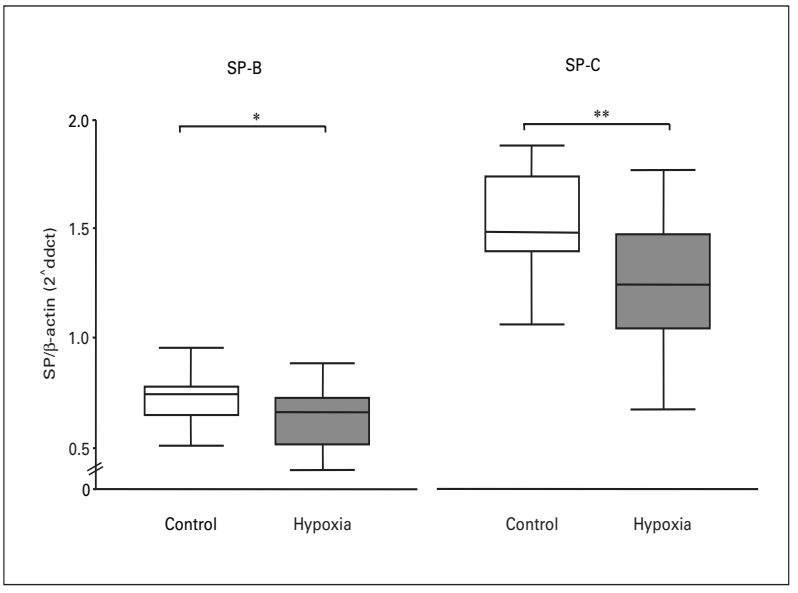

Fig. 4. Pulmonary SP-B and SP-C mRNA expression. SP mRNA expression was normalized to β-actin. Median, 95% CI and range are given. * $p < 0.05$; ** $p < 0.01$.

Discussion

Different animal models of IUGR have been established in the past (for review see [13]). Induction of IUGR in sheep by umbilical placental embolization revealed marked effects on lung development in the offspring [7, 9, 10]. The lungs of these lambs showed thickened alveolar septa and a smaller number of alveoli, with these changes still being present at 8 weeks postnatal age [14, 15]. The aforementioned changes were induced in fetal sheep during a period of lung development changing from the saccular to the alveolar period [16]. Whether these restrictions exert effects on lung function in adulthood, remains unclear, however epidemiological studies are suggestive [8, 17].

The main focus of the present study was therefore to develop a reliable animal model of IUGR, in which anatomical, histological, and molecular changes of pulmonary development can be assessed. As our group as well as others had demonstrated IUGR to increase the risk for the development of acute and chronic pulmonary disorders in preterm infants compared to age-matched appropriately grown controls [5, 8], we specifically focused on pulmonary changes secondary to chronic intrauterine hypoxia during the pseudoglandular to the terminal-saccular stage of lung maturation. Thus, the present model is close to the stage of lung development in very preterm infants and mimics in utero conditions observed in IUGR secondary to placental insufficiency leading to hypoxic conditions for the fetus. Therefore, the present model partially differs from previously introduced animal models using umbilico-placental embolization provoking not only severe hypoxemia but also affecting placental blood supply.

As shown here, a period of 3.5 days of intrauterine hypoxia is sufficient to induce a significant growth restriction with respect to body weight, length and fronto-occipital diameter in mice. Although lung weights have not been determined, previous studies, exposing pregnant rats to a longer time period of hypoxia (14–21 days of gestation), have shown lung volumes and weights to be significantly different in fetuses from hypoxic and pair-fed mothers compared to controls fed ad libitum. In relation to the body weight, however, lungs were of proportional size. Furthermore, morphometric analysis of lung tissue also showed no difference between the groups with regard to the volume of saccular air spaces and saccular/alveolar wall thickness [18, 19]. This is in agreement with our data on lung histology where the number of saccular air spaces and septal thickness were not found to be significantly different. Nevertheless, apart from auxological and pulmonary morphometric data, this report is the first to describe significant effects of solely hypoxia induced IUGR on the expression of genes encoding for SP-A, -B, -C, and -D.

As SP are known to reveal distinct developmental patterns observed in fetal lung tissue of human and rodents [20], down-regulation of SP-A, -B and -C mRNA expression potentially reflects hypoxia-induced delay in lung maturation. Clinically, a reduced synthesis of SP may

have potential implications for postnatal pulmonary adaptation and the risk for the development of chronic lung disease (BPD) in preterm neonates.

Tokieda et al. [21] observed that a complete deletion of the SP-B gene led to lethal respiratory failure shortly after birth in an animal model which is in line with findings in human neonates. Thus, a significant reduction in SP-B mRNA expression in lung tissue from fetuses subjected to intrauterine hypoxia may imply severe restrictions on postnatal pulmonary adaptation. In addition, it has recently been demonstrated that the absence of SP-C or structural effective forms could cause chronic interstitial pneumonitis or even postnatal respiratory failure due to impaired intracellular processing of other SP (for review see [22, 23]). Thus, a reduced synthesis of SP-C observed in preterm mice may lead to postnatal pulmonary disorders.

Regarding the hydrophilic SP-A and -D, these are described to be major factors of the innate pulmonary host defense [24]. Experimental deletion of the SP-A gene does not affect postnatal pulmonary adaptation, but nevertheless, it leads to an increased susceptibility for bacterial infections and the development of pulmonary emphysema [24, 25]. As pulmonary SP-A mRNA expression in preterm mice was significantly reduced, this reveals a potential role for hypoxia-induced IUGR on the pulmonary innate immune response and the development of chronic pulmonary disorders in premature infants.

The observation that IUGR was associated with a down-regulation of SP gene expression seems to be in contrast to previous studies in fetal sheep [7, 10, 26]. However, the stage of lung development investigated differs between the animal models. In the present study, mice delivered at day 17.5 of gestation (term 19–21 days; 0.87 of gestation) were found to be in the saccular stage of lung development and not yet in the transition to alveolarization [27]. Our model therefore corresponds to human fetuses of about 25 weeks gestational age. This difference also affects the stage of type II cell differentiation and thus surfactant production.

Confirming the important impact of hypoxia on fetal development previous studies have shown profound alterations of the hypothalamic-pituitary-adrenal axis as well as lung maturation processes (for review see [28]). These effects have been shown to partially depend on the stage of fetal or neonatal development. This underlines the importance of assessing the effects of intrauterine hypoxia on developmental processes in relation to the gestational age of the fetus.

As another important variable influencing lung developmental processes and overall outcome in premature infants, the effect of intrauterine endotoxin exposure (i.e. chorioamnionitis) has been demonstrated to be dependent on the stage of lung maturation [29–31]. Thus, a significant interaction between delayed lung maturation due to IUGR and the impact of other perinatal factors as chorioamnionitis on pulmonary development can be presumed in the immature preterm infant. Furthermore, the indicated effects have to be discussed against the background of the complex interaction in the fetal-maternal unit, considering a potential affection of other growth factors by hypoxia-induced IUGR. There are different essential loci where oxygen signalling pathways may cue the development of respiratory structures like mitochondrial biogenesis coupled with muted oxidative function dependent on the hypoxia-sustained production of nitric oxide as well as the proliferation and epithelial/endothelial differentiation of mesenchyme during the initiation of lung morphogenesis (for review see [32]).

In conclusion, intrauterine hypoxia-induced growth restriction in preterm mice led to significant alterations of the pulmonary surfactant system during fetal development suggesting consequences for postnatal lung adaptation and differentiation. Potential mediators of the indicated effects need to be further investigated.

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PART III

CLINICAL ASPECTS OF BRONCHOPULMONARY DYSPLASIA

chapter 7

Increased risk of bronchopulmonary dysplasia and increased mortality in very preterm infants being small for gestational age

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Increased risk of bronchopulmonary dysplasia and increased mortality in very preterm infants being small for gestational age

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Abstract *Objective:* The objective was to evaluate the impact of being born small for gestational age (SGA) on neonatal mortality and neonatal pulmonary morbidity in preterm infants <32 weeks of gestation. *Methods:* We reviewed the data reported prospectively to the quality assurance program of the Federal State of Hesse, Germany, from 1990 to 1996 of infants <32 weeks of gestation. SGA was defined as birth weight below the 10th percentile. Mann Whitney U tests were used to compare continuous variables and Fisher's exact tests to analyze differences in dichotomous variables between preterm SGA neonates and preterms born appropriate for gestational age (AGA). The effect of SGA and other potential risk factors for neonatal death and bronchopulmonary dysplasia, i.e., requiring a fraction of inspired oxygen >0.21 at day 28, was tested by multivariable analyses. *Results:* Data from 1,365 infants were analyzed. One hundred and eighty-three neonates were SGA (mean [SD] birth weight 789 [179] g; mean [SD] gestational age 28.9 [1.7] weeks) and 1,182 were AGA (mean [SD] birth weight 1,260 [348] g; mean [SD] gestational age 28.8 [2.1] weeks). Neonatal mortality and the rate of bronchopulmonary dysplasia were significantly higher in SGA neonates (23 vs. 11% and 28 vs. 14%, respectively). There was a statistically significant association of SGA with neonatal death (odds ratio [OR] = 4.54, 95% confidence interval [CI] 2.56, 8.04) and bronchopulmonary dysplasia (OR=3.80, 95% CI 2.11, 6.84). *Conclusion:* SGA neonates below 32 weeks gestation are a high-risk group regarding neonatal mortality and neonatal pulmonary morbidity.

Keywords Intrauterine growth restriction · Small for gestational age · Respiratory distress syndrome · Bronchopulmonary dysplasia · Prematurity

Introduction

Neonates born with a birth weight below the 10th percentile generally are classified as small for gestational age (SGA) infants. In most cases this condition is attributed to an intrauterine growth restriction. SGA infants represent a significant percentage of infants admitted to neonatal intensive care units. This especially holds true for SGA infants <32 weeks gestational age in whom an increased incidence of neonatal complications was reported recently [4, 8]. However, studies focusing on the influence of SGA on neonatal morbidity associated with prematurity showed controversial results.

With regard to neonatal pulmonary morbidity, data from the 1970s and 1980s suggested lower rates of respiratory distress syndrome (RDS) in very preterm SGA neonates [13]. In contrast, several recent studies revealed no increased risk of RDS [2, 8, 14] or even a significantly increased risk of RDS [4] in very preterm SGA neonates. Up to now, only two studies focusing on the risk of developing bronchopulmonary dysplasia have been published. Both demonstrate an increased risk of bronchopulmonary dysplasia in very preterm SGA infants compared with preterm neonates being born appropriate for gestational age (AGA) [2, 8]. Recently, Egreteau et al. [6] showed being born SGA to be one of the major risk factors for chronic oxygen dependency in infants born before 32 weeks gestation.

Therefore, our aim was to evaluate neonatal mortality and pulmonary morbidity in very preterm neonates being born SGA compared with AGA preterms obtained from a large representative study sample.

Materials and methods

We examined the morbidity and mortality in SGA neonates compared with AGA neonates both being born after <32 weeks of gestation using the perinatal and neonatal databases recorded by the quality assurance system of the Federal State of Hesse, Germany, from January 1990 to December 1996. All Hessian hospitals are obliged to report perinatal data to the Hessian quality assurance system center. Obstetrical data, e.g., prenatal corticosteroid administration, preterm premature rupture of membranes (PROM), Apgar score, and umbilical artery pH, have to be reported by the institution where the child was born. If a newborn has to be transferred to a pediatric hospital for further neonatal care, neonatal data have to be reported by the institution the child was transferred to, using an additional questionnaire. Thus for each child two datasets are recorded independently. Trained physicians fill out independently from each other the perinatal, as well as the neonatal datasets. Both, the neonatal and the perinatal datasets have to be sent to the Hessian quality assurance system center. We connected both different datasets. All variables that had to be recorded in the neonatal as well as in the perinatal questionnaire, i.e., date and time of birth, calculated time of birth, gestational age, single or multiple gestation, zip code, gender, and birth weight, had to coincide completely. The sets of data, which did not completely coincide in all these variables, were excluded from the study. Also, we excluded all datasets that were filled out incompletely. We expected a relatively high "drop-out" rate due to incomplete data in either the perinatal or the neonatal dataset. Thus, in a second step, we compared the variables gestational age, being SGA, RDS, bronchopulmonary dysplasia, and mortality between all infants born below 32 weeks of gestation and the infants born below 32 weeks of gestation whose perinatal and neonatal datasets could be connected. Moreover, we compared the variables gestational age, RDS, bronchopulmonary dysplasia, and mortality between all SGA infants below 32 weeks gestation and the SGA infants below 32 weeks gestation whose perinatal and neonatal datasets could be connected.

Infants with lethal malformations and chromosomal aberrations were excluded from the study. Multiple gestations were not excluded from the study, but the variables "twin" and "multiple pregnancy $\geq$3" were included in the multivariable analyses identifying risk factors for being born SGA, for neonatal mortality, and for bronchopulmonary dysplasia.

Perinatal variables were defined as follows: The calculation of gestational age was based on maternal menstrual history or early prenatal ultrasound. SGA was defined as birth weight below the 10th percentile according to the percentiles published by Voigt et al. [17] for German newborns. Preterm PROM was diagnosed when the membranes had ruptured more than 12 h before birth. Preeclampsia was diagnosed according to the criteria established by the American College of Obstetricians and Gynecologists [1]. A complete course of prenatal corticosteroids (two doses of betamethasone) was assumed if the first dose was administered $\geq$24 h before birth and less than one week from birth. Pregnancy induced hypertension was diagnosed when an arterial blood pressure >140/90 Torr was measured repeatedly. The mode of delivery, insufficiency of the placenta, preterm labor, and a pathological cardiotocogram were additionally recorded. Insufficiency of the placenta was detected by pathological flow velocity waveforms in the umbilical artery. Pathological cardiotocogram was observed and described by deceleration, lack of acceleration, and increased baseline fetal heart rate over 170 bpm.

Neonatal characteristics of study infants included the following variables: Apgar score below 7 after 5 min, umbilical artery pH below 7.1, need for intubation and days on mechanical ventilation (including continuous positive airway pressure), intratracheal surfactant administration, RDS (based on radiographic criteria [5]), duration of oxygen dependency (days), oxygen requirements or mechanical ventilation for pulmonary reasons on day 28 (i.e., bronchopulmonary dysplasia), duration of mechanical ventilation (<3 days, 3–7 days, and >7 days), intraventricular hemorrhage grades III and IV (according to the criteria of Papile et al. [11]), periventricular leukomalacia [9], necrotizing enterocolitis [3], retinopathy of prematurity diagnosed according to the criteria of the International Committee [10], and patent ductus arteriosus [7]. Probable sepsis was defined as the presence of characteristic clinical signs and typical laboratory data (positive blood culture, abnormal differential blood count or increased C-reactive protein). This definition did not allow us to distinguish between neonatal sepsis with or without positive blood culture. Furthermore, the neonatal mortality, i.e., death until day 28, and the total number of hospital days until discharge were compared between both groups.

We used the SPSS statistical program (version 8.0) for statistical analyses. To compare differences between groups, *t*-tests were used for continuous variables when normally distributed; otherwise the Mann-Whitney U-test was used. Dichotomous variables were analyzed by χ^2-test. The level of significance was set at $p<0.05$. The effect of the variables preterm labor, preterm PROM, placental insufficiency, pregnancy induced hypertension, maternal age, multiple pregnancy, and chorioamnionitis on the risk of delivering an SGA neonate were investigated by multiple logistic regression analyses. The effect of birth weight below the 10th percentile and other possible risk factors on bronchopulmonary dysplasia or death was investigated by multiple logistic regression analyses as well. The level of significance was set at $p<0.05$. Results of the multivariable analyses were expressed as odds ratios (ORs) with 95% confidence intervals (CIs).

The present study was approved by the review board of the Institute of Quality Assurance of the Federal State of Hesse, Germany.

Results

A total of 37,393 neonates were reported by Hessian pediatric hospitals between January 1990 and December 1996 to the Hessian neonatal database and 2,341 (6.3%) out of these were born before 32 weeks of gestation. Variables obtained within the perinatal as well as within the neonatal datasets coincided in 1,365 (58.3%) infants out of these 2,341. Thus, a total of 1,365 infants were included in this analysis. There were no statistically significant differences in the variables gestational age, birth weight, being born SGA, RDS, bronchopulmonary dysplasia, and mortality between all 2,341 infants born below 32 weeks of gestation and the preterm infants whose perinatal and neonatal datasets could be connected (data not shown). There were also no statistically significant differences between all SGA infants below 32 weeks gestation and those with combined peri- and neonatal datasets with respect to basic neonatal and outcome variables (mean [SD] gestational age: 28.8 [1.8] vs. 28.9 [1.7] weeks; neonatal mortality: 24.2 vs. 23%; RDS: 57.3 vs. 53.9%; bronchopulmonary dysplasia: 21.8 vs. 28.4%). Out of the total of 1,365 infants born before 32 weeks of gestation, 183 (13.4%) neonates were SGA. The basic perinatal characteristics of SGA infants and AGA infants are given in Table 1. The neonatal mortality was significantly higher in SGA infants.

Among all variables included in the multivariable analysis identifying risk factors for delivering an SGA neonate, only the variable placental insufficiency was associated with an increased risk for delivering an SGA neonate (OR 6.6, CI 4.2, 10.2).

The neonatal morbidity of the surviving infants is shown in Table 2. The mean duration of mechanical

Table 1 Basic perinatal variables in small for gestational age (SGA) infants and appropriate for gestational age (AGA) infants

	SGA infants (*n*=183)	AGA infants (*n*=1,182)	*p*
Gestational age (weeks)	28.9 (1.7)[a]	28.8 (2.1)[a]	0.731
Birth weight (g)	788.7 (178.7)[a] (380–1,230)[b]	1,259.5 (348.1)[a] (440–2,445)[b]	<0.001
Gender (male/female); *n* (%)	107/76 (59/41)	640/542 (54/46)	0.300
Maternal age (years)	28.7 (5.0)[a]	29.1 (5.7)[a]	0.889
Prenatal corticosteroids; *n* (%)	105 (57)	669 (57)	0.873
Placental insufficiency; *n* (%)	85 (46)	103 (9)	<0.001
Pregnancy induced hypertension; *n* (%)	43 (24)	85 (7)	<0.001
Preeclampsia; *n* (%)	54 (30)	128 (11)	<0.001
Preterm PROM; *n* (%)	17 (9)	279 (24)	<0.01
Pathological cardiotocogram; *n* (%)	110 (60)	330 (28)	<0.001
Preterm labor; *n* (%)	84 (46)	696 (59)	<0.01
Cesarean section; *n* (%)	171 (93)	928 (79)	<0.001
Apgar (5 min <7); *n* (%)	37 (23)	217 (20)	0.162
Umbilical artery pH <7.1; *n* (%)	8 (4)	34 (3)	0.253
Neonatal mortality; *n* (%)	42 (23)	128 (11)	<0.001

[a] Mean (SD)
[b] Ranges

Table 2 Neonatal morbidity in surviving SGA infants and AGA infants

	SGA (*n*=141)	AGA (*n*=1,054)	*p*
Respiratory distress syndrome; *n* (%)	76 (54)	583 (55)	0.735
Surfactant therapy; *n* (%)	56 (44)	359 (38)	0.858
Need for mechanical ventilation[a]; *n* (%)	112 (79)	764 (73)	0.303
Days on the ventilator[a]	16.4 (14.9)[b]	13 (14.5)[b]	<0.05
Bronchopulmonary dysplasia; *n* (%)	40 (28)	145 (14)	<0.001
Hospitalization (days)	93.3 (60.9)[b]	64.7 (32.9)[b]	<0.001
Periventricular leukomalacia; *n* (%)	1 (1)	29 (3)	1.0
Intraventricular hemorrhage grades III and IV; *n* (%)	4 (3)	54 (5)	1.0
Retinopathy of prematurity; *n* (%)	26 (18)	134 (13)	0.385
Patent ductus arteriosus; *n* (%)	24 (17)	140 (13)	0.367
Necrotizing enterocolitis; *n* (%)	12 (9)	53 (5)	0.232

[a] Includes days on continuous positive airway pressure
[b] Mean (SD)

Table 3 Multivariable analysis predicting mortality for entire cohort (*n*=1,365)

Risk factor	Odds ratio	95% CI
Birth weight <10th percentile	4.54	2.56, 8.04
Gestational age	0.56	0.50, 0.62
Multiple pregnancy (twin)	1.27	0.74, 2.20
Multiple pregnancy (≥3)	1.19	0.41, 3.41
Need for mechanical ventilation[a]	1.64	0.67, 4.00
Intraventricular hemorrhage grade III and IV	3.36	1.80, 6.30
Sex female	0.64	0.42, 0.98
Prenatal corticosteroid administration	0.94	0.75, 1.18
Pregnancy induced hypertension	0.77	0.40, 1.47
Placental insufficiency	0.89	0.40, 1.96
Preterm rupture of membrane	0.82	0.51, 1.33
Preterm labor	1.03	0.67, 1.61
Cesarean section	0.90	0.52, 1.56
Probable sepsis	1.67	1.10, 2.59
Periventricular leukomalacia	0.74	0.41, 1.35
Patent ductus arteriosus	0.30	0.16, 0.57
Surfactant therapy	2.76	1.66, 4.59

[a] Includes continuous positive airway pressure

Table 4 Multivariable analysis predicting bronchopulmonary dysplasia for survivors (*n*=1,195)

Risk factor	Odds ratio	95% CI
Birth weight <10th percentile	3.80	2.11, 6.84
Increasing gestational age (weeks)	0.67	0.60, 0.76
Gender female	0.63	0.41, 0.95
Prenatal corticosteroid administration	0.89	0.72, 1.11
Preterm premature rupture of membrane	1.16	0.74, 1.82
Preterm labor	0.93	0.61, 1.42
Cesarean section	0.83	0.48, 1.44
Days on the ventilator <3 days	7.79	0.89, 68.06
Days on the ventilator 3–7 days	15.16	1.96, 117.16
Days on the ventilator >7 days	63.88	8.51, 479.39
Probable sepsis	1.56	1.02, 2.38
Patent ductus arteriosus	1.49	0.92, 2.43
Surfactant therapy	1.57	1.01, 2.43
Multiple pregnancy (twin)	1.08	0.62, 1.87
Multiple pregnancy (≥3)	0.37	0.10, 1.43

ventilation as well as the incidence of bronchopulmonary dysplasia was significantly higher, and the mean duration of hospitalization was significantly longer in SGA infants compared with AGA infants.

By multivariable analyses, birth weight below the 10th percentile, low gestational age, need for ventilation including continuous positive airway pressure, and intraventricular hemorrhage grades 3 and 4 were identified as risk factors for an increased neonatal mortality (Table 3).

Birth weight below the 10th percentile, low gestational age, male gender, and duration of ventilation were shown to increase the risk of developing bronchopulmonary dysplasia (Table 4).

Discussion

Intrauterine growth restriction implicates an intrauterine growth pattern that deviates from the genetically determined growth percentile. Measuring intrauterine growth repeatedly by ultrasonography is the only way to prove a pathologic intrauterine growth pattern. Therefore, one has to consider that in our study as well as in other studies, neonates are defined as "SGA", although their birth weight is below the 10th percentile due to genetic reasons rather than due to a pathologic pregnancy. On the other hand, neonates with a pathologic intrauterine growth pattern not resulting in a birth weight below the 10th percentile are not defined as "SGA".

Only complete datasets from both, the perinatal and the neonatal survey have been taken in account in our final analysis. Considering this criterion, 58.3% of all datasets could be connected. We aimed to exclude the possibility that our results are biased by the high loss rate, by demonstrating that there were no statistically significant differences in main perinatal and neonatal characteristics and outcome variables between all 2,341 neonates and the 1,365 neonates, whose datasets could be connected. We further showed that there are no statistically significant differences in main perinatal and neonatal characteristics and outcome variables between all SGA neonates below 32 weeks of gestation and SGA neonates below 32 weeks of gestation whose perinatal and neonatal datasets could be connected.

The present study indicates being born SGA to result in an increased neonatal mortality in preterm infants. This finding is in accordance with several other studies [2, 4, 8, 12, 15, 16]. Our analyses showed birth weight below the 10th percentile to be associated with an about five-fold increased risk of death during the neonatal period.

Few studies investigate the impact of SGA on developing bronchopulmonary dysplasia. We therefore aimed to obtain further data focusing on pulmonary neonatal morbidity in SGA infants <32 weeks based on data derived from a large representative population-based study. The incidence of RDS and need for surfactant administration did not differ between SGA infants and AGA infants. Thus, we provide further evidence that being SGA does not protect against RDS. However, our data did show an increased pulmonary morbidity in SGA neonates below 32 weeks gestation. In addition to a significantly longer duration of ventilation, 28% within the SGA group compared with 14% within the AGA group developed bronchopulmonary dysplasia. By multivariable analyses, we identified the variable "being born SGA" to increase the risk of developing bronchopulmonary dysplasia 4-fold. This result is in accordance with a recently published study which shows intrauterine growth restriction to be one of the most important risk factors for prolonged oxygen requirements in infants born at less than 32 weeks gestation [6].

In conclusion, our data showed no differences between SGA infants and AGA infants in variables describing the early course of neonatal lung disease, i.e., RDS and need for surfactant therapy, whereas the incidence of bronchopulmonary dysplasia was significantly higher in the SGA group. SGA neonates below 32 weeks gestation thus represent a high-risk group regarding neonatal mortality and neonatal pulmonary morbidity.

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chapter 8

Bronchopulmonary dysplasia and intrauterine growth restriction

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Bronchopulmonary dysplasia and intrauterine growth restriction

The pathogenesis of bronchopulmonary dysplasia (BPD) is known to be multifactorial. We would like to add a further factor, not mentioned in the Seminar by John Kinsella and colleagues (April 29, p 1421),[1] that modulates the risk and pathogenesis of BPD.

Intrauterine growth restriction increases the risk of BPD and prolongs the duration of mechanical ventilation in preterm infants younger than 32 weeks of gestation.[2] These findings have been confirmed in population-based studies.[3,4] Preterm infants younger than 28 weeks and born small for gestational age are known to be at especially high risk compared with age-matched infants without obvious signs of impaired intrauterine growth.[3,4]

The main mechanisms to explain how intrauterine growth restriction modulates the risk of a BPD have been described in animals. For example, in experimentally growth-restricted preterm lambs, impaired growth of terminal airways and gas exchange units has been shown. Furthermore, growth restriction leads to reduced expression of surfactant protein mRNA and could induce increased inflammatory activation.[5]

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chapter 9

Prolonged mechanical ventilation induces pulmonary inflammation in preterm infants

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Prolonged Mechanical Ventilation Induces Pulmonary Inflammation in Preterm Infants

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Key Words

Chronic lung disease · Elastase · ENA-78 · Interleukin 8 · Interleukin 10 · Mechanical ventilation · Preterm infants

Abstract

Lung inflammation plays an important role in the pathogenesis of chronic lung disease in preterm infants. To test the hypothesis that prolonged mechanical ventilation induces pulmonary inflammation, we analyzed pro- and anti-inflammatory mediators in bronchoalveolar lavage fluid obtained from ventilated preterm infants having respiratory distress syndrome. Our results show a strong correlation between the duration of mechanical ventilation and the amount of proinflammatory mediators. However, the anti-inflammatory cytokine interleukin 10 remained stable during the whole period of mechanical ventilation. These data support the hypothesis that prolonged mechanical ventilation contributes to the development of chronic lung disease by the induction of lung inflammation without adequate stimulation of the counterregulatory cytokine interleukin 10 in preterm infants with respiratory distress syndrome.

Introduction

A significant proportion of preterm infants less than 28 weeks of gestational age require mechanical ventilation due to respiratory distress syndrome (RDS). Approximately 20% of the preterm infants <1,000 g birth weight develop chronic lung disease (CLD) which contributes profoundly to mortality and morbidity of preterm infants. Factors such as barotrauma, oxygen toxicity, and immaturity of lung anatomy have been implicated in the development of CLD [1]. However, there is growing evidence that inflammatory lung injury may play a key role in its pathogenesis. It has been demonstrated that elevated levels of proinflammatory cytokines in bronchoalveolar lavage (BAL) fluid (BALF) can predict the development of CLD [2, 3]. Several studies have shown that RDS and mechanical ventilation were associated with increased recruitment of inflammatory cells within the airways [4]. In adult patients with acute RDS, mechanical ventilation can induce a systemic and local inflammatory response, as demonstrated by an increase of cytokine levels in serum and BALF under the influence of injurious ventilatory strategies [5, 6].

Patients and Methods

To test the hypothesis that prolonged mechanical ventilation induces pulmonary inflammation in preterm infants, we analyzed the proinflammatory mediators interleukin (IL) 8, elastase, and epithelial neutrophil-activating peptide 78 (ENA-78) as well as the anti-inflammatory cytokine IL-10 in BALF by enzyme-linked immunoassay during the first 2 weeks of life in conventional mechanically ventilated preterm infants. Due to the fact that most infants could already be extubated after a few days of ventilation we recruited only 20 preterm infants for this study (median and range: gestational age 27 and 23–31 weeks, birth weight 980 and 460–1,570 g). Respiratory failure was caused by RDS in all infants investigated. Premature rup-

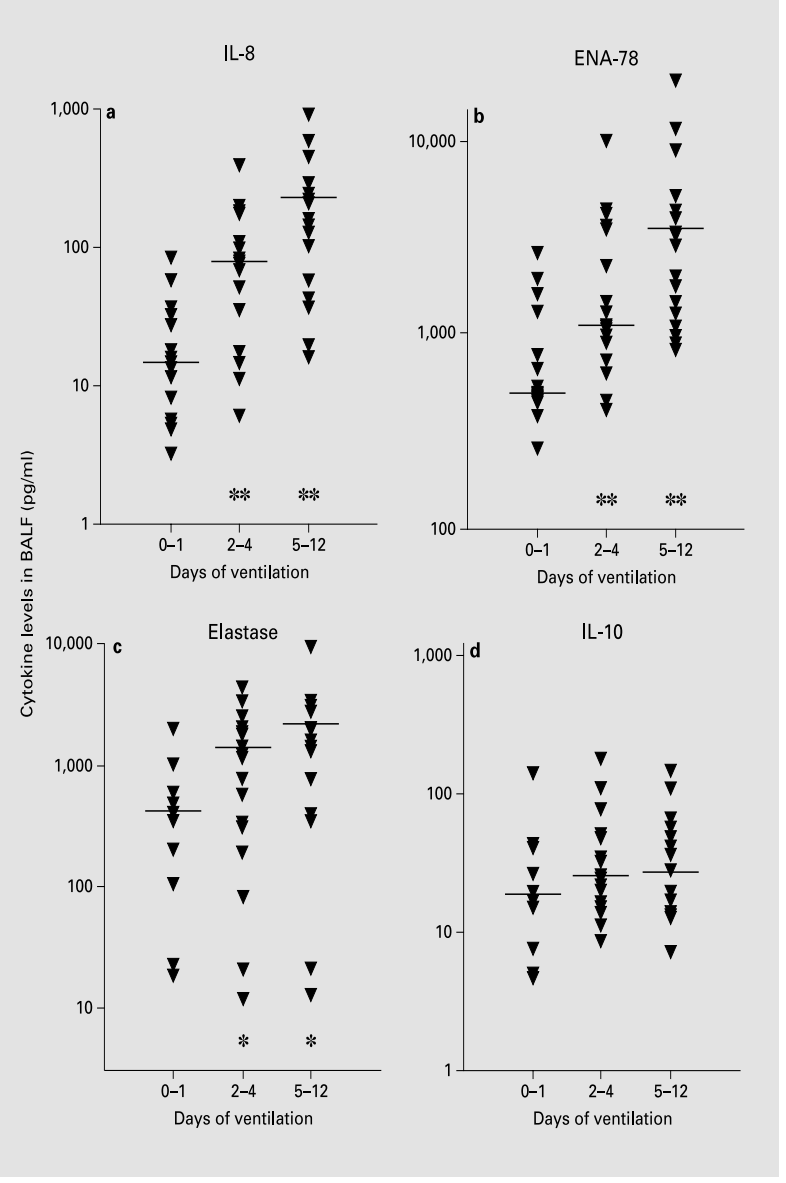

Fig. 1. Pro- and anti-inflammatory mediators in BALF during mechanical ventilation of preterm infants. The amounts of IL-8 (**a**), ENA-78 (**b**), and elastase (**c**) in BALF obtained from preterm infants steadily increased during mechanical ventilation. In contrast, the amount of IL-10 (**d**) in BALF remained stable during the whole period of mechanical ventilation. The numbers of preterm infants investigated were 18 at 0–1 days, 20 at 2–4 days, and 19 at 5–12 days. Data are presented as a vertical point plot on a logarithmic scale; the median is indicated by a line. * $p < 0.05$ and ** $p < 0.001$ as compared with ventilation on days 0–1 using the Wilcoxon signed-rank test.

ture of membranes was found in 7 out of 20 cases, an elevated C-reactive protein concentration in 9 out of 20 cases. Exclusion criterion was a ventilation period <5 days. For ethical reasons, a control group of healthy preterm or term infants could not be recruited. The BALF was obtained according to the guidelines on BAL in neonates [7]. An aliquot of 1 ml/kg of normal saline was instilled using an end-hole catheter into the right lower lobe. The fluid recovered was immediately placed on ice and processed within a few hours after collection. Supernatants were stored at −70°C until analysis. Secreted components in BALF were standardized to secretory immunoglobulin A which is not affected by gestational age, lung injury, or permeability [8]. A BAL was done only if indicated either before surfactant application or if pulmonary secretions made ventilation difficult. Consequently, most BALs were done during the first days of ventilation with greater intervals later on. Furthermore, we expected from our own in vitro data an early increase of proinflammatory mediators already after a few hours [9]. Therefore, data obtained from preterm infants were grouped into 0–1, 2–4, and 5–12 days of ventilation (fig. 1). Statistical differences were tested by the nonparametric Wilcoxon test for paired data and by the Mann-Whitney U test for unpaired data. Correlation analysis was done with a Spearman rank test. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 9.0 (SPSS, Chicago, Ill., USA). The study has been approved by the institutional ethics committee.

Results and Discussion

All proinflammatory mediators increased significantly in BALF during mechanical ventilation in preterm infants (fig. 1a–c). Furthermore, there was a strong correlation between IL-8 ($r = 0.67$, $p < 0.0001$), elastase ($r = 0.58$, $p < 0.0001$), as well as ENA-78 ($r = 0.49$, $p < 0.0001$) and the duration of ventilation in preterm infants without infection, indicating a direct relationship between mechanical ventilation and inflammation. This is of special concern because proinflammatory cytokines were related to the pathogenesis of diverse neonatal diseases such as CLD, intraventricular hemorrhage, periventricular leukomalacia, and necrotizing enterocolitis [10, 11]. Therefore, mechanical ventilation may contribute significantly to the induction of these diseases in preterm infants. Surprisingly, there was no correlation between severity of RDS as defined by Giedion et al. [12], elevation of C-reactive protein, and the level of proinflammatory mediators in BALF during prolonged ventilation (data not shown). This observation supports the role of mechanical ventilation in the induction of pulmonary inflammation independent of RDS or infection. However, a multivariance analysis could not be done due to the low number of preterm infants investigated. In contrast, the anti-inflammatory cytokine IL-10 remained stable during the whole period of mechanical ventilation in preterm infants (fig. 1d). It has been demonstrated by others that neonates have a reduced capacity to produce this anti-inflammatory cytokine in RDS [2] and after endotoxin challenge [13]. Decreased or absent IL-10 production offers a theoretical basis for dysregulated and persistent inflammation in the lungs of preterm infants with RDS and predisposes these infants toward the development of CLD.

In conclusion, we show an association between prolonged mechanical ventilation and pulmonary inflammation in ventilated preterm infants with RDS. Because a direct relationship between injurious ventilatory strategies and pulmonary inflammation has already been demonstrated in adults [5, 6] we hypothesize from our data that prolonged mechanical ventilation induces pulmonary inflammation in preterm infants as well, but fails to stimulate the anti-inflammatory cytokine IL-10 adequately. The susceptibility of the immature lung to CLD could thus be a consequence of a developmentally regulated imbalance between pro- and anti-inflammatory mediators in favor of the proinflammatory cascade with its potential to induce tissue injury.

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chapter

10

Plasma arginine and urinary nitrate and nitrite excretion in bronchopulmonary dysplasia

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Plasma Arginine and Urinary Nitrate and Nitrite Excretion in Bronchopulmonary Dysplasia

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Key Words
Bronchopulmonary dysplasia · Preterm infant · Plasma amino acids · Pulmonary hypertension

Abstract
The aim of this prospective study was to determine whether preterm infants with bronchopulmonary dysplasia (BPD) and signs of increased pulmonary artery pressure have a deficiency of plasma arginine (ARG) and systemic nitric oxide (NO) synthesis. Plasma amino acid concentrations, Doppler pulmonary systolic time intervals (ratio of acceleration time and ejection time corrected for heart rate: AT/ET_C) and urinary nitrate and nitrite concentrations were determined at the 28th day postnatal age and at 36 weeks postmenstrual age in 73 preterm infants less than 30 weeks gestational age. The AT/ET_C ratios were significantly lower in infants with BPD (n = 32) compared to controls. However, total amino acid concentrations, ARG intake as well as plasma ARG concentrations were not different between groups (median (interquartile-range) μmol/l): control: 58 (42.5–75.5) and 54.5 (42–71) at day 28 and 36 weeks; BPD: 54.5 (31.5–70.5) and 43 (35–62), respectively. Urinary nitrate and nitrite concentrations, were not different between groups at day 28, but significantly higher in infants with BPD at 36 weeks (p = 0.014). In conclusion, plasma ARG concentrations and systemic NO synthesis were not deficient in preterm infants with BPD and signs of elevated pulmonary artery pressure.

Introduction

L-Arginine (ARG) is the physiological substrate of the enzyme-catalyzed synthesis of nitric oxide (NO) by various NO synthase isoforms. NO-related functions include regulation of vascular tone and hemodynamics. NO-independent effects of ARG include synthesis of creatine, proline and polyamines, secretion of insulin, growth hormones, glucagon and prolactin, as well as effects on coagulation and immune function [1].

Low plasma ARG concentrations have been reported in acute neonatal diseases such as persistent pulmonary hypertension, respiratory distress syndrome and necrotizing enterocolitis [2–6]. Dietary ARG supplementation may represent a novel nutritional strategy for preventing and treating cardiovascular diseases of adults [7]. New insights into the pathogenesis of bronchopulmonary dysplasia (BPD), the most frequent chronic neonatal disease, indicate that BPD in preterm infants is characterized by early endothelial cell damage and impaired vascular growth, and thus, may be regarded as a vascular disease

[8]. Infants with BPD are at risk to develop pulmonary hypertension. In chronic lung disease, the increase in pulmonary artery pressure could be explained partly by a loss of the ability of the pulmonary endothelium to maintain the production of vasodilating substances [9].

The aim of this prospective study was to investigate whether plasma concentrations of ARG (as the only physiological precursor of NO) are lower in preterm infants with BPD and signs of elevated pulmonary artery pressure compared to unaffected infants. Furthermore, the renally excreted end-products of the oxidative metabolic pathway of NO, nitrate and nitrite were determined as a measure of whole-body NO synthesis.

Patients and Methods

The study was approved by the Ethics Committee of the University of Giessen and written informed parental consent was obtained.

Patients

Preterm infants with a gestational age of less than 30 weeks (assessed by obstetrical dating and/or a scoring system) were eligible for the study. Exclusion criteria were major congenital anomalies, congenital heart disease, except patent ductus arteriosus or persistent foramen ovale, inborn errors of metabolism, evidence of liver dysfunction (alanine aminotransferase greater than three times the upper limit of normal or direct hyperbilirubinemia) and exchange transfusion during the study period.

BPD was defined as the need for supplemental oxygen at the age of 28 days to reach a capillary oxygen saturation greater than 86% (Hewlett Packard CMS-Monitor with Oxisensor II^{TM}, Nellcor Incorporated; Hewlett Packard GmbH, Böblingen, Germany) and characteristic radiographic appearance of chronic lung disease [10]. The scoring system by Palta et al. [11] was used to assess the severity of BPD.

For the first time, infants were studied on postnatal day 28 to detect plasma ARG concentrations early. Again, infants were studied subsequently at 36 weeks postmenstrual age to take the sequel of BPD into account.

Echocardiography

The Doppler and echocardiographic examinations were performed using either a Hewlett Packard Sonos 2500 or Sonos 5000 ultrasound system and a 12-MHz multifrequency imaging transducer. Pulmonary artery pressure was assessed non-invasively using Doppler echocardiography. After a preliminary cross-sectional echocardiographic examination to exclude structural defects, a two dimensional image of the main pulmonary artery and pulmonary valve leaflets was obtained from a modified parasternal short axis view. A Doppler signal was recorded from the center of the main pulmonary artery immediately distal to the valve leaflets. Determinations of acceleration time (AT, time interval between the systolic Doppler waveform leaving the zero flow baseline and reaching its peak velocity) and ejection time (ET, time interval between the systolic Doppler waveform leaving and returning to the zero flow baseline) were obtained from at least five consecutive Doppler velocity waveforms [12]. The mean AT/ET ratio was calculated. The AT/ET ratio was divided by the square root of the R-R interval from the simultaneously recorded ECG (AT/ET_C) to reveal a value corrected for heart rate. Doppler examinations were performed by a single observer (M.H.) and infants were studied while asleep or during a period of quiet wakefulness.

Laboratory Analyses

Blood samples were immediately centrifuged and plasma was stored at –70°C until plasma amino acid analysis by ion-exchange column liquid chromatography (Biotronik LC 3000, Eppendorf, Hamburg Germany, with automatised data analysis by EZ Chrom Chromatography Data System).

The renally excreted end-products of the oxidative metabolic pathway of NO, nitrate and nitrite were determined as a measure of whole-body NO synthesis [13]. Spot urine specimens were collected using sterile plastic bags. After centrifugation urinary nitrite and nitrate concentrations were determined by Gas chromatography-mass spectrometry (GC-MS) as described previously [14]. The most frequently applied Griess assay may lead to an underestimation of urinary nitrate concentrations, unlike the GC-MS method, which is accurate and free of interferences [14]. Urine study samples were analyzed in three runs, concomitantly with each of three quality control (QC1, QC2, QC3) samples, which were analyzed in duplicate. QC1 sample was analyzed without external addition of nitrite and nitrate, QC2 sample was spiked with 4 μM of nitrite and 400 μM of nitrate; QC3 sample was spiked with 8 μM of nitrite and 800 μM of nitrate. The concentrations of the internal standards [^{15}N]nitrite and [^{15}N]nitrate were 8 and 800 μM in all samples. Mean recovery (accuracy) of the method for urinary nitrite and nitrate was 90 and 99% in the QC2 sample, and 89 and 101% in the QC3 sample. Method's precision (expressed in terms of relative standard deviation) was below 10.2% for nitrite and below 1.3% for nitrate in the QC samples. Creatinine was measured by the Jaffé method. A urinary tract infection was excluded by microbiological culture in infants with elevated C-reactive protein at the time of urinary collection.

Nutrition

Formula-fed infants received Humana O (Humana GmbH, Herford, Germany) or Pre-Aptamil (Milupa GmbH, Friedrichsdorf, Germany) containing 51.8 mg/100 ml (0.30 mmol/100 ml) or 50 mg/100 ml (0.29 mmol/100 ml) of ARG, respectively. In breast-fed infants arginine intake was calculated according to the arginine content of pooled breast milk from mothers with preterm infants (54 mg/100 ml equivalent to 0.31 mmol/100 ml) [15]. Additionally, FM 85 was given as a protein supplement (5% solution containing 24.2 mg/100 ml equivalent to 0.14 mmol/100 ml ARG; Nestle AG, Germany). Parenteral nutrition contained AminopädP (Baxter GmbH, Unterschleissheim, Germany) administered as a 10% amino acids mixture containing of 910 mg/100 ml (5.2 mmol/100 ml) of ARG. The study protocol did not regulate the nutritional management of the infants.

Statistics

All data were analyzed using the SAS V8 statistical package (SAS Institute Inc., Cary, N.C., USA). Data are presented as median and interquartile range, as they were not distributed normally. Amino acid concentrations and Doppler-derived variables were compared between groups by nonparametric analysis of variance (ANOVA) [16]. Multiple comparisons were made, thus, isolated significant differences should be taken as provisional. For explorative statistical analyses of qualitative and quantitative data, the Fisher exact test and the Mann-Whitney U test were used, respectively.

Table 1. Distribution of population characteristics and frequency of common neonatal complications

	Control group (n = 41)	BPD group (n = 32)	p
Gestational age, weeks	29 (28–29)#	26 (25–27)	<0.0001*
Birth weight, g	1,055 (920–1,180)#	760 (655–935)	<0.0001*
AGA/SGA	35/6	25/7	0.54**
Gender, F/M	22/19	12/20	0.24**
Prenatal steroids	22 (54%)	20 (65%)	0.47**
RDS treated with surfactant	17 (41%)	25 (78%)	0.004**
PDA requiring treatment	11 (27%)	23 (71%)	<0.0001**
NEC	4 (10%)	2 (6%)	0.69**
Intraventricular hemorrhage > II°	0	4 (13%)	0.026**
Infection at birth	11 (27%)	17 (53%)	0.03**
Hospital infection	9 (22%)	19 (59%)	0.002**

#Values presented as median (interquartile range). * Mann-Whitney U test. ** Fisher's exact test. AGA = Appropriate for gestational age; SGA = small for gestational age; RDS = respiratory distress syndrome; PDA = patent ductus arteriosus; NEC = necrotizing enterocolitis.

Table 2. Distribution (median (interquartile range)) of AT/ET_c ratios of BPD group versus control group

	Control	BPD	p
28th day	0.65 (0.49–0.80); n = 39	0.60 (0.44–0.64); n = 29	0.0018#
36 weeks	0.65 (0.54–0.76); n = 32	0.52 (0.44–0.65); n = 30	0.0034*

Statistics: Non-parametric ANOVA with gestational age# or postnatal age* as co-variables.

Results

Patients

Eighty preterm infants with a gestational age of less than 30 weeks were admitted between June 2000 and December 2001. Seventy-three of them matched the study criteria. Table 1 shows the patients' characteristics and frequency of common neonatal complications. Fourteen of 30 infants of the BPD group required supplemental oxygen at 36 weeks postmenstrual age. The Palta score was 41 (19–50) at the 28th day of life and 17 (16–20) at 36 weeks postmenstrual age [11].

Echocardiography

At both time points, infants in the BPD group had significantly lower AT/ET ratios compared to infants in the control group (table 2).

Plasma Amino Acid Concentrations and Urinary Nitrate and Nitrite Excretion

The severity of BPD, measured by the Palta score, did not correlate significantly with plasma ARG concentrations. However, there was a trend at day 28 (Spearman-rank test; r = 0.32, p = 0.078 at day 28; r = 0.10, p = 0.71 at 36 weeks).

To compare plasma amino acid concentrations between groups, nonparametric ANOVA (see Patients and Methods) with gestational age, arginine intake, total amino acid intake and hospital infection as co-variables was used at day 28 (table 3). There was a trend to slightly lower ARG concentrations in the BPD group, but the difference did not reach statistical significance (p = 0.089).

At 36 weeks, gestational age, postnatal age, arginine intake and total amino acid intake were co-variables. Hospital infection was no longer a co-variable due to its low incidence (n = 2). No significant difference in plasma ARG concentrations was found at 36 weeks.

Table 3. Distribution (median (interquartile range)) of amino acid intake and plasma amino acid concentrations [μmol/l] of BPD group versus control group

	28th day			36 weeks		
	control (n = 40)	BPD (n = 32)	p	control (n = 32)	BPD (n=32)	p
Total caloric intake, kcal/kg	99 (88–114)	103 (91–111)	0.01*	108 (102–119)	109 (94–118)	0.32*
Total AA intake, g/kg/d	2.2 (2.0–2.7)	2.6 (2.1–3.2)	0.85*	2.4 (2.2–2.8)	2.6 (2.2–2.7)	0.55*
ARG intake, mmol/kg/d	0.45 (0.39–0.61)	0.55 (0.48–0.67)	0.68*	0.51 (0.44–0.57)	0.52 (0.44–0.61)	0.76*
Arginine	58 (42.5–75.5)	54.5 (31.5–70.5)	0.089#	54.5 (42–71)	43 (35–62)	0.96##
Citrulline	18.5 (12–22)	18.5 (16–26)	0.77***	22.5 (17–31)	19.5 (16–28.5)	0.65**
Glutamine	428 (340–502)	376 (286–424)	0.77***	492 (346–686)	476 (386–626)	0.20**
Ornithine	64 (43–82)	93 (56.5–106)	0.01***	70 (59–83.5)	74.5 (56.5–103.5)	0.04**
Ornithine/citrulline	3.6 (2.8–4.6)	4.1 (3.2–5.5)	0.38###	3.1 (2.5–3.8)	3.8 (2.9 – 5.2)	0.06###
Nonessential amino acids	1,425 (1,129–1,692)	1,383 (1,137–1,728)	0.103***	1,419 (1,209–1,723)	1,480 (1,220–1,885)	0.05**
Essential amino acids	918 (798–1,080)	1,057 (884–1,259)	0.55***	961 (864–1,084)	1,028 (820–1,177)	0.22**
Plasma urea, mmol/l	6.1 (4.9–8.5)	9.2 (6.1–25.0)	0.01###	5.5 (4.3–6.1)	5.5 (4.3–7.6)	0.28###

Nonparametric ANOVA with co-variables.
* Weight; ** weight, gestational age, total amino acid (AA) intake; *** weight, gestational age, total AA intake, hospital infection.
# Weight, gestational age, ARG intake, total AA intake, hospital infection; ## weight, gestational age, ARG intake, total AA intake; ### gestational age.

Table 4. Sum of nitrate and nitrite concentrations, normalized to creatinine excretion [μmol/mmol], of BPD group versus control group

	Control	BPD	p
28th day	300 (171–441); n = 22	241 (182–533); n = 19	0.28
36 weeks	227 (197–317); n = 19	290 (173–372); n = 22	0.014

Values presented as median (interquartile range). Nonparametric ANOVA with co-variables: weight, gestational age.

Plasma concentrations of amino acids involved in the ARG pathway (i.e. citrulline, glutamine and ornithine) did not differ between groups except for a higher concentration of ornithine in the BPD group (table 3). There was a trend to a higher ratio of ornithine to citrulline, a marker of urea cycle function, at 36 weeks. The plasma level of urea was significantly higher in the BPD group compared to control infants (p = 0.01) at day 28, but not at 36 weeks postmenstrual age (p = 0.28) (table 3).

Creatinine-corrected urinary nitrate plus nitrite concentrations were not statistically different between groups at day 28, but significantly higher in the BPD group at 36 weeks (p = 0.014) (table 4). Urinary nitrite + nitrate excretion in our infants are of the same order of that in newborns reported by Tsukahara et al. [17, 18].

Nutrition

Fifty percent of the control group at day 28 (50% at 36 weeks) and 56% (41% at 36 weeks) of the BPD group (p = 0.48 and 0.80) were fed with mother's milk. The incidence of partial or total parental nutrition was low (0–25%) without significant differences between groups.

Discussion

Preterm infants with BPD had Doppler-derived signs of elevated pulmonary artery pressure, but no differences in plasma ARG concentrations and urinary nitrate + nitrite excretion rates were found compared to control infants. This holds true for ARG even after statistical adjustment for potential confounding variables like intake of ARG and total amino acids or acute disease like infections. One possible explanation for the lack of differences might be the severity of BPD in our study population, because only three (9%) infants in the BPD group were still ventilated at day 28. Thus, we cannot exclude that plasma ARG concentrations may have been altered, if a higher number of infants with a more severe pulmonary disease would have been investigated.

However, the infants in the BPD group appeared to have vascular disease in terms of Doppler derived signs of elevated pulmonary artery pressure. In our study, the ratio AT/ET was measured, which correlates inversely

with pulmonary artery pressure. This ratio showed the best correlation to pulmonary artery pressure, as estimated by tricuspid regurgitation compared to other variables (r = –0.84) [12]. Only one infant of the BPD group had a significant tricuspid regurgitation, which is in accordance with the recently reported low incidence of tricuspid regurgitation in preterm infants [19].

The plasma ARG concentrations of our infants were at the lower reference range reported for term breast-fed infants [20], although the ARG intake was 25–50% higher than reported for term breast-fed infants [21]. One could speculate that the lack of difference in plasma ARG concentrations in our study population reflects a metabolic adaptation to a higher ARG demand in a chronic disease. Endogenous ARG synthesis, which plays an important role during the neonatal period [1], may be upregulated. In adult vascular disease, plasma ARG concentrations do also not appear to be altered, but oral administration of ARG was nevertheless found to be beneficial in those patients [7]. That elevation of plasma ARG concentrations can enhance systemic and vascular NO production and also repair NO-related endothelial dysfunction [22] is a paradox, because ARG is found in the mammalian organism at concentrations by far exceeding the half-saturating ARG concentrations [1] for all known NO synthase isoforms [22]. Thus, plasma ARG concentrations may play an important role in regulating endothelial NO synthesis, with plasma ARG concentrations exceeding the physiological levels being beneficial.

Elevated ornithine and urea concentrations and trend to a higher ratio of ornithine to citrulline in the BPD group possibly indicates an increased metabolism of ARG via the arginase I pathway [1]. Upregulation of this enzyme could limit availability of ARG as a substrate for NO synthetase without detectable changes in plasma ARG concentrations. However, because ARG metabolism involves multiple organs and complex compartmentalization [1], and since only a very small portion of ARG is metabolized via the ARG/NO pathway [23], it is not possible to determine the status of the ARG/NO pathway by simply comparing plasma concentrations of other amino acids involved in the multiple metabolism of ARG.

Systemic NO production can be assessed by measuring the urinary excretion of nitrite + nitrate [13, 17, 18] or measuring plasma cGMP, the second messenger of NO [24], whereas analyzing exhaled NO represents NO production from the upper and lower respiratory tract [25]. NO synthesis is differentially regulated in different tissues by three isoforms of NO synthase. Measuring the urinary excretion of nitrite + nitrate reflect whole body NO synthesis but cannot represent certain tissues or isoforms of NO synthase. A study by Castillo et al. [23] using stable isotope-labelled amino acids (*L*-[$^{15}N_2$,$^{2}H_2$]arginine and *L*-[$^{2}H_3$]leucine) in the neonate with persistent pulmonary hypertension indicated that there was a decreased arginine utilization for NO production and a decreased urinary excretion of NO products during the acute vasoconstrictive state. In contrast, urinary nitrate and nitrite excretions were increased in preterm infants with respiratory distress syndrome [17]. In our study, no difference in urinary excretion of these NO metabolites was found between BPD and control infants at day 28. At 36 weeks, urinary excretion of NO metabolites was significantly higher in the BPD group, which may reflect persisting pulmonary inflammation or may be indicative of an upregulated compensatory mechanism. Finally, exposure to high concentrations of inspired oxygen could also contribute to an increase of endogenous NO synthesis.

In conclusion, we found similar plasma ARG concentrations and similar or even higher urinary nitrate + nitrite excretion in preterm infants with BPD, who had signs of elevated pulmonary artery pressure, as well as in control infants, suggesting that NO synthesis was not impaired in the preterm infants with BPD. Healthy and ill infants received comparable amounts of ARG by food. With respect to NO synthesis, this moderate ARG intake could have been more beneficial in the preterm infants with BPD than in the control group. With regard to the low plasma ARG concentrations in preterm infants in general and the promising findings of ARG supplementation [7, 26, 27], our findings do not exclude a potential benefit from ARG supplementation in preterm infants with BPD. Future studies should also take into account indirect NO-dependent or NO-independent and hormone-related effects of ARG supplementation on cardiovascular diseases in infants. They also should include markers like nitrotyrosine and 8-hydroxy-2′-deoxyguanosine to elucidate the role of NOx as activated oxygen species in the pathogenesis of BPD as well as asymmetric dimethylarginine, the endogenous inhibitor of endothelial nitric oxide synthase.

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PART IV

SURFACTANT TREATMENT IN ARDS IN CHILDREN

chapter

11

Treatment with bovine surfactant in severe acute respiratory distress syndrome in children: a randomized multicenter study

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Treatment with bovine surfactant in severe acute respiratory distress syndrome in children: a randomized multicenter study

Abstract *Objective:* To determine whether bovine surfactant given in cases of severe pediatric acute respiratory distress syndrome (ARDS) improves oxygenation. *Design:* Single-center study with 19 patients, followed by a multicenter randomized comparison of surfactant with a standardized treatment algorithm. Primary endpoint PaO_2/FIO_2 at 48 h, secondary endpoints: PaO_2/FIO_2 at 2, 4, 12, and 24 h, survival, survival without rescue, days on ventilator, subgroups analyzed by analysis of variance to identify patients who might benefit from surfactant. *Setting:* Multicenter study in 19 reference centers for ARDS. *Patients:* Children after the 44th postconceptional week and under 14 years old, admitted for at least 4 h, ventilated for 12–120 h, and without heart failure or chronic lung disease. In the multicenter study 35 patients were recruited; 20 were randomized to the surfactant group and 15 to the nonsurfactant group. Decreasing recruitment of patients led to a preliminary end of this study. *Interventions:* Administration of 100 mg/kg bovine surfactant intratracheally under continuous ventilation and PEEP, as soon as the PaO_2/FIO_2 ratio dropped to less than 100 for 2 h (in the pilot study increments of 50 mg/kg as long as the PaO_2/FIO_2 did not increase by 20%). A second equivalent dose within 48 h was permitted. *Results:* In the pilot study the PaO_2/FIO_2 increased by a mean of 100 at 48 h (n=19). A higher PaO_2/FIO_2 ratio was observed in the surfactant group 2 h after the first dose (58 from baseline vs. 9), at 48 h there was a trend towards a higher ratio (38 from baseline vs. 22). The rate of rescue therapy was significantly lower in the surfactant group. Outcome criteria were not affected by a second surfactant dose (n=11). A significant difference in PaO_2/FIO_2 in favor of surfactant at 48 h was found in the subgroup with an initial PaO_2/FIO_2 ratio higher than 65 and in patients without pneumonia. *Conclusions:* Surfactant therapy in severe ARDS improves oxygenation immediately after administration. This improvement is sustained only in the subgroup of patients without pneumonia and that with an initial PaO_2/FIO_2 ratio higher than 65

Keywords Acute respiratory distress syndrome · Surfactant · Children · Ventilation · Oxygenation · Pneumonia

Introduction

Acute respiratory distress syndrome (ARDS) is defined by radiographic diagnosis of diffuse bilateral alveolar infiltrates, the degree of hypoxemia, lung function, and histopathology. It is the final generalized inflammatory response of the lung to catastrophic events of various pulmonary and nonpulmonary origins and occurs in all age groups. The diagnostic criteria have been established by an American-European Consensus Conference [1, 2, 3]. ARDS in children is still associated with a high mortality. Mortality is correlated with the severity of the underlying disease [4, 5]; however, it is still generally accepted that the degree of hypoxemia predicts outcome [5, 6, 7, 8]. Mortality in children with a PaO_2/FIO_2 ratio lower than 100 in central Europe is still greater than 50% [5]. Another outcome criteria is the aggressiveness of ventilatory support, reflected in the peak inspiratory pressure (PIP), mean airway pressure, and ventilation index [9, 10]. The enforcement of standardized ventilation protocols and the lower incidence of both multiple trauma and sepsis in central Europe have caused a continuous decrease in severe ARDS incidence in children [11, 12, 13].

As the mortality is still high in children with profound hypoxemia and severe underlying conditions such as immunosuppression [4, 5, 8, 13], many other therapies in addition to baro- and volutrauma preventing ventilation strategies have been reported. Nevertheless, randomized controlled studies evaluating new therapeutic strategies for the treatment of severe hypoxemic ARDS as nitric oxide, high-frequency oscillatory ventilation (HFOV), extracorporeal membrane oxygenation (ECMO), and surfactant treatment are lacking in the pediatric age group [14, 15, 16, 17, 18]. Exogenous surfactant improves oxygenation in neonates not only with respiratory distress syndrome but also in other conditions with secondary surfactant deficit such as meconium aspiration syndrome and congenital pneumonia, comparable with ARDS [19]. Oxygenation improved after surfactant administration in several case reports on adult ARDS patients [11, 20] and in a controlled study in moderate pediatric ARDS [10]. A retrospective and prospective survey of pediatric ARDS in all major German pediatric intensive care facilities demonstrated that the mortality and chronic illness after ARDS in patients with PaO_2/FIO_2 ratios above 150 is very rare, and that most patients with a PaO_2/FIO_2 ratio around 200 are not even ventilated [5]. For this reason the German-Austrian working group on ARDS performed a controlled, randomized study in severe, hypoxemic ARDS in children, associated with a very high mortality, and no evidence-based treatment options available.

Patients and methods

A single-center pilot study was carried out in 19 pediatric patients with ARDS and PaO_2/FIO_2 ratio lower than 100, aimed at dose finding and calculating the number of patients required. This pilot study treated patients with ARDS defined by consensus conference criteria and aged between the 44th postconceptional week and 14 years of age. They were included if their PaO_2/FIO_2 ratio was below 100 for at least 2 h. Ventilation in the pilot study was in accordance with the ventilation algorithm established by the German Working Group on Pediatric ARDS (Fig. 1). A bovine surfactant (Alveofact, Boehringer, Ingelheim, Germany) was administered in 50 mg/kg increments (intratracheal bolus under continuous ventilation and PEEP over maximally 5 min) as long as the PaO_2/FIO_2 ratio did not increase by 20% or decrease by 10%. The PaO_2/FIO_2 ratio was determined at 48 h The increase/decrease from baseline was evaluated using the Mann-Whitney *U* test. No other exclusion criteria were applicable [17, 19]. This pilot study as the following mulitcenter study were approved by the ethics review board of the principal investigators' institution (Medical University of Lübeck).

The subsequent multicenter study was an open, randomized, parallel group comparison performed at 19 German pediatric intensive care units between May 1997 and November 1999. These units were referral centers for children with severe respiratory failure. All children were randomized if they fulfilled the following criteria: ARDS Consensus Conference criteria, lung injury score [21] of at least 2, ventilation between 12 and 120 h, age between 44th postconceptional week and 14 years, admission for at least 4 h, no echocardiographically detectable left heart failure, and a PaO_2/FIO_2 ratio lower than 100. Written informed consent was obtained from the parents or legal guardians. Patients were excluded if they were under other investigational or experimental therapies: nitric oxide, high frequency ventilation with current disease, liquid ventilation, prostaglandins, steroids for therapy of ARDS, ECMO; chronic lung disease such as bronchopulmonary dysplasia or cystic fibrosis, participation in other clinical studies except treatment protocols and studies for oncological diseases. In addition, patients with severe hypoxemia, i.e., PaO_2 lower than 50 after 4 h of treatment in the referral center were excluded.

Thirty-eight patients were recruited over the 18-month period (mean age 3.9 years), but three randomized patients were not included in the study, as their PaO_2/FIO_2 ratio improved to 100 or higher before the start of treatment. All patients in the participating centers with ARDS in the specified age group who were not included as they did not fulfill all entry criteria were recorded and evaluated as "intended to treat patients" if their PaO_2/FIO_2 ratio was below 100. All patients in the participating centers were ventilated according to a ventilation algorithm (Fig. 1) [4], blood pressure was kept above the 50th percentile for age, fluid intake between 90% and 100% maintenance [22], and hemoglobin levels above 12 g/dl. As the PaO_2/FIO_2 ratio was less than 100, patients were randomized either to receive 100 mg/kg bovine surfactant (Alveofact, Boehringer-Ingelheim, Germany), administered under continuous PEEP and ventilation as a bolus over not longer than 5 min to the distal tip of the endotracheal tube (n=20), or be continuously treated based on the ventilation algorithm and concomitant therapy outlined above (n=15). In the surfactant group an additional dose of 100-mg/kg surfactant during the 48-h observation period was allowed if the PaO_2 decreased by 20% from the maximum level reached. Diagnoses, age, weight and other demographic characteristics are depicted in Table 2, including the ratio of immunosuppressed patients (after bone marrow transplant or under chemotherapy), PRISM III score, and pneumonia; there were no significant differences between the two groups in regard to these factors.

PaO_2, FIO_2, $paCO_2$, pH, peak inspiratory pressure, PEEP, ventilatory rate, tidal volume, blood pressure, heart rate, hemoglobin,

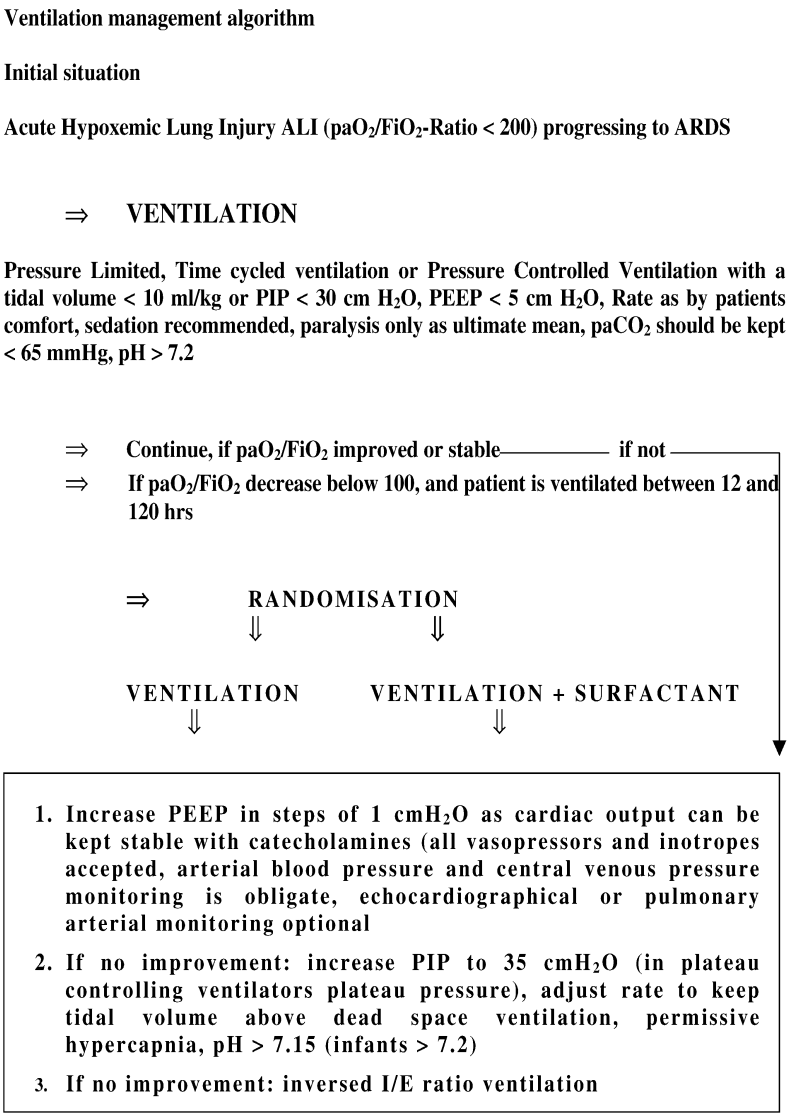

Fig. 1 Algorithm for ventilatory management in the study centers before and after randomization

Table 1 Reasons for not randomizing patients with ARDS and PaO_2/FIO_2 ratio less than 100 ("intended to treat"). The survival rates were not compared statistically because of the small number of patients in each section of the intended to treat patients; the overall survival rate was 36%

	n	Age range (months)	Nonsurvivors	
			n	%
Admission under resuscitation/death at arrival	6	8–152	6	100
On ventilator for longer than 120 h	6	3–152	5	80
Chronic lung or left heart disease	10	2.5–160	3	33
Under rescue therapy before study	11	3–156	8	72
No informed consent	6	3–145	3	50

all medications administered, fluid balance, and the derived variables (PaO_2/FIO_2, Hallman oxygenation index, ventilatory index, mean airway pressure) were recorded 2, 4, 12, 24, and 48 h after randomization. The patients were followed to day 30 or to discharge/transfer from the referral center to document outcome data: survival, rescue therapy, days on ventilator, days in the ICU, Pediatric Risk of Mortality (PRISM) III score [23], lung injury (Murray score) [21], and days in hospital.

Random allocation was performed centrally by telephone (24 h coverage). The randomization schedule was designed to achieve a 1:1 randomization at each participating center. If the PaO_2 decreased to below 50 mmHg for at least 1 h in any patient, all rescue therapies considered appropriate by the principal investigator of the center were allowed (e.g., NO, HFOV, additional surfactant, ECMO, vasodilators). Rotational therapy or prone positioning was obligatory.

Table 2 Patient characteristics in surfactant and nonsurfactant groups

	Surfactant	Controls	Overall	Comments
Randomized	22	16	38	–
Treated	20	15	35	–[a]
Age (range; years)	3.5 (0–13)	4.5 (0–12)	3.9 (0–13)	n.s.
Female	7 (35%)	7 (46.7%)	14 (40%)	–
Body weight (kg)	15.7±10.4	22.4±20.7	18.6±15.8	n.s.
Time since FIO_2 >0.5 (h)	35.2±25.5	49.8±44.5		n.s.
Time since PIP >30 cmH_2O (h)	24.9±21.8	34.1±32.9		n.s.
Causative diagnosis pneumonia	15 (68.2%)	11 (68.7%)		n.s.
Causative diagnosis sepsis	7 (31.8%)	5 (31.3%)		n.s.
Under immunosuppression	9	7		n.s.
Rescue ECMO	0	2		–[b]
Rescue NO	4	4		–[b]
Rescue HFOV	2	3		–[b]
Rescue surfactant	0	4		–[b]
Rescue vasodilators	1	1		–[b]
Nonsurvivors/mortality	8 (44%)	9 (60%)		p=0.29
Death and/or rescue	11 (56%)	12 (80%)		p=0.13
Ventilator-free (alive and without ventilator)				n.s.
0 days	12 (63.2%)	9 (64.3%)		
10–20 days	3 (15.8%)	3 (21.4%)		
>20 days	4 (21.1%)	2 (14.3%)		
PRISM III at randomization, median	11.5	11		n.s.
Lung injury score (Murray) at randomization	3.0	3.3		n.s.
PaO_2/FIO_2 at baseline	71.3±13.7	64.3±16.2		n.s.
2nd surfactant dose	11	–		

[a] Three patients improved within the 2 h between reaching a PaO_2/FIO_2 <100 and final randomization
[b] Rescue therapy after the study surfactant medication was given

The study was conducted as a multicenter, open, randomized parallel comparison. The primary variable was the change from baseline in the PaO_2/FIO_2 ratio at 48 h after the first administration of surfactant or randomization to the control group. Secondary endpoints were: peak inspiratory pressure, positive end-expiratory pressure, mean airway pressure, in- and expiration time, respiratory rate, FIO_2, PaO_2, $paCO_2$, SaO_2, pH, heart rate, and blood pressure 2, 4, 12, 24, 48, and 120 h after randomization. In addition Murray Score, PRISM III score at baseline, 48 and 120 h, clinical status at 30 days after randomization, days on ventilator, days in intensive care, days on supplemental oxygen, mortality at day 30, ventilator-free days at day 30, and the necessity of rescue therapy as ECMO, HFOV, NO, or rescue surfactant. The primary hypothesis defined was tested by the Mann-Whitney U test at an error level of $\alpha \leq 0.05$. A secondary analysis was performed to detect changes from baseline for all other time points up to 48 h by the same test procedure.

For all other variables the same procedures were performed. The number of deaths was compared between groups by means of Fisher's exact test, as were patients who received rescue therapy (each rescue therapy was summarized by frequencies). In a third analysis the combined event death and/or rescue therapy was investigated. We performed multiple regression analyses for changes in the oxygenation index at 2, 4, 12, 24, and 48 h to analyze differences between special subgroups. We used a linear model for repeated measurements. For this post hoc analysis the following subgroups were evaluated: baseline PaO_2/FIO_2 less than vs. 65 or higher, baseline PRISM III score less than vs. 12 or higher, age under 1 year vs. 2 years or older, girls vs. boys, body weight less than vs. 12 kg or higher, time since FIO_2 being higher than 0.5 at randomization shorter than 24 vs. 24 h or longer, time since PIP being higher than 30 cmH_2O shorter than 30 h or longer, pneumonia vs. no pneumonia, sepsis vs. no sepsis, and immunosuppression vs. no immunosuppression.

Results

In the pilot study the average increase in PaO_2/FIO_2 was 54 at 4 h and 103 after 48 h (p<0.01). Seven patients died despite improved oxygenation. The average dose of surfactant given was 94 mg/kg (Table 3). This led to the multicenter study dose of 100 mg/kg.

Regarding the primary variable, change in PaO_2/FIO_2 over the 48-h observation period, PaO_2/FIO_2 was significantly higher in the surfactant group 2 h after the first surfactant dose (p<0.003). Even after 48 h the surfactant group patients still showed a greater, albeit not significantly greater, increase in PaO_2/FIO_2 ratio (Fig. 2). Using the Hallman oxygenation index as oxygenation parameter produced similar results; oxygenation was significantly improved after 2 and 4 h (p<0.00222 and p<0.05). Considering secondary endpoints, mortality and mortality and/or rescue therapy was lower in the surfactant group, however not significantly so at all times during the study period. Mortality in both groups was considerably lower than that in the "intended to treat" patients (64%). Patients in the surfactant group received significantly less rescue therapy than those in the nonsurfactant group (p<0.05).

There was a reduction in mean airway pressures used in the surfactant group after 2 and 24 h (p<0.05, p<0.007; (Fig. 3, Tables 4, 5) – there was no difference

Table 3 Data of the pilot study in 19 patients, their diagnoses, age, body weight and PaO_2/FIO_2 ratio at baseline, 4 and 48 h (*RSV* respiratory syncytial virus)

Patient no.	Diagnosis	Age (months)	Weight (kg)	PaO_2/FIO_2			Outcome
				0 h	4 h	48 h[a]	
1	Meningococcemia	24	15	84	111	444	Survived
2	Pneumonia	26	10.3	31	109	160	Survived
3	Pneumonia	18	8	48	148	242	Survived
4	RSV bronchiolitis	13	8	38	115	151	Survived
5	Pertussis pneumonia	14	7.2	116	99	145	Died
6	Near drowning	38	12	43	174	240	Survived
7	Liver failure	9	4	65	96	108	Died
8	Sepsis	2	3	61	242	50	Died
9	Sepsis	7	8	32	57	–	Died
10	Pneumonia	38	9	64	53	–	Died
11	Burns	54	17	61	84	65	Died
12	RSV bronchiolitis	2	2.5	59	94	137	Died
13	Pneumonia	54	15	64	100	113	Died
14	Pneumonia	3	2.5	64	96	92	Survived
15	Near drowning	69	23	63	176	290	Survived
16	Aspiration	10	8	47	66	109	Survived
17	Aspiration	12	10	46	87	105	Survived
18	Pneumonia	3	3.2	60	128	122	Survived
19	Aspiration	6	6	63	88	160	Survived
	Mean±SD	21±20	9±5	58±19	112±46	161±97	$p<0.01$

[a] n=17

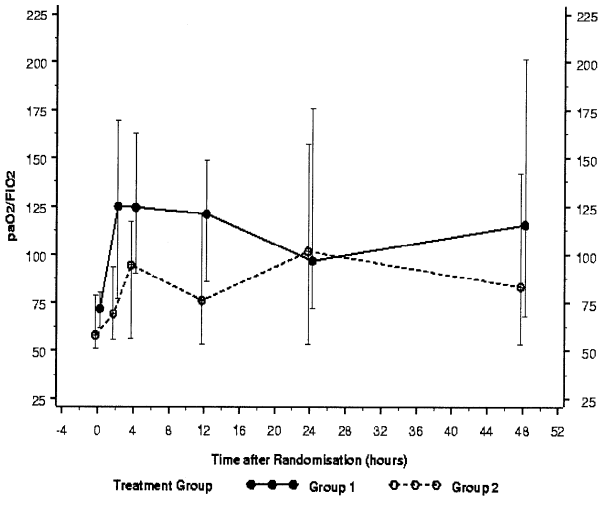

Fig. 2 Medians and interquartile ranges (25–75 percentiles) of the oxygenation index (PaO_2/FIO_2) in the 48-h observation period of the surfactant group (*group 1*) and controls (*group 2*)

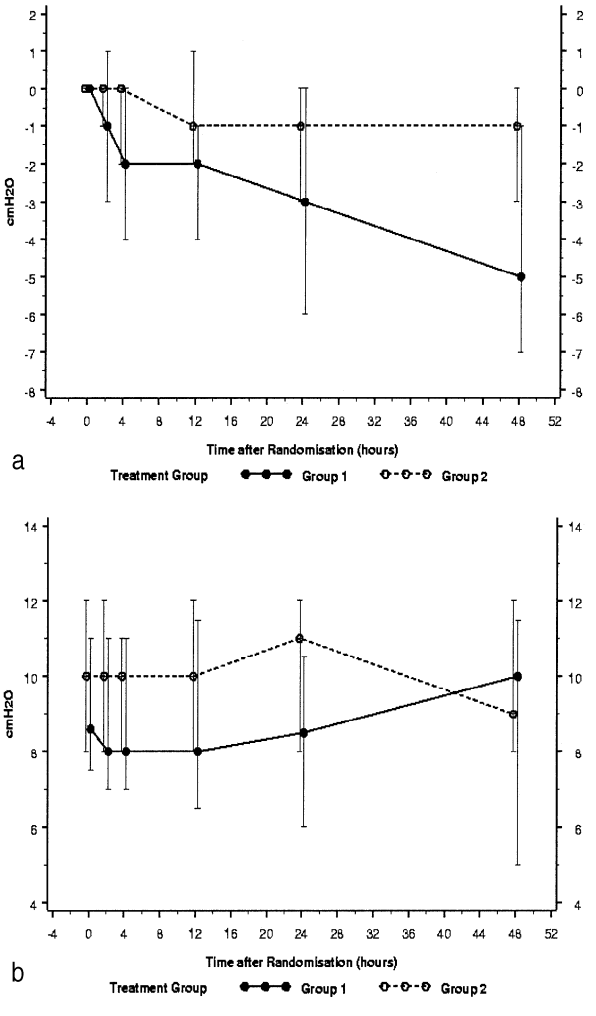

Fig. 3 Medians and interquartile ranges (25–75 percentiles) of changes of peak inspiratory pressure in cmH_2O (**a**) and positive end-expiratory pressure (**b**) over the 48-h observation period in the surfactant group (*group 1*) and controls (*group 2*)

Table 4 Physiological and ventilation variables at baseline and after 2, 4, 12, 24, and 48 h (changes from baseline): median (25th/75th percentiles) or mean ±SD (*RR* ventilatory/respiratory rate, *OI* oxygenation index, *CVP* central venous pressure)

	Surfactant						Controls					
	Baseline	2 h	4 h	12 h	24 h	48 h	Baseline	2 h	4 h	12 h	24 h	48 h
Oxygen. index PaO_2/FIO_2	71.3	+54 (16/90)	+57 (19/92)	+39 (19/72)	+24 (3/106)	+38 0/122)	64.3	+9 (0/25)*	+20 (1/50)	+20 (0/47)	+42 (0/98)	+22 (0/78)
Hallman OI		−10 (−17/−7)	−11 (−16/−7)	−12 (−15/−4)	−7 (−19/−2)	−9 (−18/−3)		−2 (−7/1)	−4 (−9/0)	−4 (−12/0)	−6 (−12/−2)	−5 (12/−2)
(cmH_2O/mmHg)												
PaO_2 (mmHg)		+14 (7/38)	+10 (0/37)	+9 (−5/22)	+5 (−1/9)	+5 (−4/25)		+4 (−2/11)*	+3 (−2/25)	+6 (−4/19)	+6 (−2/33)	+6 (−2/19)
$paCO_2$ (mmHg)		−4 (−9/5)	−4 (−7/9)	+2 (−7/14)	+7 (−1/14)	+2 (−8/13)		−3 (−12/4)	−4 (−12/8)	−5 (−12/6)	−4 (−12/2)	−1 (−10/5)
RR (1/min)		0.0 (−5/0)	−1.5 (−4.5/0)	−3 (−4/0)	−3 (−12/0)	−2.8 (−9/0.5		0 (0/0)	0 (0/0)	0 (0/3)	0 (−2/3)	0 (−1/4)
PIP (cmH_2O)		−1 (−3/1)	−2 (−4/0)	−2 (−4/−1)	−3 (−6/0)	−5 (−7/−1)		0 (−1/0)	0 (−2/0)	−1 (−2/1)	−1 (−3/0)	−1 (−3/0)
PEEP (cmH_2O)		0.0 (−0.9/0)	0.0 (−0.9/0)	0.0 (−1/0)	0.0 (−1.6/0)	−0.5 (−2/0)		0.0 (0/0)	0.0 (0/0)	0.0 (−2/1)	0.0 (−1/1)	0.0 (−1/1)
Mean airway pressure (cmH_2O)		−1 (−1.7/0)	−1 (−2.7/0.1)	−1 (−3.2/0.5)	−1.5 (−5/−0.9	−1 (−6/1.3)		0.4 (0/1.5)*	0.4 (−2/2)	0.4 (−2/2)	0.0 (−2/2)*	0.0 (−2/1.5)
Ventilatory index (RR×PIP×pCO_2/1000)		−7 (−12/−1)	−5 (−11/1)	−3 (−12/5)	−5 (−15/5)	−5 (−16/5)		−9 (−18/−1)	−10 (−22/1)	−10 (−25/5)	−10 (−24/3)	−6 (−19/6)
BP systolic (mmHg)	109±19	109±13	108±31	109±19	104±24	105±16	97±13	100±21	102±24	112±14	112±26	110±18
BP diastolic (mmHg)	56±18	61±13	55±12	60±13	58±16	57±12	51±9	52±11	51±12	60±11	58±12	56±11
CVP (mmHg)	11±5	11±5	11±5	10±5	10±4	9±5	13±6	11±5	12±5	11±6	11±5	12±7
Heart rate (1/min)	143±29	134±32	136±31	133±27	134±22	117±34	138±28	139±26	133±24	139±24	130±22	130±22
Hemoglobin (g/dl)	11.9±2.3	12.4±2.4	12.0±2.0	12.2±1.6	13.1±2.1	13.5±1.8	11.1±1.8	11.0±1.7	11.3±1.6	12.5±1.9	12.2±2.1	11.7±1.8

Fig. 4 *Z* statistic of the primary endpoint, change in PaO_2/FIO_2 ratio at 48 h between the surfactant group (*group 1*) and controls (*group 2*)

between $paCO_2$ or pH in the two groups at any time of the 48-h observation period. Tidal volumes were kept below 10 ml/kg in all cases as specified in Fig. 1. Lung injury and PRISM III scores decreased from 0 to 48 h; however, only the decrease in PRISM III reached the level of significance (p 0.05). No other significant differences in secondary outcome criteria were detected. Eleven patients in the surfactant group received the possible second dose of 100–mg/kg surfactant; no significant increase in PaO_2/FIO_2 after this second dose was observed at any time. No treatment associated adverse events were observed in the surfactant group; however, the expected risk of intermittent obstruction of the endotracheal tube with a short time deterioration in oxygenation was observed in three patients.

In a post hoc analysis of the PaO_2/FIO_2 changes from baseline between 2 and 48 h considering various patient characteristics as additional information no significant differences were found regarding Murray Score PRISM III score, age, sex, time with FIO_2 longer than 0.5, PIP higher than 30 cmH_2O, sepsis vs. no sepsis, or immunosuppression vs. no immunosuppression (Table 6). However, a significant difference in PaO_2/FIO_2 increase was found between those whose initial ratio was higher than 65 and those whose initial ratio was less and 65 and group 2 patients (p<0.028, Fig. 4); patients with the lower ratio had a 100% mortality in both groups even when rescue therapy was applied. In addition, surfactant patients without pneumonia had significantly better oxygenation after 48 h than nonsurfactant patients with pneumonia (p<0.0017, Fig. 5). In addition, a trend to efficacy of surfactant was seen in patients weighing at least 12 kg (p=0.055).

The study had to be stopped earlier than originally planned due to increasing recruitment difficulty. To demonstrate that the trial was not being stopped at a

Table 5 PRISM III and Murray score at baseline, 48, and 120 h (no significant differences)

	Surfactant			Controls		
	Baseline	48 h	120 h	Baseline	48 h	120 h
PRISM III	11.5±6.5	11.55±6.5	11.0±6.7	11.0±4.5	11.0±7.0	11.0±4.0
Murray score	3.0±0.7	2.8±0.4	2.6±1.0	3.3±0.4	3.1±0.7	3.1±0.5

Table 6 *p* values in linear models for the change from baseline in the PaO_2/FIO_2 ratio between 2 and 48 h including various additional factors of clinical concern. The significant interactions between treatment and morbidity characteristics (e.g., pneumonia, PaO_2/FIO_2<65) indicate that the difference between treatment groups depends on the baseline value of the oxygenation index and on the presence of pneumonia as a causative diagnosis for ARDS. These differences are clearly demonstrated by the median profiles which are displayed separated for the respective subgroups

Factor of interest	Surfactant treatment	Factor of interest (morbidity characteristics)	Treatment by factor
No	0.079	–	–
Baseline PaO_2/FIO_2 (<65 vs. >65)	0.220	0.019	0.028
Baseline lung injury score (<3.1 vs. >3.1)	0.103	0.958.	0.426
Baseline PRISM III (<12 vs. >12)	0.069	0.232	0.786
Age (<1 vs. ≥2 years)	0.076	0.291	0.783
Sex	0.139	0.727	0.285
Body weight (≤12 vs. >12 kg)	0.059	0.570	0.055
Time since FIO_2 >0.5 before randomization (<24 vs. >24 h)	0.122	0.617	01.77
Time since PIP >30 cmH_2O before randomization (<30 vs. >30 h)	0.096	0.269	0.893
Pneumonia (primary pulmonary ARDS)	0.001	0.047	0.002
Sepsis	0.123	0.085	0.965
Under immunosuppression	0.135	0.135	0.743

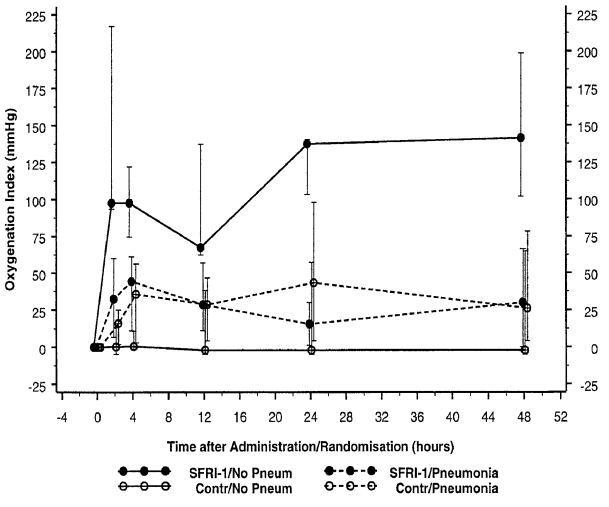

Fig. 5 PaO_2/FIO_2 changes from baseline in the group of patients with an initial PaO_2/FIO_2 ratio less than and greater than 65 (*dotted lines*) in the surfactant group (*group 1*) and controls group (*group 2*)

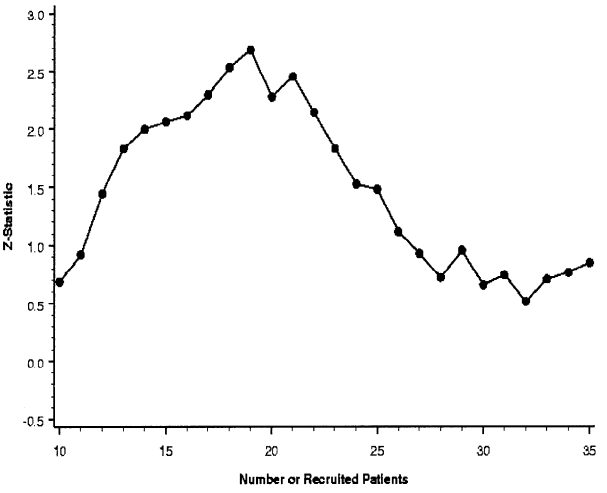

Fig. 6 PaO_2/FIO_2 changes from baseline in the group of patients with (*dotted lines*) and without pneumonia in the surfactant group (*group 1*) and controls (*group 2*)

prejudicial time point the primary comparison between treatments was performed sequentially, post hoc beginning with the first ten evaluable patients. The resulting *Z* statistics, which are approximately normally distributed, are depicted graphically vs. the number of patients (Fig. 4).

Discussion

In patients with ARDS less endogenous surfactant is produced, and this is inactivated, modified, and not reused, thus causing an absolute and relative surfactant deficiency [20, 24, 25, 26, 23]. As surfactant is a major biological factor for alveolar recruitment, surfactant deficit is a key problem in ARDS, and substitution of surfactant in

ARDS could be an important therapeutic tool [19, 23, 26]. As early as 1989 it was hypothesized that surfactant could be of therapeutic value not only in premature infant's respiratory distress syndrome but also in ARDS [24]. In that year the first case report of a successfully surfactant-treated child with severe hypoxemic ARDS was published [27]. Surfactant therapy in preterm infants is now based on sound data (e.g., [28, 29, 30]). In respiratory failure of term infants, in some ways resembling ARDS, a randomized study demonstrated a significant reduction in the need for ECMO [31]. Several uncontrolled studies have been published on adult ARDS [32, 33]; a large controlled study using small doses of aerosolized synthetic surfactant found no advantage of surfactant [33], and a smaller administering a bolus reported improved oxygenation and slightly increased survival in the treatment group [34]. In the pediatric population surfactant has been used in several case reports and in small uncontrolled studies of near fatal ARDS [17, 19, 24, 35]. In mild to moderate ARDS (Hallman oxygenation index less than 10) a randomized study demonstrated improved oxygenation immediately after surfactant administration and most ventilation-associated parameters [36]. In patients with severe hypoxemia we observed a short period of improved oxygenation, as in the study by Willson et al. [36] in patients with less severe hypoxemia.

The originally planned number of patients, however, could not be recruited in the time period scheduled. This might be due to the fact that the overall incidence of ARDS in children decreased dramatically in central Europe during the study period [13, 37]. The study was stopped as the recruitment dropped in this obviously high mortality group. As a sequential analysis of the primary endpoint (normalized *Z* statistics) demonstrated, the study was not stopped at a point at which there was a significant difference in respect of the primary outcome criteria; this would have been true at an earlier time point.

The overall mortality rate in this study is comparable to that of other studies carried out in severe hypoxemic pediatric ARDS [4, 5, 13]. The trend towards a decreased mortality and need for rescue medication in such a high mortality population might nevertheless be important as no other evidence-based treatment option exists. Surfactant may be only one factor in improving oxygenation in severe ARDS; it enhances the benefits of the "open lung concept," prone positioning, and NO-induced pulmonary vasodilatation [25, 38, 39]. The improvement in oxygenation in the control group, which was managed only on a strictly enforced ventilation algorithm and blood pressure and fluid intake protocol, suggests that standardized treatment alone using conventional methods leads to an overall improvement in patient outcome. This is in line with similar findings by Steinhard et al. [12] who reported improved patient outcome simply by introducing enforced ventilatory management protocols. Surfactant might also be directly involved in improving the balance of pro- and anti-inflammatory mediators in ARDS and therefore be a causative approach [39, 40, 41]. The design of modified surfactant solutions being more prone against inactivation by different proteins in the course of ARDS could enhance and prolong the effect of surfactant on oxygenation [42].

As patients without pneumonia showed a significant improvement and patients weighing more than 12 kg had a benefit from surfactant, it can be speculated that these small infants, in whom pneumonia is the principal cause of ARDS, would require much higher surfactant doses. This would be in accordance with experimental results in which in pneumonia surfactant inactivation was increased, compared with other causes of ARDS [38]. Higher doses of the surfactant preparation used cannot be applied as they would obstruct the major airways. A second dose showed no benefit in our study, either in the data analysis of primary and secondary endpoint criteria or in the post hoc analysis of subgroups. If at all, higher initial doses in selected patients could be considered if a preparation were available with lower volume, lower viscosity, and perhaps more prone to inactivation. Surfactant treatment with a listed price of € 200–400/100 mg is still very expensive.

Conclusions

This study reveals the difficulty in obtaining conclusive results from randomized studies in an intensive care setting and patients with a high mortality. Many "intended to treat" patients could not be randomized as they had underlying lung or heart disease, were dying at arrival in the ICU, or had been ventilated for more than 5 days in regional hospitals. An additional difficulty for such a study is the decreasing incidence of ARDS in children. Our study confirmed results of a previous randomized study by Willson et al. [38] reporting improved oxygenation in pediatric patients with mild to moderate ARDS. The improvement in oxygenation was sustained for patients without pneumonia as underlying disease and with a PaO_2/FIO_2 ratio greater than 65. The latter subgroup does not seem to benefit from any other available rescue tool at this moment. Ventilation variables could be reduced in the surfactant group. The devastating consequences of aggressive ventilation in these children with severe ARDS can probably be ameliorated with surfactant treatment. We conclude that surfactant treatment in severe pediatric ARDS might offer benefits to the patients.

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PART V

EXPERIMENTAL ASPECTS OF NATURAL AND SYNTHETIC SURFACTANT TREATMENT IN INFANTS

chapter

12

Effects of pulmonary surfactant on TNF-α-activated endothelial cells and neutrophil adhesion in vitro

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Effect of pulmonary surfactant on TNF-α-activated endothelial cells and neutrophil adhesion in vitro

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Abstract

Pulmonary surfactant given to infants and adults with respiratory failure is metabolized and recycled to a large extent. A small proportion also enters the circulation in cases of increased permeability of the alveolar-capillary membrane. We therefore investigated whether exogenous surfactants such as a natural bovine (natSF) or a synthetic (synSF) preparation had an impact on inflammatory conditions involving the adhesion of neutrophils to endothelial cells. Human umbilical cord vein endothelial cells (HUVEC) were plated on coverslips until confluence, activated by tumor necrosis factor-alpha and incubated with or without surfactant in the media. Human neutrophils passed the HUVEC layer in a flow chamber and interactions were visualized using a video microscope. To test if surfactant affected the expression of cell adhesion molecules, RT-PCR analyses were performed for E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Using concentrations between 50 and 300 μg/ml of surfactant in the pre-incubation media the number of adherent neutrophils increased by 10–20% at the higher concentration of the natSF ($^{*}P < 0.05$) whereas the synSF had no effect. Increased neutrophil adhesion was associated with a significant up-regulation of mRNA levels for E-selectin and VCAM-1; mRNA levels for ICAM-1, however, were not affected by the presence of surfactant. These observations indicate that natSF but not synSF might have pro-inflammatory effects when higher amounts of the exogenous dose reach the circulation. This might be explained by different fatty acid profiles, e.g. the presence of arachidonic acid in the natSF or higher concentrations of surfactant-associated protein-C in the synSF.

Keywords: Surfactant; Neutrophil; Adhesion

Abbreviations: dNTP, desoxynucleotide triphosphate; DTT, dithiothreitol; FAME, fatty acid methyl ester; GAP-DH, glyceraldehyde 3-phosphate dehydrogenase; HUVEC, human umbilical cord vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; natSF, natural surfactant; NF-κB, nuclear factor kappa B; RDS, respiratory distress syndrome; SP, surfactant-associated proteins; synSF, synthetic surfactant; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule 1.

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Introduction

Surfactants are routinely used to treat preterm infants with respiratory distress syndrome (RDS), and are being evaluated for the treatment of infants and adults with acute respiratory distress syndrome (ARDS) (Jobe, 1993; Ware and Matthay, 2000). ARDS is characterized by an impaired pulmonary gas exchange secondary to atelectasis and disruption of the alveolar-capillary membrane. In consequence, small amounts of exogenous pulmonary surfactant may directly enter the circulation (Hallman et al., 1994; Lachmann et al., 1987). There is emerging evidence that natural and synthetic surfactant preparations exert different effects on immunological reactions such as the accumulation of neutrophils in the lung alveoli (Kukkonen et al., 2000; Speer and Groneck, 1998; Tegtmeyer et al., 1996).

Surfactant consists of a mixture of phospholipids, the composition of which differs depending on its origin. For example, fatty acid composition in natural surfactant derived from bovine or porcine lung is more heterogeneous than in synthetic preparations also containing long chain omega-6 and omega-3 fatty acids.

It has also been shown that the presence of certain surfactant-associated proteins (SP) especially SP-A has immune modulating properties (Kramer et al., 2001; LeVine et al., 1999; Rosseau et al., 1999). However, in surfactant preparations for therapeutic use, SP-B and SP-C, but not SP-A, are present in natural surfactants of bovine or porcine origin, and their recombinant variants in synthetic products. These natural surfactants but also some protein-free synthetic surfactants have recently been shown to inhibit intracellular signaling events in leukocytes. As a consequence, they decrease the risk of tissue destruction and chronic inflammation (Brenner et al., 2000). However, other groups reported SP-B to enhance the induction of immunoglobulin M immune responses (Van Iwaarden et al., 2001). Most recently, Augusto et al. (2003) observed that SP-C used CD14 on macrophages as immunological targets.

Endothelial immune responses are triggered by activation via cytokines leading to an increased expression of cell adhesion molecules such as selectins, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the cell surface. Interactions of these molecules with their counterpart ligands on leukocytes mediates adhesion and extravasation at the site of inflammation (Vestweber and Blanks, 1999). These reactions are also major contributors to the development of chronic lung disease (Mulligan et al., 1991; Sarafidis et al., 2001).

We therefore investigated whether natural or synthetic surfactant administered to patients with acute lung injury has a direct effect on neutrophil adhesion to tumor necrosis factor-alpha (TNF-α) activated and non-activated endothelial cells. These experiments were performed under flow conditions thus mimicking the dynamic behavior of neutrophils on stimulated endothelium. Furthermore, the influence of endothelial cells with regard to the expression of adhesion molecules was also included.

Methods

Surfactant preparations

In the present study, two surfactant preparations were used one of which was a commercially available phospholipid preparation from bovine lung (natSF) purchased from Boehringer (Ingelheim, Germany). The second surfactant was a synthetic product (synSF) provided by Altana Pharma (Konstanz, Germany) which has not been released for therapeutic use in RDS yet. The protein fraction of natSF consisted of SP-B and SP-C in a proportion of about 1% each (related to dry weight). In synSF, however, recombinant SP-C (2% of the dry weight) represents the only constituent of the protein fraction. The lipid portion of the two surfactants was individually analyzed as described in the following section.

Determination of free and phospholipid-bound fatty acids

Lipids were extracted using methanol/chloroform according to the method of Bligh and Dyer (1959). Free fatty acids were converted to fatty acid methyl ester (FAME) using diazomethane and purified by means of silica thin layer chromatography with toluol as developing solvent. FAMEs were identified using primuline (Wright, 1971) and extracted using chloroform. Phospholipids were separated from neutral lipids by thin layer chromatography with chloroform/methanol/water (65/25/4 v/v/v) as mobile phase and isolated with chloroform/methanol (2/1 v/v). Phospholipid-bound fatty esters were converted to FAME using 2 mol/l HCl/methanol and subjected to gas chromatography as previously described (Schmidt et al., 2001). Briefly, gas chromatographic separation was performed using a silica capillary column (CP-Sil 88, 50 mm × 0.25 mm, Chrompack, Frankfurt, Germany), with the detector (FID) at 300 °C and hydrogen as carrier gas (flow rate 1.0 ml/min). A linear gradient was used from 120 to 220 °C at 3 °C/min and 5 min at 220 °C. FAMEs were identified by comparison with the retention times of commercial standards (Sigma, Deisenhofen, Germany). The resulting peak areas were corrected against blanks by means of the internal standard and treated with empirically determined mass/response factors.

Neutrophil isolation

Human neutrophils were isolated from 50 ml heparinized peripheral blood by agglutinating erythrocytes using dextran (Sigma, St. Louis, USA) for 30 min, followed by density gradient centrifugation on a Ficoll gradient. Neutrophils were treated with ammonium chloride to remove remaining erythrocytes, washed twice in PBS and suspended in RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (Gibco BRL, Karlsruhe, Germany) and 1/100 (v/v) penicillin/streptomycin (Gibco BRL, Karlsruhe, Germany). Cells were tested for purity (above 90%) and viability (above 95% throughout the study) by flow cytometry and trypan blue staining, respectively.

Preparation of endothelial cells

Endothelial cells were obtained from human umbilical veins according to a method described by Jaffe et al. (1973). Human umbilical cord vein endothelial cells (HUVEC) were used for further experiments after the first passage when they had reached confluence on fibronectin (Biochrom KG, Berlin, Germany) coated coverslips. The purity and the characteristics of the endothelial cell populations were determined by indirect immunofluorescence using a monoclonal antibody for van Willebrand factor (DAKO Diagnostica GmbH, Hamburg, Germany).

Neutrophil adhesion assay

Neutrophil adhesion was determined as described recently (Krüll et al., 1999; Mayer et al., 2002). Confluent HUVEC monolayers were stimulated with 10 ng TNF-α (RandD Systems, Minneapolis, MN, USA) per milliliter of cell culture media for 4 h prior to the experiments. One hour prior to the flow experiment, HUVEC were incubated with the surfactant preparations. It was assumed that secondary to a severely disturbed integrity of the alveolar-capillary membranes up to 10% or more of the surfactant applied exogenously (100 mg/kg body weight) may enter the circulation (N. Lachmann, pers. comm.). Thus, concentrations of 50–300 µg surfactant per milliliter of media were used to incubate the HUVEC layer prior to contact with neutrophils.

HUVEC-coated coverslips were then set into a parallel plate flow chamber (Keutz, Reiskirchen, Germany). Neutrophils (2×10^6 cells in 0.5 ml) were allowed to pass the HUVEC layer at a flow rate of 6.7 ml/h revealing a shear force of 1 dyne/cm^2. Experiments were evaluated using a phase contrast microscope (Type DMJL from Leica, Wetzlar, Germany) and videotaped over the entire time of neutrophil perfusion. After all neutrophils had passed the flow chamber, cell adhesion was determined by counting the number of adherent neutrophils at eight randomized areas of the coverslip (Mayer et al., 2002).

Expression of cell adhesion molecules

RNA from HUVEC was extracted by a guanidinium thiocyanate method according to the method described by Chomczynski and Sacchi (1987) with slight modifications followed by first-strand cDNA synthesis. For each sample, 5 µg RNA was added to first-strand buffer (50 mmol/l Tris–HCl, 75 mmol/l potassium chloride, 3 mmol/l magnesium chloride, 20 mmol/l dithiothreitol (DTT)), 0.5 mmol/l of each desoxynucleotide triphosphate (dNTP), 20 U RNaseOUT^{TM} ribonuclease inhibitor, 100 ng oligo(dT)$_{15}$-primer (MBI Fermentas, St. Leon-Roth, Germany) and 200 U SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). First-strand cDNA synthesis was carried out in a total volume of 20 µl at 42 °C for 1 h and was inactivated by heating at 70 °C for 15 min.

PCR amplification of sequence specific fragments was carried out with those amounts of cDNA derived from the linear range of amplification in *Taq* buffer (20 mmol/l Tris–HCl, 50 mmol/l potassium chloride), 1.5 mmol/l magnesium chloride, 0.2 mmol/l of each dNTP, 7.5 µmol/l of each primer (see Table 1) and 0.4 U *Taq* DNA polymerase (Promega, Mannheim, Germany) in a total volume of 50 µl. PCR was performed with cycle conditions of 94 °C for 4 min followed by 30 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, primer extensions at 72 °C for 45 s and end synthesis at 72 °C for 10 min. RT-PCR products were separated on a 2% agarose gel and visualized by ethidium bromide. A θX174 DNA/*Hae*III marker was used in all PCR experiments as a size control of the amplified products. No products were obtained for any genes without reverse transcription indicating the specificity of mRNA determination.

The amplified products were photographed, and the intensity of the bands was analyzed by densitometry (SigmaGel software). The area under the curve (AUC) for E-selectin, ICAM-1 and VCAM-1 was set against that for the standard gene glyceraldehyde 3-phosphate dehydrogenase (GAP-DH).

Statistics

The effects of the different surfactant preparations were determined by comparing the numbers of neutrophils that adhered to stimulated HUVEC with and without surfactant incubation. The effects on cell adhesion are given as mean $\pm$ SD of at least three

Table 1. Primer specifications for the housekeeping gene GAP-DH and cell adhesion molecules

Name	Position	Size	Orientation	Sequence (5′→3′)
GAP-DH	558–1010	452 bp	Sense	GACCACAGTCCATGCCATCACT
			Antisense	TCCACCACCCTGTTGCTGTAG
ICAM-1	849–1087	237 bp	Sense	TATGGCAACGACTCCTTCT
			Antisense	CATTCAGCGTCACCTTGG
VCAM-1	—	513 bp	—	[a]
E-selectin	—	485 bp	—	[a]

[a]Sequence not published (primers were purchased from R&D Systems, Minneapolis, MN, USA).

independent sets of experiments with five-fold determinations for each surfactant preparation and concentration tested. For the effects on the molecular basis, at least three independent experiments were carried out for each type of cell adhesion molecule. Data are given as mean ± SD. For all experiments the results for the treatment of TNF-α-activated HUVEC was set to 100% in order to compare data within different experimental days. The results were compared using Student's *t*-test for the evaluation of different treatments. Effects were considered to be significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$.

Results

The composition of the surfactant preparations investigated in the present study differed with regard to lipids and proteins. The lipid fraction of both surfactant preparations mostly consisted of dipalmitoylphosphatidylcholine. NatSF and synSF contained 2.1 ± 0.2% and 2.6 ± 0.2% total free fatty acids (in percent of total lipids, w/w), respectively. The pool of free fatty acids in synSF consisted of palmitic and oleic acid only, whereas the natSF contained a variety of fatty acids including the omega-6-fatty acids linoleic and arachidonic acid in relative amounts of 4.2% and 1.9%, respectively (Fig. 1). Phospholipid fatty acids of synSF also consisted exclusively of palmitic and oleic acid. The main fatty acids of natSF were palmitic acid (41.9 ± 1.9% of all phospholipid fatty acids), oleic acid (35.4 ± 0.6%) and stearic acid (10.3 ± 1.0%). The omega-6-fatty acids linoleic acid and arachidonic acid had a relative distribution of 2.9 ± 0.5% and 1.2 ± 0.0%, respectively (Fig. 1).

Endothelial cells were activated using TNF-α which revealed a significant increase in the number of adherent neutrophils to about 200–300 cells per high magnification field compared to 20–30 cells per field on resting, i.e. non-activated HUVEC (Fig. 2A and B). The incubation

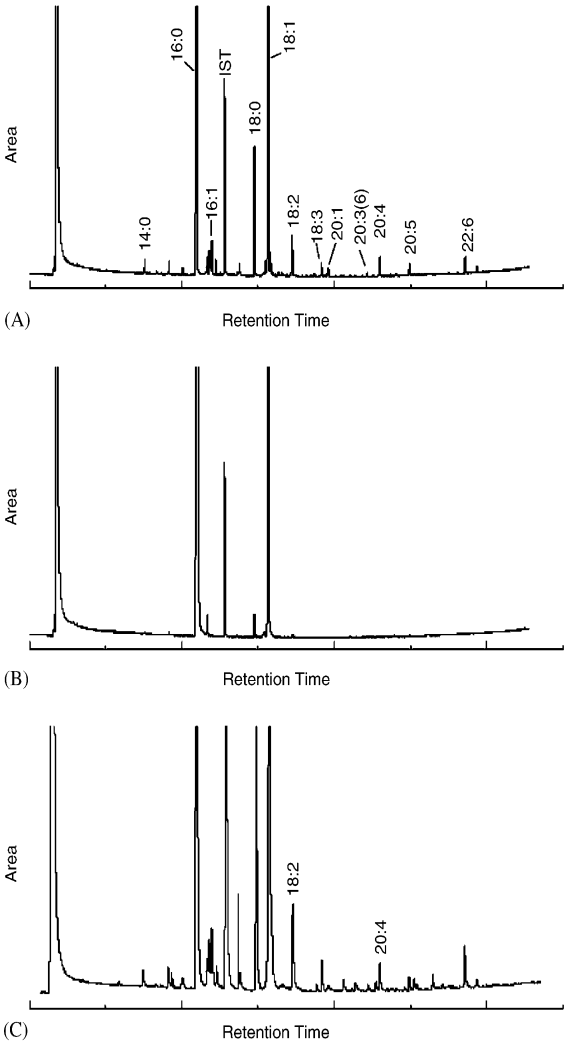


Fig. 1. Gas chromatographic analyses of surfactant fatty acid profiles: (A) natSF free fatty acids; (B) synSF free fatty acids and (C) natSF phospholipid-bound fatty acids. Definitions of abbreviations: 14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 16:1 = palmitoleic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = linolenic acid; 20:3(6) = eicosatrienoic acid; 20:4 = arachidonic acid; 20:5 = eicosapentaenoic acid, 22:6 = docosahexaenoic acid; IST = internal standard.

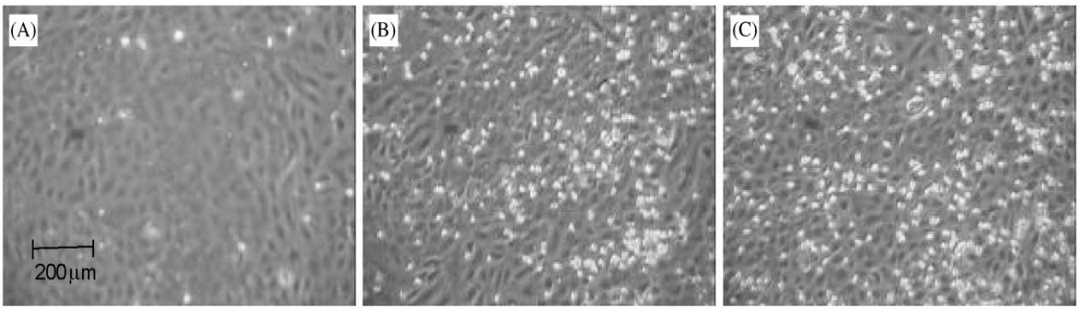


Fig. 2. High magnification field (× 20) from videotape showing adherent neutrophils (light colored) on a HUVEC monolayer. A total of 4×10^6 neutrophils was injected into the flow system. (A) Control experiment using non-stimulated HUVEC. (B) HUVEC were incubated with hrTNF-α (10 ng/ml) for 4 h prior to the flow experiment. (C) HUVEC were pre-incubated with, e.g. 200 µg/ml surfactant for 1 h prior to the flow experiment.

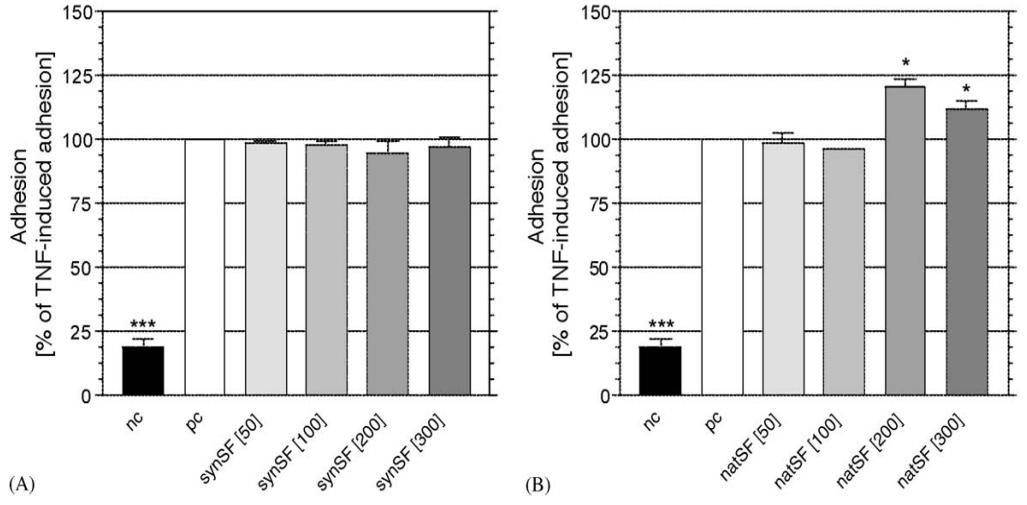
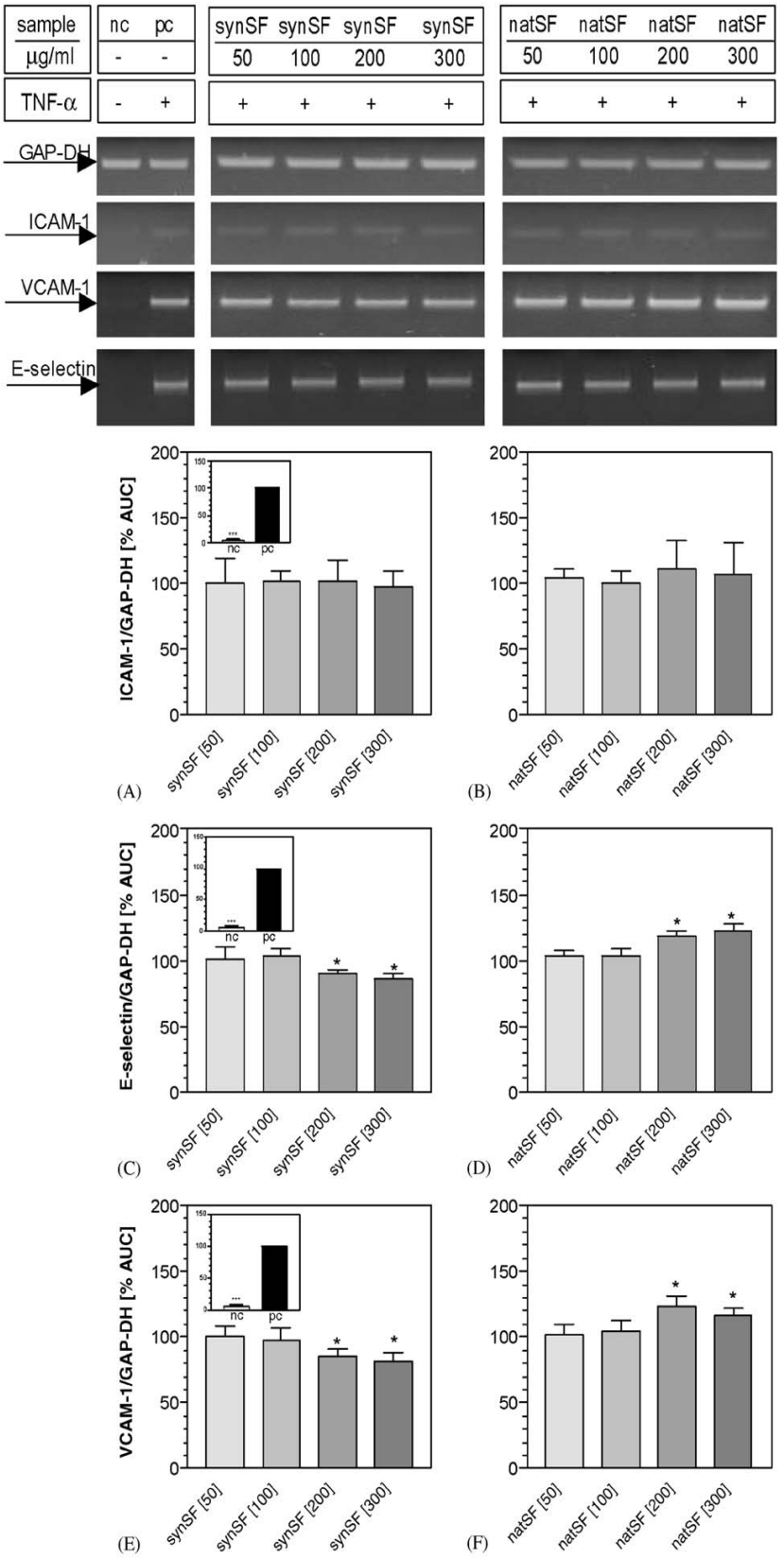


Fig. 3. Effects of surfactant on neutrophil adhesion to TNF-α-stimulated cells: (A) synSF and (B) natSF. HUVEC were stimulated with 10 ng/ml TNF-α for 4 h and co-incubated for 1 h prior to the flow experiment with 50–300 µg/ml of surfactant or unstimulated (negative control, nc). Cell adhesion was determined by an analysis of eight random high magnification fields (× 20) from videotape. Adhesion of neutrophils to TNF-α-activated HUVEC was set at 100% for each experiment (positive control, pc). Values given are means ± SD of three independent experiments each including 4–6 replications ($^*P < 0.05$ and $^{***}P < 0.005$).

with 50 and 100 µg/ml natSF did not further increase neutrophil adhesion. However, a concentration of 200 and 300 µg/ml natSF resulted in a significant enhancement of neutrophil adhesion ($^*P < 0.05$) by 20.0 ± 0.5% and 12.1 ± 2.5%, respectively (Figs. 2C and 3). SynSF had an only minor effect on neutrophil adhesion by slightly reducing the number of adherent cells by 3.5 ± 3.7% ($P < 0.25$; Fig. 3).

To test whether surfactant had a direct effect on endothelial cells, mRNA levels of the most important cell adhesion molecules E-selectin, VCAM-1 and ICAM-1 were determined using semiquantitative RT-PCR. Non-activated HUVEC expressed low mRNA levels for ICAM-1, VCAM-1 and E-selectin with 5.1 ± 1.8, 6.7 ± 1.7 and 5.1 ± 3.6% of the positive control, respectively (Fig. 4, insert nc). After cytokine stimulation, mRNA levels of all three cell adhesion molecules increased significantly compared to non-stimulated HUVEC. These values were subsequently used as positive controls (Fig. 4, insert pc). No further effect on gene expression was observed when only 50 and 100 µg/ml natSF were used in the pre-incubation media. However, after additions of 200 and 300 µg/ml natSF, VCAM-1 (118.9 ± 3.9% and 122.7 ± 5.1%, respectively) and E-selectin mRNA levels (123.0 ± 8.9% and 116.5 ± 6.1%, respectively) increased significantly compared to the positive control (Fig. 4D and F); only ICAM-1 mRNA levels remained in the range observed for the positive control (Fig. 4B). Incubating HUVEC with the same concentration of synSF revealed the


sample
µg/ml
TNF-α
nc
pc
synSF 50
synSF 100
synSF 200
synSF 300
natSF 50
natSF 100
natSF 200
natSF 300
GAP-DH
ICAM-1
VCAM-1
E-selectin
ICAM-1/GAP-DH [% AUC]
E-selectin/GAP-DH [% AUC]
VCAM-1/GAP-DH [% AUC]
synSF [50]
synSF [100]
synSF [200]
synSF [300]
natSF [50]
natSF [100]
natSF [200]
natSF [300]
(A)
(B)
(C)
(D)
(E)
(F)

opposite effect. A significant decrease of the mRNA levels for VCAM-1 (90.3±2.3% and 85.7±4.3% of the positive control, respectively) and E-selectin (85.3±5.6% and 81.4±6.7%) was found compared to the control (Fig. 4C and E) at 200 and 300 µg/ml whereas at 50 and 100 µg/ml natSF, the expression levels remained unchanged. Similar to natSF, synSF had no influence on ICAM-1 mRNA levels and stayed in the same range as the positive control (Fig. 4A).

The observed effects of synSF and natSF on neutrophil adhesion and mRNA levels seemed to be dependent on TNF-α stimulation because in both cases the application of surfactant alone (300 µg/ml) failed to affect neutrophil adhesion to endothelial cells (Fig. 5) and mRNA expression levels of adhesion molecules in unstimulated HUVEC (Fig. 6).

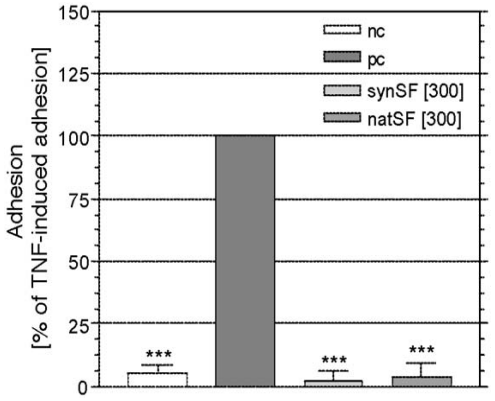


Fig. 5. Effects of surfactant on neutrophil adhesion to unstimulated cells. HUVEC were incubated for 1 h with 300 µg/ml surfactant prior to the flow experiment. Cell adhesion was determined by an analysis of eight random high magnification fields (×20) from videotape. Adhesion of neutrophils to TNF-α-activated HUVEC (pc) was set at 100% for each experiment. Values given are means±SD of three independent experiments each including 4–6 replications (*** $P < 0.005$).

Discussion

Both, synthetic and natural surfactants have been well documented to improve oxygenation in preterm infants with RDS and experimental models of acute lung injury (Spragg et al., 2000). Despite this treatment, a significant number of non-responders to surfactant treatment is explained by an influx of inflammatory cells into the alveolar space due to an increased permeability of the alveolar-capillary membrane (Ware and Matthay, 2000).

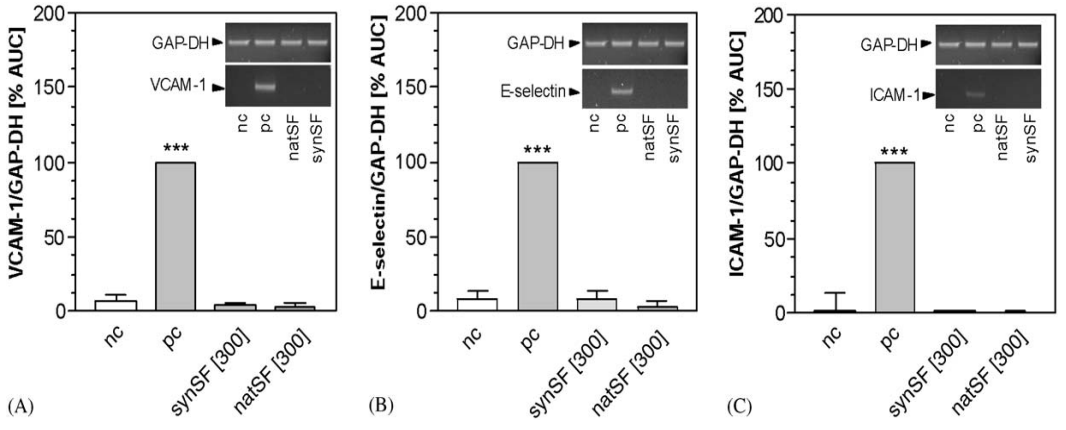


Fig. 6. Effect of natSF and synSF on the expression of ICAM-1, VCAM-1 and E-selectin mRNA in unstimulated HUVEC by semiquantitative RT-PCR. HUVEC were incubated with 300 µg/ml natSF or synSF, whereas the negative control was only supplemented with media and the positive control was activated by hrTNF-α (10 ng/ml). RNA was extracted, reverse transcribed and amplified by PCR using specific primers for GAP-DH (as a constitutively expressed standard gene), VCAM-1, ICAM-1 and E-selectin. Ratio of target gene to standard gene in controls and treated cells for (A) VCAM-1, (B) E-selectin and (C) ICAM-1 ($n = 3$; *** $P < 0.005$). (Inserts) Photographs of the UV-illuminated gel electrophoresis of amplified cDNA products from a representative experiment.

Fig. 4. Effect of natSF and synSF on TNF-α-stimulated expression of ICAM-1, VCAM-1 and E-selectin mRNA in HUVEC by semiquantitative RT-PCR. HUVEC were pre-incubated with (positive control, pc) or without (negative control, nc) TNF-α (10 ng/ml) for 4 h and co-incubated with the indicated concentrations of natSF and synSF for 1 h. RNA was extracted, reverse transcribed and amplified by PCR using specific primers for GAP-DH (as a constitutively expressed standard gene), VCAM-1, ICAM-1 and E-selectin. (Upper panel) Photographs of the UV-illuminated gel electrophoresis of amplified cDNA products from a representative experiment. (Lower panel, A–F) Ratio of target gene to standard gene in treated cells. (Inserts) Ratio of target gene to standard gene in controls ($n = 3$; * $P < 0.05$ and *** $P < 0.005$).

Therefore, surfactants might influence different processes with both positive as well as potentially negative effects. Recent observations indicate that surfactants do have an impact on cell–cell interactions or cell signaling events (Vestweber and Blanks, 1999). We therefore addressed the questions whether natural and synthetic preparations have an influence (a) on the adhesion of neutrophils to unstimulated and TNF-α-stimulated endothelial cells, and (b) on the endothelial cells themselves affecting the expression of various cell adhesion molecules. In addition, the different fatty acid compositions of natSF and synSF, e.g. palmitic and oleic acid, or the presence of omega-3 (e.g. eicosapentaenoic acid and docosahexaenoic acid) and omega-6 fatty acids (e.g. linoleic acid and arachidonic acid) were investigated since they may be involved in regulating various transcription pathways.

Inflammatory processes in general are mediated by leukocyte–endothelial cell interactions. An increased neutrophil transmigration, however, is associated with tissue damage and the development of chronic inflammation.

The contribution of surfactant constituents (phospholipids, free fatty acids and SP) with immune modulating properties within the inflamed lung tissue is not well understood. We therefore proposed that up to 10% or more of the exogenously applied surfactant dose (100 mg/kg body weight) may enter the blood circulation when the permeability is increased due to edema associated with acute respiratory failure (Lachmann et al., 1987). In order to mimic the conditions of leukocytes in the systemic blood circulation we used a flow model where shear stress forces were set accordingly. Under these conditions, a pre-incubation of TNF-α-activated endothelial cells with natSF at concentrations of 200 and 300 μg/ml lead to an increase in the number of adherent neutrophils of about 12–20% compared to the positive controls where HUVEC were exposed to TNF-α only. The same concentrations of synSF had a slightly reducing, but non-significant effect on cell adhesion, lower concentrations had no impact. Reports from other groups with respect to immune modulating effects of a natural surfactant on human monocytes may seem contradictory (Pinot et al., 2000; Walti et al., 1997). However, both groups used a porcine surfactant which might differ from the preparations used in our study regarding its composition and the concentrations they had applied. Furthermore, the effects they had observed were limited to monocytes. Van Iwaarden et al. (2001) showed that the administration of SP-B in combination with surfactant lipids enhanced the immune responses in sera of mice whereas SP-C had no effect. This is in accordance with our findings for the synSF containing only SP-C as no increase in neutrophil adhesion was observed. In addition, it has been shown recently that SP-C itself had an effect on the immune response, not by enhancing leukocyte infiltration but through their affinity toward bacterial endotoxins (Augusto et al., 2003).

A second set of experiments addressed the question whether surfactant directly caused a stimulation of the expression of cell adhesion molecules in endothelial cells. In the present study, the addition of TNF-α to human HUVEC led to an increased mRNA expression for E-selectin, VCAM-1 and ICAM-1.

The application of natSF not only caused a rise in the number of adherent neutrophils but also markedly enhanced TNF-α-induced VCAM-1 mRNA and E-selectin mRNA expression in a dose-dependent manner. However, it needs to be emphasized that an effect was only observed at high concentrations of natSF, i.e., 200 and 300 μg/ml whereas the lower concentrations had no impact. The synSF preparation, on the other hand, selectively decreased E-selectin and VCAM-1 mRNA expression which was associated with a slight but not significant reduction of the number of adherent neutrophils on endothelial cells. The data presented demonstrated that surfactant plays a particular role in the modulation of TNF-α-stimulated neutrophil adhesion and expression of adhesion molecules because it did not have an impact on neutrophil adhesion and expression levels when applied to unstimulated HUVEC. Thus, both surfactant preparations only modulated the TNF-α-stimulated cell adhesion and mRNA expression.

One can speculate that polar lipid components which were shown to activate endothelial cells might be the factors for the increased expression of surface adhesion molecules (Ochi et al., 1995; Subbanagounder et al., 2002). Both, incorporation of the phospholipids into cellular membranes and uptake of free fatty acids in the cell affect physicochemical properties and activities of, e.g., enzymes and receptors (Houliston et al., 2001; Menendez et al., 2004). Because of the existence of multiple cellular targets for TNF-α, it is difficult to readily decipher the mechanisms involved in neutrophil adhesion and adhesion molecule expression. The overall effect could be a direct action on intracellular signaling pathways which are induced by TNF-α (e.g. activation of transcription factors such as nuclear factor kappa B (NF-κB)) or an effect of TNF-α itself (e.g. activation on receptor expression). It is most likely that our findings may be explained by the different composition of both surfactants with regard to the fatty acid profiles. In synSF, the predominant fatty acids are palmitic acid and oleic acid whereas the natSF contained a mixture of fatty acids including arachidonic acid and linoleic acid. Although the role of fatty acids in adhesion events is controversial our data are in accordance with recently published reports (Hii et al., 1999; Paine et al., 2000). In both studies, it was clearly shown that, e.g. arachidonic acid is able to serve as an intracellular second messenger

and to induce adhesion processes by influencing different signal transduction cascades resulting in a modulation of the potency of the TNF-α signal pathway with the consequence of changeable expression levels of adhesion molecules. Recently, Moghaddami et al. (2003) showed that arachidonic acid stimulates the expression of receptors for TNF in different cell lines. Thus, an increase in TNF-receptor expression could result in a more pronounced effect of TNF-α with the consequence of activating signal transduction pathways and up-regulating the expression of adhesion molecules as we have observed. In contrast to natSF, synSF selectively attenuated TNF-α-stimulated VCAM-1 and E-selectin expression and adhesion of neutrophils to HUVEC. This effect could be explained by the fatty acid profile, i.e. palmitic and oleic acid, the predominant fatty acids in synSF. Oleate is known to decrease the adhesion of monocytes to endothelial cells and is accompanied by a decreased expression of adhesion molecules like VCAM-1 and E-selectin (Carluccio et al., 1999; De Caterina and Libby, 1996). A possible explanation of the pre-translational effect of oleate to decrease adhesion molecule expression could be the extent of NF-κB activation after endothelial cell stimulation.

Interestingly, the incubation of HUVEC with natSF or synSF had an impact on E-selectin and VCAM-1 expression, but no effect on the mRNA expression for ICAM-1. An explanation might be that the VCAM-1 promoter consists of two NF-κB binding sites, whereas that for E-selectin has three sites and that for ICAM-1 just one (Collins, 1993; Van de Stolpe and Van der Saag, 1996). Thus, distinct pathways may regulate the expression of these proteins and surfactant may affect only the signaling pathways involved in VCAM-1 and E-selectin regulation.

In conclusion, although the concentration of free fatty acids (e.g. arachidonic acid) in the natSF was found to be in a physiological range it may be high enough to induce acute changes with regard to leukocyte–endothelial cell interactions. It is further notable that natural surfactants in general are not standardized with the consequence that the concentrations of fatty acids and other constituents may vary within batches. Therefore, the effects seen in vitro should be further investigated and may be taken into consideration when high risk patients with apparent inflammatory symptoms are to be treated with surfactant.

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chapter 13

Synthetic and natural surfactant differentially modulate inflammation after meconium aspiration

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Synthetic and natural surfactant differentially modulate inflammation after meconium aspiration

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Abstract *Objective:* Meconium aspiration syndrome remains a relevant cause of neonatal respiratory failure and is associated with severe pulmonary changes including surfactant inactivation and pronounced inflammatory changes. The present study investigated the effect of two different surfactant preparations—recombinant surfactant protein C surfactant (rSP-C Surf) and natural bovine surfactant—on pulmonary gas exchange and inflammatory response. *Design and subjects:* Twenty-three newborn piglets were intubated, mechanically ventilated, received 5 ml/kg 20% sterile meconium for induction of lung injury, and were randomized thereafter for controls (n=7), rSP-C Surf (n=8), or natural surfactant (n=8). Surfactants were given as an intratracheal bolus (75 mg/kg) and animals were further ventilated. *Measurements and results:* Lung function variables, arterial blood gas samples and lung tissues were obtained. Histological evaluation was performed in right lung tissue using an established score. Cytokine mRNA expression (left lung tissue) was quantified using TaqMan real-time PCR (ΔΔCT method, normalized to controls). In addition to significant improvement in gas exchange and lung function, histological evaluation showed significantly lower sum scores in the rSP-C Surf group than in controls). Cytokine mRNA expression of IL-1β in whole lung tissue was significantly lower after administration of rSP-C Surf than in natural surfactant and controls whereas IL-10 mRNA expression was significantly induced in both surfactant groups. *Conclusions:* Surfactant administration improved both gas exchange and pulmonary inflammatory cytokine transcription. Mechanisms underlying the differential inflammatory response in both surfactant preparations need to be further addressed.

Keywords Cytokines · Respiratory insufficiency · Newborn · Inflammation · Meconium aspiration · Pulmonary surfactants

Introduction

Meconium aspiration syndrome (MAS) remains a relevant cause of respiratory failure in term neonates, and despite intensive perinatal treatment it is still associated with high neonatal mortality and morbidity [1]. MAS-induced pulmonary changes include typical histological characteristics [2], inflammatory cell infiltration with release of vasoactive substances [3], airway obstruction, and surfactant dysfunction and inactivation [4, 5]. Experimental models of MAS have therefore been established for studying the pathogenesis of respiratory failure in neonates. A number of studies have been performed addressing the pulmonary response to surfactant in MAS [6, 7]; however, results still remain controversial [8]. Type, dosage, and mode of surfactant administration are

under investigation. Meconium constituents such as lysophosphatidylcholine, bilirubin, bile salts, and proinflammatory agents and the presence of hemoglobin in the lung are able to disrupt the surfactant monolayer formation and stabilization thus interfering with surface tension-lowering properties i.e., surfactant inactivation. Therefore the need for repetitive administration of natural surfactant to overcome its inactivation has been reported [9, 10], whereas recent studies revealed serious precautions against the lavage technique [11].

In addition to the improvement in MAS-induced airway obstruction and atelectasis following exogenous surfactant, its potential in influencing the pulmonary inflammatory response is discussed. As increased levels of tumor necrosis factor (TNF) α, interleukin (IL) 1β, 6, and 8, and low levels of IL-10 are known to be present in lung tissue after meconium aspiration [12, 13], and in vitro studies demonstrating an altered inflammatory response of polymorph nuclear leukocytes following surfactant incubation [14] seem to be of great potential.

Therefore the objective of the present study was to evaluate the effect of bolus administration of two different surfactant preparations-a natural bovine surfactant containing surfactant protein B (SP-B) and surfactant protein C (SP-C) and a synthetic surfactant containing recombinant SP-C (rSP-C Surf) on (a) variables of lung function and gas exchange, (b) lung histology, and (c) inflammatory cytokine transcription in a piglet model of MAS.

Materials and Methods

Animals

Twenty-three newborn piglets of either sex and a median age of 6 days (range 1–11) and a median weight of 2200 g (range 1900–2500) were studied. Animal experiments were approved by the State Ethics Committee for Animal Eexperiments of the state of Hesse, Germany, and performed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Protocol

Intravenous anesthesia was induced with ketamine (2 mg/kg body weight, b.w.; Inresa, Freiburg, Germany) and midazolam (0.2 mg/kg b.w., Roche, Grenzach-Wyhlen, Germany) after an intramuscular bolus of 50 mg/kg b.w. ketamine. Following relaxation with vercuronium (0.2 mg/kg b.w.; Inresa), the piglets were intubated with a cuffed endotracheal tube (3.0 mm outer diameter; Rüsch, Kernen-Rommelshausen, Germany). Continuous infusion with midazolam (1 mg/kg b.w. per hour), ketamine (2 mg/kg b.w. per hour), and vecuronium (0.6 mg/kg b.w. per hour) provided sedation, analgesia and muscular relaxation. Ringer's solution was given intravenously at a rate of 5 ml/kg b.w. per hour. Animals were ventilated using a pressure controlled mode (Servo 900, Siemens, Germany), with a positive inspiratory pressure (PIP) of 15 cmH_2O, a positive endexpiratory pressure (PEEP) of 2 cmH_2O, and a respiratory rate of 30 cycles/min (rate of inspiration to expiration 30:70) at 100% oxygen (FIO_2 1.0). Under these conditions normocapnia was observed. Body temperature was maintained in an art-specific range (38°–39°C) with radiant warmers and heating pads. An arterial catheter (20 G; Arrow, Erding, Germany) was placed into the right common carotid artery and a central venous line (4.0 F; Arrow) in the right femoral vein. Arterial blood gases (paO_2, $paCO_2$, and pH; ABL Radiometer 555, Diamond Diagnostics, Mass., USA), heart rate, blood pressure, central venous pressure, and electrocardiography (78354A Hewlett-Packard) were monitored before and during induction of lung injury, immediately before treatment and every 30 min thereafter. Tidal volumes were detected by pneumotachograph (CO_2SMO plus 8100, Novametrix Medical Systems, USA) at the end of the endotracheal tube and ventilation efficiency index (VEI) was further calculated as: $3{,}800/(\delta P \times \delta R \times Pa_{CO2})$ [15]. After a stabilization period of 30 min ventilation pressures were increased (PIP 25 cmH_2O, PEEP 4 cmH_2O) and remained unchanged during the residual ventilation period. Lung injury was induced in all animals by a bolus administration of 5 ml/kg b.w. of 20% meconium given in aliquots of 2–4 ml via the endotracheal tube under ongoing ventilation according to Soukka et al. [16]. Thirty minutes after induction of lung injury piglets were prospectively randomized to one of the following groups: group 1 (*n*=7) received intratracheal (i.t.) administration of 1.5 ml/kg b.w. physiological saline (Braun, Melsungen, Germany); group 2 (*n*=8) received 75 mg rSP-C Surf/kg b.w. intrathecally (Altana Pharma, Constance, Germany); and group 3 (*n*=8) received 75 mg bovine (SP-B and SP-C) surfactant/kg b.w. intrathecally with 75 mg corresponding 1.5 ml each. After another observation period of 330 min (360 min after induction of lung injury) piglets were killed using an overdose of potassium chloride and phenobarbitone. All animals survived the whole observation period with the exception of one control animal dying from severe respiratory failure. Immediately after death the chest was opened and a cannula was placed into the pulmonary artery, and the left atrium was opened and the lung was perfused in situ with Ringer's lactate containing procaine (250 mg/l), eparine (20 U/ml), and calcium chloride (2.2 mmol/l) for 10 min. PIP was increased for 15 s to 25 cmH_2O and thereafter reduced and left at 10 cmH_2O. Thereafter tissue samples of the superior and inferior lobe of the left lung were extracted and immediately snap-frozen in liquid nitrogen for real time polymerase chain reaction (PCR) analyses. Afterwards right lungs were perfused with 300 ml of a formaldehyde (4.6%) and glutaraldehyde (0.5%) solution for approx. 10 min. Finally, the trachea was clamped at a PEEP of 10 cmH_2O, and right lungs were removed and submersed in the above solution for histomorphological analyses.

Preparation of pooled meconium

Meconium was collected from healthy full-term neonates with history of neither perinatal complications nor maternal drug administration during pregnancy and delivery. Probes were tested for sterility, pooled, and further lyophilized and diluted with sterile saline to a 20% concentrated solution, equivalent to 65 mg/ml lyophilized meconium [16].

Therapeutic surfactants

Experiments were performed using a recombinant SP-C based surfactant (Altana) or a natural bovine surfactant. In the rSP-C Surf phenylalanine replaces cystein in positions 4 and 5 and isoleucin replaces methionin in position 32 within the recombinant human 34 amino acid SP-C sequence. The final surfactant contained 2% rSP-C (wt:wt) in phospholipids (dipalmitoylphosphatidylcholine and palmitoyloleoylphosphatidyl-glycerol at a 70:30 ratio (wt:wt), 50 mg/ml), plus 5% palmitic acid, which was added to facilitate the preparation of this surfactant product. The rSP-C Surf surfac-

tant was delivered as lyophilized powder and was suspended in 0.9% sterile saline (Braun) to a concentration of 50 mg/ml phospholipids. The natural bovine surfactant, also provided as lyophilized powder, was resuspended in sterile saline 0.9% (Braun) to a final concentration of 50 mg/ml. Its composition has been described previously [17].

Lung histology

Tissue slides were obtained from dependent and nondependent parts of the right lung of each animal as described above and stained with hematoxylin-eosin to perform histological analyses. Lung histology was evaluated by a pathologist (M.E.), blinded to the animal's group assignment, according to a previously described histological score [18]. Variables scored for histological evaluation were atelectasis, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis, and overdistension. The variables were scored using a five-point scale with no injury corresponding to 0 points and 4 points indicating maximum injury.

Measurements of cytokine mRNA expression by real-time PCR

Cytokine mRNA expression of IL-1β, IL-6, IL-8, IL-10, and transforming growth factor (TGF) β was measured in tissue of the left lung lobe, obtained as described above and stored at −80°C, using the real-time reverse-transcriptase PCR technique (TaqMan). To normalize gene expression mRNA of the housekeeping genes β-actin (A) and hypoxanthine-guanine-phosphoribosyl-transferase (H) was quantified. Cytokine mRNA expression was analyzed both for whole left lung tissue and in each left lung lobe separately.

For this purpose lung-tissue was homogenized in liquid nitrogen and total RNA was then extracted using the acid guanidinium-thiocyanate-phenolchloroform method (Roti Quick, Roth, Germany). The RNA concentration was measured spectrophotometrically and quality was estimated by agarose gel electrophoresis with ethidium bromide staining; 0.5 µg RNA was reverse transcribed in a volume of 100 µl at 42°C for 50 min after a denaturation step at 65°C with random hexamer primers and deoxyribonucleoside triphosphates (all chemicals from Gibco BRL, Calif., USA).

TaqMan real-time PCR

Real-time PCR has been confirmed to be a sensitive and precise tool for the quantification of nucleic acids and thus for gene expression studies [19]. Primers and probes for IL-1β, IL-6, IL-8, IL-10, TGF-β, hypoxanthine phosphoribosyltransferase (HPRT) and β-actin were designed using Primer Express software (Applied Biosystems) with reference to Hardt et al. [20] following a fixed set of recommendations to allow the application of standardized cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles for 15 s at 95°C and 1 min at 60°C). Control PCRs showed no signal for pure genomic DNA, confirming mRNA specificity. Primers were purchased from Roth and probes from Applied Biosystems. The reaction (total volume 25 µl) contained approximately 5 ng cDNA, primers and probes in a final concentration of 300 nM and 200 nM, respectively, as well as commercial reagents (Universal Master Mix, Applied Biosystems). Relative quantification of gene expression was performed using the ΔΔCt method, which results in a ratio of target gene expression to equally expressed housekeeping genes. The ratio found in a calibration sample that may be arbitrarily chosen serves as a reference point and is set to be 1. In this study control samples were subsumed for this purpose. HPRT and β-actin were chosen as housekeeping genes. To fulfill the requirements for the ΔΔCt method, PCR efficiency was determined by running cDNA dilution series (seven steps of 1:2 dilution performed in duplicate). PCR efficiency varied between 95.7% (IL-6) and 105.4% (IL-10).

Primer and TaqMan probes

- Hypoxanthine-guanine-phosphoribosyl transferase:
- Forward: 5′-TGGAAAGAATGTCTTGATTGTTGAAG-3′
- Reverse: 5′-ATCTTTGGATTATGCTGCTTGACC-3′
- TaqMan probe: 5′(VIC)-ACACTGGCAAAAVAATGCAAAC-CTTGCT-(TAMRA)3′
- β-Actin:
- Forward: 5′-TCATCACCATCGGCAACG-3′
- Reverse: 5′-TTCCTGATGTCCACGTCGC-3′
- TaqMan probe: 5′(VIC)-CCTTCCTGGGCATGGAGTCCTG-C-(TAMRA)3′
- Interleukin 1β:
- Forward: 5′-GGTTTCTGAAGCAGCCATGG-3′
- Reverse: 5′-GATTTGCAGCTGGATGCTCC-3′
- TaqMan probe: 5′(FAM)-AAAGAGATGAAGTGCTGCACC-CAAAACCTG-(TAMRA)3′
- Interleukin 6:
- Forward: 5′-GGGTAGGGAAGGCAGTAGCC-3′
- Reverse: 5′-GAATCCCTCTCCACAAGCG-3′
- TaqMan probe: 5′(FAM)-CTTCAGTGGAGTCGCCTTCTCCCTAA-(TAMRA)3′
- Interleukin 8:
- Forward: 5′-TTCTGCAGCTCTCTGTGAGGC-3′
- Reverse: 5′-GGTGGAAAGGTGTGGAAGTC-3′
- TaqMan probe: 5′(FAM)-TTCTGGCAAGAGTAAGTGCAG-AACTTCGATG-(TAMRA)3′
- Interleukin 10:
- Forward: 5′-TTGGAGCTTGCTAAAGGCACT-3′
- Reverse: 5′-CGGCGCTGTCATCAATTTCT-3′
- TaqMan probe: 5′(FAM)-CACCTCCTCCACGGCCTTGCTC-TT-(TAMRA)3′
- Transforming growth factor β:
- Forward: 5′-TACGCCAAGGAGGTCACCC-3′
- Reverse: 5′-CAGCTCTGCCCGAGAGAGC-3′
- TaqMan probe: 5′(FAM)–CTAATGGTGGAAAGCGGCAACCAAATGTA-(TAMRA)3′

Free fatty acid analysis of therapeutic surfactant preparations

Lipids in rSP-C Surf and natural bovine surfactant were extracted using methanol/chloroform according to the method of Bligh and Dyer [21]. Free fatty acids were converted to fatty acid methyl ester using diazomethane and then purified by means of thin layer chromatography (Silica 60 plates, Merck, Darmstadt, Germany), with toluol as developing solvent. Fatty acid methyl ester was identified using primuline [22] and subjected to gas chromatography as previously described [23, 24].

Statistical analysis and data presentation

Histological results are given as geometric mean ±standard deviation with 95% confidence intervals. Results of real-time PCR analyses are given as geometric mean and dispersion factor (lognormal standard deviation). Data were normalized to the housekeeping gene and further to the control values. Data analysis was performed using SPSS for Windows. The *t* test or the Mann-Whitney *U* test was used to compare two groups and analysis of variance or the Kruskal-Wallis test to compare of more than three groups, as appropriate. In the case of significance, Bonferroni's and Dunn's posthoc tests were applied, respectively. Significance was considered with a p value less than 0.05.

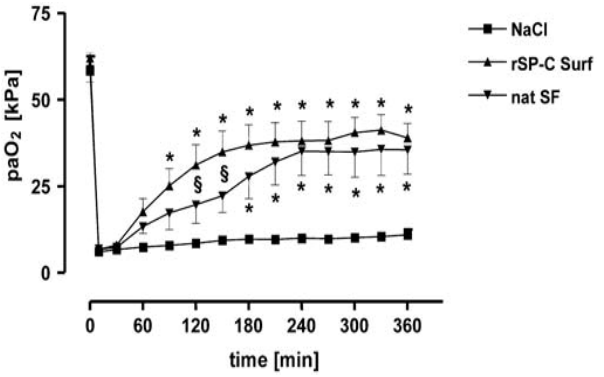

Fig. 1 Levels of paO_2 (kPa) before and after meconium and following surfactant administration (rSP-C or natural bovine surfactant, *nat SF*) or physiological saline (*controls*). *p<0.02 vs. controls, §p<0.05 vs. natural bovine surfactant

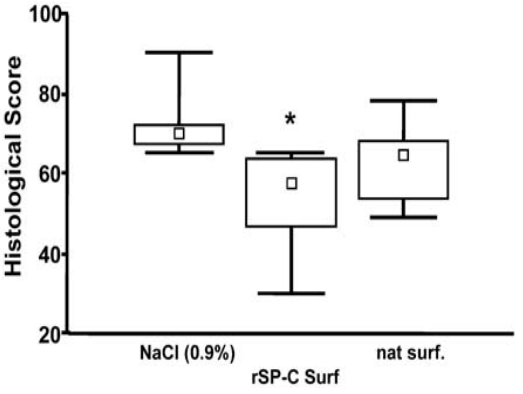

Fig. 2 Lung histology sum scores for alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, alveolar edema, interstitial edema, overdistension, and necrosis in the rSP-C Surf (75 mg/kg b.w.) and the natural surfactant group (*nat SF*; 75 mg/kg b.w.) compared to controls. Data given as boxplots, geometric mean, standard deviation, and 95% confidence interval. *p<0.05 vs. controls

Table 1 Lung function variables in MAS before and after surfactant administration

	Control (n=6)	rSP-C Surf (n=8)	nat SF (n=8)
Tidal volume (min)			
–30	23.4±4.2	21.5±4.8	20.8±4.9
0	13.5±4.1	13.7±4.5	12.4±8.1
30	14.9±3.02	17.98±4.7	16.3±6.8
330	13.5±1.2	22.3±3.9*	19.5±6.7*
VEI (min)			
–30	1.17±0.25	1.03±0.2	1.05±0.46
0	0.55±0.17	0.46±0.2	0.46±0.29
30	0.56±0.13	0.67±0.29	0.58±0.31
330	0.44±0.08	0.89±0.15*	0.89±0.3*

Results

Gas exchange and lung function

Prior to induction of acute respiratory failure by administration of meconium an adequate oxygenation was observed in all groups. After administration of meconium the arterial pO_2, tidal volumes, and VEI were reduced with high significance in all animals (see Table 1; Fig. 1). After intratracheal administration of either rSP-C Surf or natural bovine surfactant (75 mg/kg b.w.) arterial pO_2, tidal volumes, and VEI significantly improved in surfactant treated animals as compared to controls. Initial effects were observed 30 min and maximum effects 330 min after bolus administration of rSP-C Surf and natural bovine surfactant (p<0.0001 vs. control; Table 1).

Histology

Data from 22 piglets were available for histological evaluation of tissue samples from the superior and inferior right lung lobes. Gross macroscopic examination of the lungs revealed severe dystelectasis without differences between left and right lungs. A differing degree of histological alterations was observed in each study group. Sum scores, including atelectasis, alveolar and interstitial inflammation, hemorrhage, edema, necrosis, and overdistension were significantly lower in the rSP-C Surf group (53±12; n=8) than controls (72±9; n=7; p<0.01; Fig. 2). There also was a trend towards lower sum scores in the natural bovine surfactant group (62±10; n=8) than in controls.

Cytokine and growth factor expression

Lung tissue of 12 piglets (control n=3; rSP-C Surf n=5, natural bovine surfactant n=4) was available for real time PCR analyses; material of one piglet had to be excluded because of poor mRNA quality with degradation. Tissue samples of the superior and inferior left lung lobe showed significantly lower IL-1β mRNA expression in the rSP-C Surf group than in controls [p<0.02, normalized to HPRT (H) and β-actin (A)] and to natural bovine surfactant treated animals (p<0.05, H, A; Table 2; Fig. 3). IL-6, IL-8, and TGF-β mRNA expression tended to be higher in natural bovine surfactant treated animals than in rSP-C Surf and controls (Table 2). Cytokine mRNA expression of IL-10 was significantly higher in both surfactant groups than in controls in whole lung tissue (p<0.05, H, A; Table 2; Fig. 4).

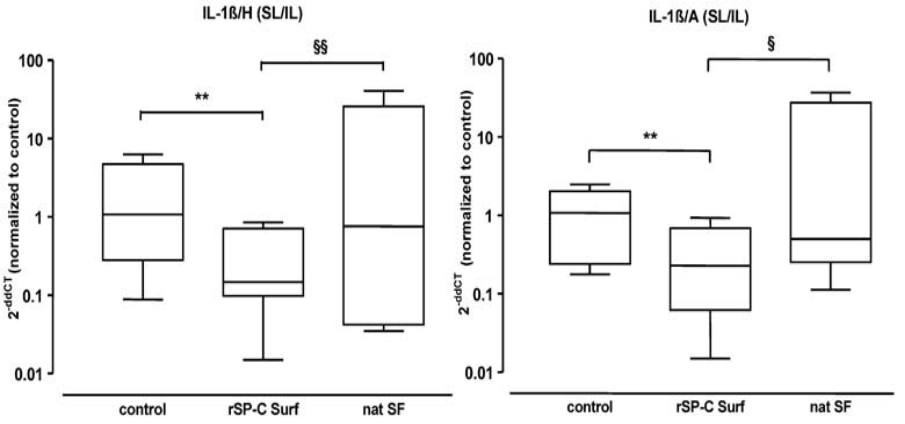

Fig. 3 Pulmonary IL-1β mRNA expression normalized to HPRT (*H*, *left panel*) or β-actin (*A*, *right panel*) in the superior (*SL*) and inferior (*IL*) lobe of the left lung in newborn piglets with experimentally induced MAS (5 ml/kg meconium) after administration of rSP-C Surf, natural bovine surfactant (*nat SF*), or physiological saline. Results are normalized to controls, logarithmic scale. $^{**}p<0.02$ vs. controls, $^{\S}p<0.05$, $^{\S\S}p<0.02$ vs. natural bovine surfactant

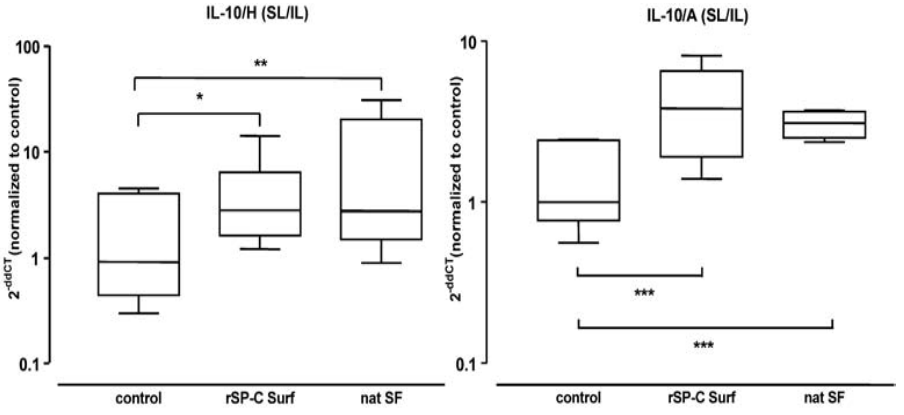

Fig. 4 Pulmonary IL-10 mRNA expression normalized to HPRT (*H*, *left panel*) or β-actin (*A*, *right panel*) in the superior (*SL*) and inferior (*IL*) lobe of the left lung in newborn piglets with experimentally induced MAS (5 ml/kg meconium) after administration of rSP-C Surf, natural bovine surfactant (*nat SF*), or physiological saline. Results are normalized to controls, logarithmic scale. $^{*}p<0.05$, $^{**}p<0.02$, $^{***}p<0.01$ vs. controls

Table 2 Cytokine mRNA expression in superior and inferior left lung lobes and in inferior left lung lobe; normalization to HPRT (*H*) and β-actin (*A*) and further to mRNA expression in control subjects. Data given as geometric mean and dispersion factor

	Control (n=3)	Superior, inferior		Inferior	
		rSP-C Surf (n=5)	nat SF (n=4)	rSP-C Surf (n=5)	nat SF (n=4)
IL-1β/H	1±0.19	0.17±0.30**,5*	0.77±0.05	0.17±0.30**,5*	0.77±0.05
IL-1β/A	1±0.33	0.19±0.27**,4*	0.63±0.1	0.19±0.27**,4*	0.63±0.1
IL-6/H	1±0.19	1.92±0.29	4.61±0.09	1.92±0.29	4.61±0.09
IL-6/A	1±0.33	2.11±0.38	3.78±0.2	2.11±0.38	3.78±0.2
IL-8/H	1±0.14	0.35±0.30	1.34±0.07	0.35±0.30	1.34±0.07
IL-8/A	1±0.22	0.38±0.16	1.1±0.11	0.38±0.16	1.1±0.11
IL-10/H	1±0.35	3.19±0.47*	3.61±0.28**	3.19±0.47*	3.61±0.28**
IL-10/A	1±0.56	3.5±0.54***	2.96±0.8***	3.5±0.54***	2.96±0.8***
TGF-β/H	1±0.21	0.88±0.4	2.52±0.23	0.88±0.4	2.52±0.23
TGF-β/A	1±0.42	0.97±0.24	2.06±0.58	0.97±0.24	2.06±0.58

Analyzing cytokine mRNA expression in both lung lobes separately, significantly lower levels of IL-1β and IL-6 mRNA were observed in the lower lung lobe from rSP-C Surf treated animals than in natural bovine surfactant ($p<0.05$, H, A) and controls (IL-1β, $p<0.05$, H; Table 2). Again in the natural bovine surfactant group a tendency to higher IL-6 and TGF-β mRNA expression levels was observed. IL-10 mRNA expression was significantly increased in the rSP-C Surf group in the upper and lower left lung lobe ($p<0.05$, A, vs. controls; Table 2), a trend to higher expression levels was observed for natural bovine surfactant.

Free fatty acid analysis

Chromatographic analyses revealed nearly 2% (w/w) arachidonic acid referring to total free fatty acid concentrations in the natural bovine surfactant samples. The fraction of free fatty acids in natural bovine surfactant was

nearly 3% (wt:wt) compared to total phospholipids and significant amounts of ω-3 fatty acids eicosapentaenoic acid (1%) and docosahexaenoic acid (0.6%) were found in the analyzed natural bovine surfactant probes. The profiles of the tested natural bovine surfactant charges (*n*=3) were almost identical. In contrast the recombinant SP-C surfactant contained no arachidonic acid, and free fatty acids were composed mainly of palmitic acid (82%) and oleic acid (12%). Again, the profiles of different charges (*n*=3) were stable in the analyses.

Discussion

The present study investigated lung function and inflammatory pulmonary response in a well established piglet model of MAS [16] after bolus administration of two different surfactant preparations. Response to surfactant administration was determined by monitoring pulmonary gas exchange, tidal volumes, and pulmonary inflammatory reaction. Following the early phase of MAS with predominantly mechanically impaired gas exchange, expression of proinflammatory cytokines in meconium aspiration is the second step of the pathophysiological cascade leading to neonatal lung failure resembling acute respiratory distress syndrome (ARDS) [12, 13, 16]. Surfactant administration is a therapeutic option previously used in several studies on treatment strategies for ARDS-like lung failure in neonates [6, 10, 18, 25]. Therefore surfactant preparations with different protein contents and different administration techniques gained increasing interest [26, 27, 28, 29].

In the present study bolus administrations of two different surfactant preparations—rSP-C Surf and natural bovine surfactant—used in a piglet model of MAS showed an adequate response with improved gas exchange and tidal volumes. As recent studies revealed serious concerns against surfactant lavage techniques, bolus treatment was chosen for this experimental setting and confirmed for both surfactant preparations as an efficient and safe intervention in MAS. Apart from these "classical variables," surfactant effects on the inflammatory response in MAS have not been studied systemically yet. As cytokines themselves play a crucial role in altering the surfactant system [30], interactions between these two pathophysiologically important opponents seem to be of considerable interest.

Because mRNA expression precedes protein formation, mRNA expression of early cytokines was quantified using TaqMan real-time PCR as a highly sensitive approach. It is a well established fact, lung maturation to show apicobasal pattern in the neonatal period. Furthermore initial distribution of meconium in MAS has been described to differ vertically involving more severely the lower lung areas [3, 31]. Therefore cytokine mRNA expression was analyzed both in whole lung tissue and for upper and lower lung lobe separately.

Administration of rSP-C Surf led to significantly lower proinflammatory cytokine mRNA expression in whole lung tissue than in natural bovine surfactant and controls. With the natural bovine surfactant mRNA expression of the proinflammatory cytokines even tended to be higher than in controls. As an indicator of direct anti-inflammatory properties of exogenous pulmonary surfactant preparations IL-10 mRNA expression was found to be induced after administration of both surfactant preparations. As IL-10 with its strong anti-inflammatory properties has been discussed as a potential therapeutic option for treatment of chronic inflammatory pulmonary disorders of the newborn [32, 33, 34], the present findings might implicate important clinical potentials.

The differential modulation of the inflammatory response was even more evident, when results were analyzed for upper and lower lung lobe separately, rSP-C Surf revealing most pronounced anti-inflammatory properties. Differing results in pro- and anti-inflammatory cytokines in the superior and inferior left lung lobe may be due to the above differences in maturation and regional distribution of meconium as well as exogenous surfactant. The trend towards a differential cytokine mRNA expression concerning Il-1β and IL-6, more evident in whole left lung tissue, may be due to time dependence in cytokine activation and to their differential role in the inflammatory cascade. Analyses of dynamics in cytokine expression could further explain the extent to which meconium and especially different surfactant preparations affect the complex system of inflammatory pulmonary response in the time course. Furthermore there is evidence in pulmonary inflammatory response a hierarchic regulation of cytokines to be apparent. Studies have shown IL-1 β to induce IL-8 expression in human bronchial epithelial cells [35].

Although anti-inflammatory properties of rSP-C Surf and natural bovine surfactant could be explained by a reduction in ventilator-associated lung injury, identical tidal volumes in both surfactant groups strongly contradict this explanation. Therefore differing qualitative and quantitative surfactant protein and phospholipid compositions must be considered as potential modifiers of the inflammatory response. Therefore effects of the recombinant surfactant might be due to its increased content of SP-C, whose dysfunction was shown to cause severe interstitial lung disease by dysregulation of intracellular surfactant processing [36]. Furthermore, SP-C was shown to interact with inflammation-stimulating agents such as lipopolysaccharides [37] and may thus have impact on pulmonary inflammatory processes. The above properties of SP-C and differing phospholipid compositions of surfactant preparations also may explain the results of other studies that have demonstrated different biophysical interactions [24, 29], gas exchange, and lung mechanics [28] after administration of various surfactant preparations. In contrast, recent reports found no differ-

ence in the inflammatory response after administration of four different surfactant preparations in a model of ventilator-associated lung injury [27].

Furthermore interactions between additional contents of the surfactant preparations and meconium itself or lung tissues must be considered. Phospholipase A_2, present in meconium [38], has been discussed as being responsible for morphological alterations in lung injury [39] and additionally has been demonstrated to inactivate natural surfactant. These interactions might be different for various surfactant preparations, as it has been shown recently that resistance towards inactivation mediated by meconium of the rSP-C Surf is superior to animal lung's derived surfactant preparations [29]. Furthermore, the rate of surfactant subtype conversion induced by meconium [40] may be altered by differing protein and phospholipid contents.

Another important property of phospholipase A_2 is the generation of arachidonic acid, known to be a major precursor of eicosanoids, which mediate various inflammatory processes in both premature and adult lungs [41]. This precursor was found in the natural bovine surfactant preparation in contrast to the studied recombinant product as demonstrated chromatographically. This might explain previously reported activating properties of natural surfactant preparations [42].

Having established gas exchange and tidal volumes to be improved after bolus administration of both surfactant preparations, differences between the rSP-C and the natural bovine surfactant were demonstrated with respect to their immunomodulatory properties. Since there is evidence that persistently high levels of inflammatory cytokines are a pathophysiological mainstay for the development of chronic lung disease in newborns [33, 34], the evaluation of immunomodulatory surfactant properties seems to be of considerable interest. As the exact sequence of the reduced inflammatory response following surfactant in the present model of MAS is unclear, further studies are mandatory to address potential underlying mechanisms.

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chapter 14

Effects of a recombinant surfactant protein-C-based surfactant on lung function and the pulmonary surfactant system in a model of meconium aspiration syndrome

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Crit Care Med. 2006;34:203-210
An editorial was written about this paper (see Appendix A)

Effects of a recombinant surfactant protein-C-based surfactant on lung function and the pulmonary surfactant system in a model of meconium aspiration syndrome*

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Objective: **Meconium aspiration syndrome (MAS) remains a relevant cause of neonatal respiratory failure and is characterized by severe impairment of pulmonary gas exchange, surfactant inactivation, and pronounced inflammatory changes. Surfactant administration has been shown as an effective treatment strategy in MAS. The present study aimed at investigating the impact of a recombinant surfactant protein (SP)-C-based surfactant on pulmonary gas exchange and lung function in this model of neonatal lung injury. Furthermore, SP-B and -C were determined on the transcriptional and protein level.**

Design: **Laboratory experiment.**

Setting: **University laboratory.**

Subjects: **Twenty three newborn piglets (median age 6 days, weight 1900–2500 g).**

Interventions: **Piglets were intubated and mechanically ventilated and then received 20% sterile meconium (5 mL/kg) for induction of lung injury. After 30 mins, animals were randomized for control (n = 7, MAS controls), recombinant SP-C surfactant (n = 8), or natural surfactant (n = 8). Surfactant preparations were administered as an intratracheal bolus (75 mg/kg), and animals were ventilated for another 330 mins. Nonventilated newborn piglets at term (n = 28; median weight 1484 g, range 720–1990 g) served as a healthy reference group (healthy controls).**

Measurements and Main Results: **Lung function variables, arterial blood gas samples, and lung tissues were obtained. Expression of SP-B and -C messenger RNA was quantified in left lung lobe tissue using real-time polymerase chain reaction. Protein concentrations were determined by enzyme-linked immunosorbent assay. Scanning electron microscopy and transmission electron microscopy were performed in tissue samples of the right lung lobe. Compared with healthy controls, SP-B messenger RNA expression was significantly increased in MAS ($p < .02$), whereas SP-C messenger RNA expression was found to be significantly reduced ($p < .001$). SP concentrations, however, were not significantly different. Although a significant improvement of gas exchange and lung function was observed after surfactant administration in both groups, surfactant messenger RNA expression and protein concentrations were not significantly altered. Scanning and transmission electron microscopy showed severe pulmonary ultrastructural changes after meconium aspiration improving after surfactant treatment.**

Conclusions: **Impairment of lung function in MAS, associated with marked changes in SP messenger RNA expression, can be sufficiently treated using recombinant SP-C-based or natural surfactant. Despite improved lung function and gas exchange as well as pulmonary ultrastructure after treatment, pulmonary SP messenger RNA expression and concentrations remained significantly affected, giving important insight into the time course following surfactant treatment in MAS. (Crit Care Med 2006; 34:203–210)**

Key Words: pulmonary surfactant; surfactant proteins; respiratory insufficiency; newborn; meconium aspiration

Meconium aspiration syndrome (MAS) remains a relevant cause of respiratory failure in term neonates despite intensive perinatal treatment (1). MAS-induced pulmonary changes include, among others, typical histologic findings (2), inflammatory cell infiltration with release of vasoactive substances (3), airway obstruction, and surfactant dysfunction and inactivation (4, 5).

Besides the evaluation of treatment strategies such as anti-inflammatory drugs, high-frequency ventilation, inhaled nitric oxide, and liquid ventilation, a number of clinical and animal studies have been performed addressing the pulmonary response to surfactant in MAS (6–9). Nevertheless, despite remarkable treatment effects, the type of surfactant preparation, its dosage, and the mode of administration are under current investigation. Repetitive administration of natural surfactant seems needed to overcome its inactivation induced by meconium constituents such as lysophosphatidylcholine, bilbirubin, bile salts, and inflammatory processes (10, 11). Recent reports further suggested synthetic recombinant surfactant protein (rSP)-C-based surfactant preparations, a safe and efficient therapy in acute lung injury and acute respiratory distress syndrome (ARDS) (12–14), to be less suscep-

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tible to inactivation mediated by meconium compared with natural surfactant preparations (15). In contrast, synthetic but apoprotein-free surfactant preparations such as Exosurf or Pumactant were revealed to be much more sensitive to inactivation by plasma proteins and meconium constituents (16, 17). Thus, the objective of the present study was to evaluate whether bolus administration of the rSP-C-based surfactant is capable of reconstituting lung function in a piglet model of MAS compared with a standard treatment regime.

In addition, to investigate whether exogenous surfactant preparations display an impact on the recovery of pulmonary function by affecting pulmonary surfactant protein expression, surfactant protein (SP)-B and -C were determined on the transcriptional and protein level. To visualize fundamental changes in lung tissue at an ultrastructural level (e.g., morphologic features of alveolar epithelial cells and alveolar septa), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed in pulmonary tissue.

MATERIALS AND METHODS

Animal. Twenty-three newborn piglets, at a median age of 6 days (range 1–11 days) and a median weight of 2200 g (range 1900–2500 g), were studied in a model of experimentally induced MAS. Pulmonary healthy, nonventilated newborn piglets at term (healthy controls, n = 28, median weight 1484 g, range 720–1990 g) served as a control group to define physiologic levels of SP messenger RNA (mRNA) expression and pulmonary protein concentrations. Animal experiments were approved by local authorities and performed according to the *Guidelines for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Protocol. The experimental protocol has been previously published (18). In brief, intravenous anesthesia was induced with ketamine and midazolam after an intramuscular bolus of ketamine. The piglets were intubated with a cuffed endotracheal tube (3.0 mm outer diameter; Rüsch, Kernen-Rommelshausen, Germany), and continuous infusion with midazolam (1 mg/kg of body weight per hour), ketamine (2 mg/kg of body weight per hour), and vecuronium (0.6 mg/kg of body weight per hour) provided sedation, analgesia, and muscular relaxation, respectively. Animals were ventilated using a pressure-controlled mode (Servo 900, Siemens, Germany), with a positive inspiratory pressure of 15 cm H_2O, a positive end-expiratory pressure of 2 cm H_2O, and a respiratory rate of 30 cycles/min (rate of inspiration to expiration 30–70) at 100% oxygen (F_{IO_2} 1.0). Under these conditions, normocapnia was observed. An arterial catheter (20-gauge, Arrow, Erding, Germany) was placed into the right common carotid artery and a central venous catheter (4.0-Fr, Arrow) in the right femoral vein. Arterial blood gases (Pa_{O_2}, Pa_{CO_2}, and pH; ABL Radiometer 555, Diamond Diagnostics, Holliston, MA), heart rate, blood pressure, central venous pressure, and electrocardiogram (78354A Hewlett-Packard) were monitored before and during induction of lung injury, immediately before treatment, and every 30 mins thereafter. Tidal volumes were detected by pneumotachograph (CO_2SMO plus 8100, Novametrix Medical Systems) at the end of the endotracheal tube, and ventilation efficiency index (VEI) was further calculated (19) as well as dynamic compliance. Lung function variables were related to body weight. After a stabilization period of 30 mins, ventilation pressures were increased (positive inspiratory pressure 25 cm H_2O, positive end-expiratory pressure 4 cm H_2O) and were left unchanged during the remaining ventilation period. Lung injury was induced in all animals by a bolus administration of 5 mL/kg of body weight of meconium 20% given in 2- to 4-mL aliquots via the endotracheal tube under ongoing ventilation according to Soukka et al. (20). Thirty minutes after induction of lung injury, piglets were prospectively randomized to one of the following groups: group 1 (MAS control; n = 7) received intratracheal administration of 1.5 mL/kg of body weight physiologic saline (Braun, Melsungen, Germany); group 2 (rSP-C SF; n = 8) received 75 mg of rSP-C surfactant/kg of body weight intratracheally (Venticute; ALTANA Pharma, Konstanz, Germany); group 3 (natural SF, n = 8) received 75 mg of bovine (SP-B and SP-C containing) surfactant (Alveofact; Boehringer Ingelheim, Ingelheim, Germany)/kg of body weight intratracheally, at a concentration of 75 mg/1.5 mL.

After another observation period of 330 mins (360 mins after induction of lung injury), piglets were killed. All animals survived the whole observation period with the exception of one animal of the MAS control group dying from severe respiratory failure. Immediately after death, the chest was opened and a cannula was placed into the pulmonary artery, the left atrium was opened, and the lung was perfused *in situ* with Ringer's lactate containing procaine (250 mg/L), heparin (20 units/mL), and calcium-chloride (2.2 mmol/L) for 10 mins. After perfusion of the lung, positive inspiratory pressure was first increased for 15 secs to 25 cm H_2O and then reduced to 10 cm H_2O. Thereafter, tissue samples of the superior and inferior lobe of the left lung were extracted and immediately snap-frozen in liquid nitrogen for real-time polymerase chain reaction (PCR) and protein analyses. In the next step, right lungs were perfused with 300 mL of a formaldehyde (4.6%)/glutaraldehyde (0.5%) solution for approximately 10 mins. Finally, the trachea was clamped at a positive end-expiratory pressure of 10 cm H_2O, and right lungs were removed and submersed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for TEM and SEM. To estimate physiologic pulmonary surfactant mRNA expression levels and protein concentrations, 28 nonventilated newborn piglets were killed according to the standardized procedure described here and served as a healthy control group. Tissue samples of the left lung lobe were obtained for mRNA and protein analyses.

Preparation of Pooled Meconium. Meconium was collected from healthy full-term neonates with neither history of perinatal complications nor maternal drug administration during pregnancy and delivery and prepared as described previously (20).

Therapeutic Surfactant Preparations. Experiments were performed using an rSP-C-based surfactant (Venticute) or a natural bovine surfactant extract (Alvofact). The rSP-C surfactant contains 2% of a human recombinant nonpalmitoylated SP-C in phospholipids (dipalmitoylphosphatidylcholine and palmitoyloleoyl-phosphatidylglycerol in a 70:30 ratio (weight/weight), 50 mg/mL, plus 5 % palmitic acid). Alveofact® is an organic calf lung lavage extract containing the hydrophobic apoproteins SP-B and SP-C (approximately 1.5%) as described previously (21). Both surfactant preparations were provided as lyophilized powder and resuspended in sterile saline 0.9% (Braun) to a final concentration of 50 mg/mL.

Measurements of Surfactant Protein B and C mRNA Expression by Real-Time PCR. Expression of SP-B and -C mRNA was measured in tissue of the left upper and lower lung lobe using the real-time reverse transcriptase (RT)-PCR technique (TaqMan). Primers and probes for SP-B, SP-C, 18-S, and β-actin were designed using Primer Express software (Applied Biosystems, Foster City, CA) following a fixed set of recommendations to allow the application of standardized cycling conditions (2 mins at 50°C, 10 mins at 95°C, followed by 45 cycles for 15 secs at 95°C, and 1 min at 60°C). Control PCRs showed no signal for genomic DNA proving mRNA specificity. Primers were purchased from Roth (Karlsruhe Germany) and probes from Applied Biosystems. Lung tissue homogenization was performed in liquid nitrogen, and total RNA was then extracted using the acid guanidinium-thiocyanate-phenolchloroform method (Roti Quick, Roth). Total RNA isolation, random primed reverse transcription, and real-time PCR were performed following a standardized protocol as described previously (18). Primer and TaqMan probe sequences are depicted next. Relative quantification was performed using the ΔΔCt method, which results in a ratio of target gene expression and the expression of a housekeeping or reference gene.

As the validity of this method depends on the constant expression of the housekeeping gene throughout the set of samples studied, we analyzed gene expression ratios of 18S (S)

and β-actin (A) as well as the total mRNA content of each sample. As 18S showed both no differences between different experimental groups and the lowest variation in all samples, it was chosen as reference gene for further analysis.

Primer and TaqMan probes.

18S:

Forward primer (FP): 5'–GCC GCT AGA GGT GAA ATT CTT G–3'

Reverse primer (RP): 5'–CAT TCT TGG CAA ATG CTT TCG–3'

Probe: 5'(FAM)–CCG GCG CAA GAC GGA CCA GA–(TAMRA)3'

β-Actin:

FP: 5'–TCA TCA CCA TCG GCA ACG–3'

RP: 5'–TTC CTG ATG TCC ACG TCG C–3'

Probe: 5'(FAM)–CCT TCC TGG GCA TGG AGT CCT GC–(TAMRA)3'

Surfactant protein B (SP-B):

FP: 5'–TCC GCT GGT CGT TGA TCA C–3'

RP: 5'–GTT TGC ACA GGC CCA AGT G–3'

Probe: 5'(FAM)–CAG AGC CAA ATG AAC CTG AAG GCC ATC–(TAMRA)3'

Surfactant protein C (SP-C):

FP: 5'–CAC CTT CTC CAT TGG CTC TAG TG–3'

RP: 5'–ATA CTC TGC GGA GAC ATC TTC ATG–3'

Probe: 5'(FAM)–TGA CTA CCA GCG GCT CCT GAT TGC C–(TAMRA)3'

Quantification of Surfactant Proteins in Lung Homogenate. SP-B and SP-C were quantified in lung homogenates of the left upper and lower lung lobe by enzyme-linked immunosorbent methods as described earlier (22, 23) with slight modifications. In brief, for quantification of SP-B, lung homogenate was diluted 1:1000 with phosphate-buffered saline (PBS)/1-propanol 1:1, pH 7.4 and for quantification of SP-C lung homogenate was diluted 1:500 with 80% 2-propanol, pH 3.5. Samples were transferred to a microtiter plate and the proteins were allowed to bind. After selective removal of phospholipids (iisopropylether/butanol [3:2, volume/volume] wash for SP-B, methanol wash for SP-C) and saturation of free binding sites, plates were incubated with primary antibodies (anti-SP-B, 8B5E, raised against porcine SP-B, 5 μg/mL in PBS/1% bovine serum albumin; anti-SP-C, polyclonal antiserum raised against human rSP-C and cross-reacting with porcine SP-C, 1:2000 dilution [volume/volume] in PBS/1% bovine serum albumin). Microtiter plate-bound antibodies were detected using secondary species-specific biotinylated antibodies (SP-B, antimouse immunoglobulin G; SP-C, antirabbit immunoglobulin G [Amersham Biosciences, Freiburg, Germany], each 1:1000 in 1% bovine serum albumin/PBS) with subsequent application of AB-complex and ABTS as substrate. Absorbance was measured at 450 nm with an enzyme-linked immunosorbent assay photometer (Tecan, Crailsheim, Germany). SP content was calculated with respect to the dry lung weight employing cubic-spline-interpolated standard curves obtained from isolated human SP-B and recombinant human dipalmitoylated SP-C, respectively.

Scanning Electron Microscopy. Fixation for SEM was carried out in formaldehyde 3.5%. Material was prepared out of the lung tissue, dried using the critical point method,

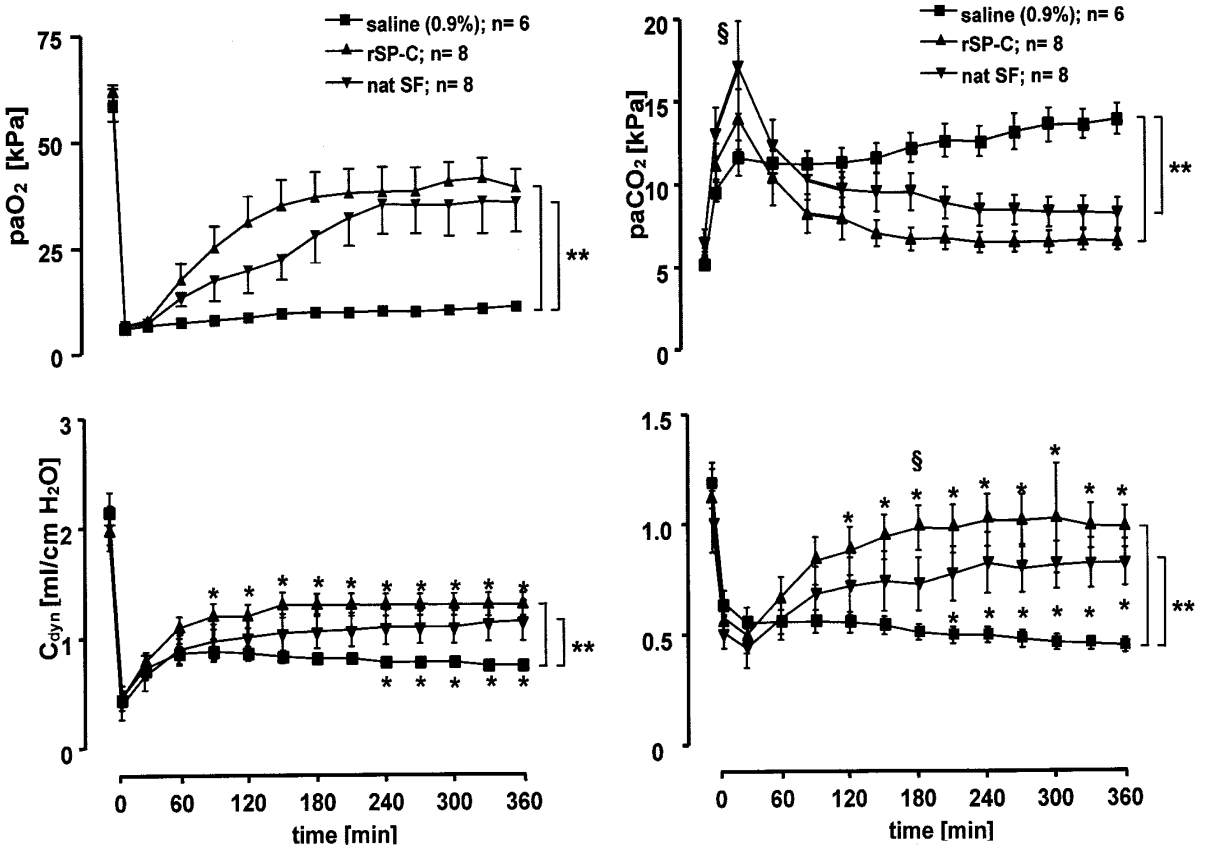

Figure 1. Levels of Pa_{O_2} (kPa; *left upper panel*), P_{CO_2} (kPa; *right upper panel*), dynamic compliance (C_{dyn}; mL/cm H_2O; *left lower panel*), and ventilation efficiency index (*VEI; right lower panel*) before and after meconium and following either surfactant administration (recombinant surfactant protein C [*rSP-C*] or natural bovine surfactant [*nat SF*]) or physiologic saline (meconium aspiration syndrome [MAS] controls), respectively. Results are given as mean and SEM; $^{**}p < .001$ vs. MAS controls.

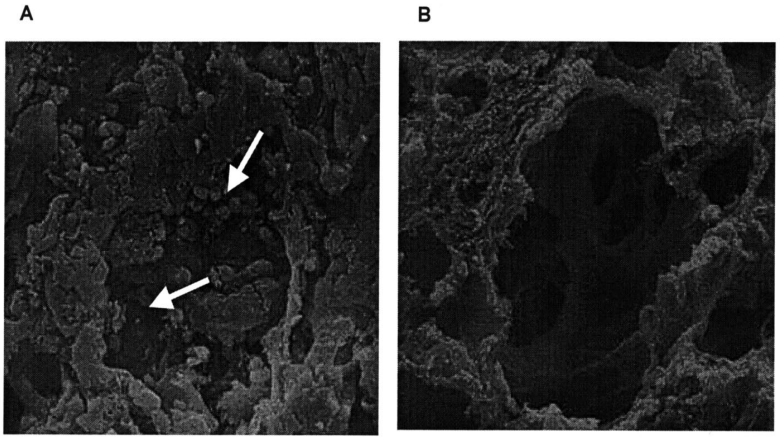

Figure 2. Scanning electron microscopy (SEM) before (*A*) and after administration of recombinant surfactant protein C (rSP-C) surfactant in a piglet model of meconium aspiration syndrome (*B*; rSP-C surfactant, 75 mg/kg body weight). *A*, lung tissue of a control animal. Thickened alveolar septa, fibrin deposition, and numerous intra-alveolar granulocytes indicate lung injury (*arrow*, SEM, magnification ×500). *B*, lung tissue after therapy with rSP-C surfactant. The alveolar structure is intact and septa are thin. No inflammatory cells could be shown (SEM, magnification ×360).

and sputtered with gold after mounting. Measurements were carried out using the scanning electron microscope Zeiss DSM 950 (Zeiss, Jena, Germany) with the pathologist blinded for the animals' group assignment. Tissue samples for SEM were collected from areas with macroscopic changes, as, for example, atelectases, significant changes in consistency, and/or conspicuous color of the lung tissue. From lungs with no visible macroscopic alterations, specimens from peripheral segments of the upper and lower lobes and central parts of the lung tissue were selected for analysis.

Transmission Electron Microscopy. For TEM, tissue samples were fixed for 2 hrs in 2.5% glutaraldehyde in phosphate buffer (pH 7.2). After postfixation in osmiumtetroxide and block contrast with uranyl acetate, they were dehydrated in an increasing alcohol concentration and embedded in Epon 812 (Shell Chemicals, Houston, TX). Thin sections were cut using a Reichert Om U3 Ultramicrotome (Vienna, Austria), stained with methylene blue for 2 mins, and evaluated by light microscope. Ultrathin sections of selected tissue areas were stained with lead citrate and examined by TEM (Zeiss EM 900, Jena, Germany). Tissue sampling for TEM was performed as described for SEM analysis.

Statistical Analyses and Data Presentation. Results of lung function testing are given as mean and SEM. The effect of surfactant treatment on lung function variables over the study period was tested by a two-way analysis of variance procedure with repeated measurements as normal distribution could be assumed.

Results of real-time PCR analyses (arbitrary units) and protein measurements are given as median and range and are represented in the figures as box plots, as the data are not normally distributed. Real-time PCR results were normalized to two housekeeping genes. For further statistical analysis and comparison of groups, data were logarithmically transformed and one-way analysis of variance was performed with *post hoc* Scheffé. Data analysis was performed using SPSS for Windows, version 11.5. Calculated *p* values are given in the Results section.

RESULTS

Gas Exchange and Lung Function. All animals survived the whole observation period with the exception of one animal of the MAS control group dying from severe respiratory failure. After induction of acute respiratory failure by administration of meconium, Pa_{O_2}, dynamic compliance, and VEI were significantly reduced in all animals, whereas Pa_{CO_2} significantly increased up to two- to three-fold (Fig. 1). Intratracheal administration of either rSP-C surfactant or natural bovine surfactant (each 75 mg/kg of body weight) significantly improved Pa_{O_2}, dynamic compliance, and VEI and significantly decreased Pa_{CO_2} in surfactant-treated animals compared with MAS controls ($p < .001$, two-factor analysis of variance; Fig. 1). There was a tendency toward faster and more sustained effects regarding Pa_{O_2}, Pa_{CO_2}, dynamic compliance, and VEI in the rSP-C surfactant-treated group after induction of lung failure compared with animals treated with natural surfactant, although there was no statistically significant difference (Fig. 1).

Scanning and Transmission Electron Microscopy. Pulmonary ultrastructural changes were screened using SEM and TEM in right lung lobe tissue samples. In lungs of newborn piglets following meconium aspiration (MAS controls), significant structural changes were found with intra-alveolar granulocytes identified by typical cytoplasmic patterns. In these sampled lung areas, changing amounts of neutrophils could be found in the majority of alveoli analyzed, and >50% of the alveolar septa showed a marked thickening. About 30% of the thickened alveolar septa showed fibrin deposition (Fig. 2). After rSP-C treatment, no influx of inflammatory cells could be detected, and alveolar structures were intact with thin alveolar septa (Fig. 2).

TEM revealed that MAS controls had structural changes in the cytoplasm of alveolar epithelial cells as well as intra-alveolar granulocytes with cell detritus (Fig. 3). After surfactant treatment, intact alveolar septa and capillaries as well as alveolar epithelial cells could be shown (Fig. 3).

SP-B and C mRNA Expression. Lung tissue from 18 piglets was available for real-time PCR analyses. Material of one piglet in the saline and two piglets in the natural surfactant group had to be further excluded because of poor mRNA quality due to degradation (MAS controls, n = 5; rSP-C SF, n = 6; natural SF, n = 4). Control samples of the left lung lobe from 28 healthy newborn piglets (healthy controls) were available for examination. There was no significant difference in SP-B and -C mRNA expression when analyzing SP mRNA expression in upper and lower lung lobes separately. Therefore, means were calculated and used for further analysis. In the figures, results are given normalized to 18S; normalization to β-actin showed comparable results.

Tissue samples of the superior and inferior left lung lobe showed a significantly increased SP-B mRNA expression after induction of lung injury with meconium and mechanical ventilation (MAS controls) compared with healthy controls ($p < .001$, Fig. 4). In contrast, SP-C mRNA expression was significantly decreased after induction of lung injury (MAS controls) compared with healthy controls ($p < .001$; Fig. 5). After treatment with either synthetic rSP-C or natural bovine surfactant, SP-B mRNA expression was found to remain significantly increased ($p < .001$ vs. healthy controls; Fig. 4), and SP-C mRNA expression significantly decreased compared with healthy controls (rSP-C vs. healthy con-

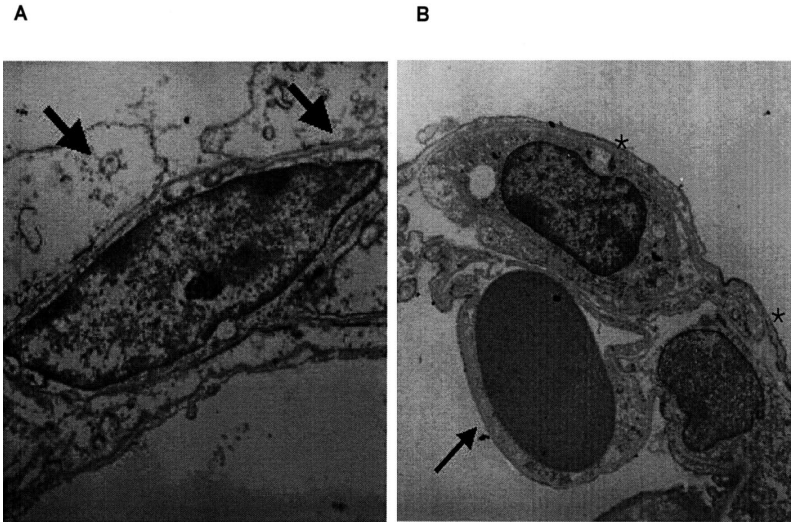

Figure 3. Transmission electron microscopy (TEM) before (*A*) and after administration of recombinant surfactant protein C (rSP-C) surfactant in a piglet model of meconium aspiration syndrome (*B*; rSP-C surfactant, 75 mg/kg body weight). *A*, lung tissue of a control animal. In the center, an alveolar septum with an oval nucleus can be seen. The cytoplasm of the alveolar type I cells show severe edema and loss of organelles (*arrows*, TEM, magnification ×7000). *B*, lung tissue after therapy with rSP-C surfactant. The alveolar septum shows two distinct nuclei. Cytoplasm of alveolar type I (*stars*) cells is regularly arranged; furthermore, an intact capillary is seen (*arrow*, TEM, magnification ×7000).

trols $p < .001$; natural SF vs. healthy controls $p = .02$; Fig. 5). No significant differences between SP-B or SP-C mRNA expression were observed with regard to the different treatment regimes, although there was a trend toward elevated SP-B mRNA expression in the surfactant-treated groups compared with MAS controls.

SP-B and C Concentrations. Lung tissue from 18 piglets was available for analysis; material of one piglet in the saline and two piglets in the natural surfactant group had to be further excluded because of protein degradation (MAS controls, n = 5; rSP-C SF, n = 6; natural SF, n = 4). Control samples of the left lung lobe from ten healthy newborn piglets (healthy controls) were available for examination. There was no significant difference in SP-B and -C concentrations in lung homogenates when analyzing SP concentrations in upper and lower lung lobes separately. Therefore, means were calculated and used for further analysis. SP-B and -C were detectable in homogenates of the left lung and were related to lung dry weight for comparison. Pulmonary SP-B and -C protein contents were not significantly altered after induction of lung injury by meconium (MAS controls) when compared with pulmonary healthy controls. Nevertheless, a trend toward decreased SP-B concentrations could be observed in lung homogenates after induction of lung injury ($p = .2$). Surfactant treatment did not alter pulmonary SP-B and -C concentrations significantly (Figs. 4 and 5).

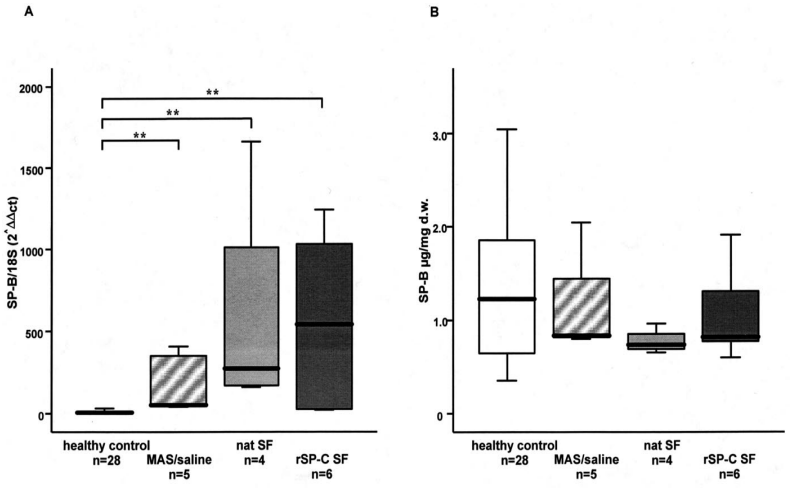

Figure 4. Pulmonary surfactant protein-B (*SP-B*) messenger RNA expression normalized to 18S (*A*) and SP-B protein concentrations in lung homogenate (dry weight; *B*) in the left lung lobe of newborn piglets with experimentally induced meconium aspiration syndrome (*MAS*; 5 mL/kg meconium) following administration of physiologic saline, recombinant surfactant protein C (*rSP-C*), or natural bovine surfactant (*nat SF*). Means were calculated from values of upper and lower lung lobe. Median, interquartile range (box limits, 25th and 75th percentiles), and extreme values (error bars) are indicated in the figure. $^{**}p < .001$ vs. healthy controls.

DISCUSSION

This is the first report to describe the response of exogenous surfactant administration on pulmonary gas exchange and lung function variables as well as ultrastructural changes to the alveolar-capillary unit and effect on the pulmonary surfactant system in an established piglet model of MAS (20) after bolus administration of either an rSP-C or a natural bovine SP-B- and SP-C-containing surfactant.

Meconium aspiration leading to neonatal ARDS-like lung failure through mechanically impaired gas exchange and induction of pronounced pulmonary inflammatory changes (20, 24, 25) significantly responds to exogenous surfactant administration (26). In the past decade, different administration techniques and alteration of protein contents in surfactant preparations gained increasing interest to improve the treatment response in ARDS-like lung failures of the neonate (15, 27–29).

As rSP-C surfactant revealed to be efficient in premature lambs and rabbits suffering from respiratory distress syndrome (19) as well as in different models of acute lung injury (13, 30), the present model of MAS addressed its potencies in a model of lung injury mainly characterized by severe inflammatory changes. Serious concerns about the surfactant lavage techniques, which have been raised from recent studies (31), formed the rationale for bolus treatment in this experimental setting and confirmed both sur-

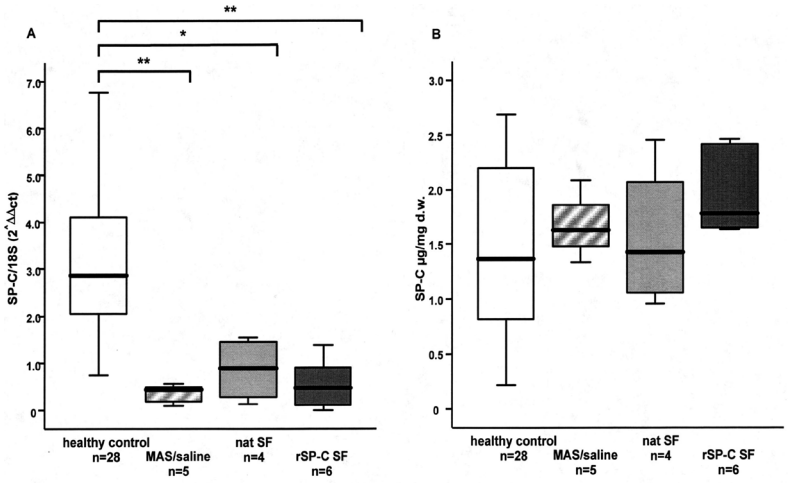

Figure 5. Pulmonary surfactant protein-C (*SP-C*) messenger RNA expression normalized to 18S (*A*) and SP-C protein concentrations in lung homogenate (dry weight; *B*) in the left lung lobe of newborn piglets with experimentally induced meconium aspiration syndrome (*MAS*; 5 mL/kg meconium) following administration of physiologic saline, recombinant surfactant protein C surfactant (*rSP-C SF*), or natural bovine SF (*nat SF*). Means were calculated from values of upper and lower lung lobe. Median, interquartile range (box limits, 25th and 75th percentiles), and extreme values (error bars) are indicated in the figure. $*p = .03$; $**p < .001$ vs. healthy controls.

factant preparations as efficient and safe treatment strategies in MAS.

For rSP-C surfactant, an adequate response with significantly improved gas exchange, dynamic compliance, and ventilation efficiency index can be shown in this piglet model of MAS. Time and extent of pulmonary recovery were at least comparable to effects in the group treated with natural surfactant, with a tendency toward faster and more sustained effects regarding Pao_2, $Paco_2$, dynamic compliance, and VEI in the rSP-C surfactant-treated group. The effects of rSP-C treatment in MAS, supporting findings of other investigators mentioned previously (13, 30), can be partially attributed to the well-known properties of SP-C facilitating the adsorption of surfactant phospholipids to an air-water interface and stabilizing the interfacial lipid layer during film compression (32, 33). Although SP-C has fewer effects on the adsorption rate of phospholipids than SP-B, there are important functions attributed to SP-C, such as film stabilization and respreading after film collapse (34). As there is no consensus about the relative importance of the specific SP for optimal function of exogenous surfactant preparations, we hypothesized that rSP-C surfactant would affect the recovery of pulmonary function by affecting the pulmonary surfactant system and either stimulate endogenous SP RNA expression or mobilize intracellular surfactant protein pools as a potential mechanism for the recombinant SP-C-based surfactant to compensate for its lack in SP-B. The importance of the hydrophobic SP-C in normal lung function is highlighted by the fact that dysfunction of SP-C has been shown to cause severe interstitial lung disease in newborns, older children, and adults (35, 36). As dysregulation of intracellular SP processing is known to be causally related to the pathophysiology of the disease and furthermore *in vitro* studies showed SP-C but not SP-B to enhance the uptake of isopropylether liposomes by type II cells (37), an important role of SP-C regarding surfactant metabolism and function can be assumed. Therefore, in the present study SP-B and C were determined on the transcriptional and protein level in lung homogenates after exogenous surfactant administration. For quantification of mRNA expression in pulmonary tissue, TaqMan real-time PCR was used as a highly sensitive approach. Compared with control animals, the induction of lung injury by meconium resulted in a significant reduction of SP-C mRNA expression when compared with pulmonary healthy controls. This indicates severe changes to alveolar type II cells, as SP-C has so far been detected in alveolar type II cells of the mature lung only (38). Indeed, signs of an impaired structure of alveolar epithelial cells in MAS controls could be detected by using SEM and TEM. In accordance to our findings, previous studies revealed reduced SP-C concentrations in bronchoalveolar lavage specimens of patients suffering from ARDS (22). SP-B mRNA expression could be shown to be significantly up-regulated and potentially represents compensatory or repair mechanisms of type II cells in the alveolar compartment. Vayrynen et al. (39) found alterations of SP-B and -C expression by lipopolysaccharide and cytokines to be dependent on the degree of lung maturation. Lungs of term neonates revealed reduced SP-B and -C expression in response to lipopolysaccharide, whereas SP-B was shown to be induced by inflammatory stimuli in the premature lung, indicating differential regulation of surfactant protein expression. Divergent effects observed in our study regarding SP-B mRNA expression may be due to the short observation period or differences between *in vitro* and *in vivo* conditions. Furthermore, corresponding pulmonary protein concentrations were determined and revealed no significant differences in SP-B and-C concentrations after induction of lung injury, although SP-B levels showed a trend to lower levels compared with pulmonary healthy controls. This finding may indicate compensatory mechanisms in a formerly healthy lung exposed to pronounced changes leading to severe lung injury.

Regarding the effect of exogenous surfactant administration on the pulmonary surfactant system, neither treatment regime led to significant differences in pulmonary SP-B and -C mRNA expression and protein concentration when compared with saline-treated controls after induction of lung injury by meconium (MAS controls). Nevertheless, SP-B mRNA expression tended to be higher in surfactant-treated animals, potentially indicating regenerating mechanisms in alveolar epithelial cells. As results of TEM and SEM could demonstrate structural reconstitution of the alveolar-capillary compartment following surfactant administration, these findings may represent the time course of pulmonary recovery following severe mechanical and inflammation-induced lung injury. Thus, improved gas exchange and lung function were followed by structural reconstitution. This leads to recovery of the surfactant system, not entirely acquired in the study setting. In accordance with these findings, other studies have shown recovery of the pulmonary surfactant system indicated by SP-B and -C concentrations reaching baseline levels not earlier than 4 hrs after induction of lung injury (40). In animal mod-

Impairment of lung function in meconium aspiration syndrome, associated with marked changes in surfactant protein messenger RNA expression, can be sufficiently treated using recombinant surfactant protein-C-based or natural surfactant.

els of ARDS, even up to 48 hrs have been reported for an increase of surfactant proteins after lung failure (41).

As recent studies revealed resistance of synthetic rSP-C-based surfactant preparations to inactivation by meconium (15) as well as anti-inflammatory potencies of rSP-C (18), a regulatory or protective effect of rSP-C on type II cells or different parts of the alveolar-capillary compartment has to be considered. Nevertheless, the differing phospholipid content of both surfactant preparations also has to be taken into account as a possible additional mechanism influencing their effects on lung function and surfactant protein expression.

As there are limitations to the study regarding the number of subjects studied, results have to be interpreted on an explorative level; further studies are needed addressing the ability of different surfactant preparations to interfere with alveolar type II cell metabolism as a possible cause for individual effects of surfactant preparations exceeding their various biophysical properties.

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chapter

15

Positive end-expiratory pressure modifies response to recombinant and natural exogenous surfactant in ventilated immature newborn rabbits

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Positive End-Expiratory Pressure Modifies Response to Recombinant and Natural Exogenous Surfactant in Ventilated Immature Newborn Rabbits

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Key Words
Respiratory distress syndrome · Rabbit · Natural bovine surfactant · Recombinant SP-C surfactant · Positive end-expiratory pressure

Abstract
Background and Objectives: Different types of surfactant preparations were shown not to exert uniform response in preterm infants suffering from respiratory distress syndrome (RDS). Therefore, the effects of a recombinant surfactant protein C (rSP-C) based preparation and a natural surfactant were compared applying different levels of positive end-expiratory pressure (PEEP) in experimental RDS. ***Methods:*** Preterm rabbits (n = 7–14 per group; 27 days gestation; term 30 days) were randomized for receiving either 100 mg/kg rSP-C or natural bovine surfactant and were compared with saline treated controls. Animals were ventilated for 30 min with either 0.3 or 0 kPa PEEP at standardized tidal volumes and lung mechanics were measured as well as lung histology and mRNA expression of surfactant associated proteins B and C by real-time PCR. ***Results:*** The PEEP level applied (0.3 vs. 0 kPa) largely influenced dynamic compliance after administration of rSP-C surfactant (4.45 vs. 2.58 ml/kg), whereas natural surfactant improved compliance regardless of the PEEP applied (4.86 vs. 4.24 ml/kg) compared to controls (2.41 vs. 1.55 ml/kg). Accordingly, administration of PEEP significantly increased alveolar count in all groups as well as SP-C mRNA expression, whereas SP-B expression and protein content both remained unchanged. ***Conclusion:*** Response to rSP-C surfactant depends on the PEEP level applied in our model of neonatal RDS. These findings should be considered for the conception of clinical trials regarding treatment strategies in neonatal RDS.

The results were presented in part at the meeting of the Society for Pediatric Research 2002 in Baltimore and 2003 in Seattle, USA.

Introduction

Natural surfactant therapy and administration of positive distending airway pressure both are standards in treatment regimes for neonatal respiratory distress syndrome (RDS) in preterm infants.

Introduced into clinical routine use as second generation surfactants in the beginning 90s, surfactant prepara-

tions are either organic lavage or minced lung extracts containing the hydrophobic surfactant proteins (SP) B and C. Both SP are known for their surface tension lowering property, essential for adequate postnatal pulmonary adaptation and maintenance of lung physiology. Underscoring their important function, the complete absence of SP-B has been shown to lead to progressive lethal pulmonary failure during the neonatal period and is additionally associated with a decreased concentration of mature SP-C pro-protein [1–3]. SP-C, a highly active peptide enhancing the movement of phospholipids to the air–water interface, inherits important functions as the facilitation of re-spreading after surface film collapse and maintenance of film stability [3, 4].

As third generation surfactants protein-free synthetic phospholipids and lipid mixtures containing recombinant SP-C (rSP-C) or a synthetic SP-B mimetic peptide have been developed [5]. Very recently, studies have been published, describing multicenter randomized clinical trials showing SP-B mimetic based third generation surfactant to be effective in prevention of acute and chronic pulmonary disorders in preterm infants [6, 7]. Furthermore, experimental studies on a rSP-C based surfactant preparation demonstrated adequate biophysical properties and increasing lung volumes in preterm rabbits comparable to natural surfactant preparations [8–10]. Accordingly, rSP-C based surfactant was effective in animal models of surfactant deficiency induced by saline lung lavage and in experimental meconium aspiration [11, 12]. Nevertheless, there is up to now no consensus about the relative importance of the specific surfactant associated proteins for optimal function of exogenous surfactant preparations and the composition of an 'ideal' surfactant preparation for treatment of neonatal RDS.

With respect to ventilation strategies in neonatal RDS, the administration of continuous distending airway pressure or positive end-expiratory positive airway pressure (PEEP) has been demonstrated to improve lung function and furthermore, has been described to strongly modify the effects of exogenously administered surfactant preparations in experimental settings [13]. Although the application of PEEP in ventilation strategies for preterm infants is clinical routine, high-mean airway pressure resulting from increasing PEEP can result in reduced cardiac output and therefore may predispose to various neonatal complications especially in the extremely preterm infant [14, 15]. The approach of administering inadequately high levels of PEEP using either nasal continuous positive airway pressure systems or a combination with mechanical ventilation strategies may thus expose infants to an increased risk of acute cerebral hemodynamic compromise [15–17].

Therefore, the comparison of a second generation natural surfactant being in clinical use with a third generation preparation at different levels of PEEP in a neonatal model of RDS may provide further insights into differential surfactant properties and lead to implications for clinical practice.

Materials and Methods

The experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by local authorities of the animal investigation committee.

Twelve pregnant New Zealand White Rabbits (Bauer, Neuenstein-Lohe, Germany) on day 27 of gestation (physiologic gestation 30 days) were anesthetized with ketamine (10 mg/kg) and xylazine (20 mg/kg), body temperature was kept within the physiologic range by heating pads and tracheotomy was performed followed by mechanical ventilation. Preterm rabbits were sequentially delivered by cesarian section and given ketamine (10 mg/kg) and xylazine (20 mg/kg) by intraperitoneal injection to provide analgesia and sedation. After a tube made from an 18-gauge stainless-steel needle was secured into the trachea, the newborn rabbits (birth weight 27.4 ± 3.6 g; mean ± standard deviation) were randomized to receive either saline or surfactant preparations. Ventilated control animals received 2 ml/kg saline, whereas animals of the surfactant groups obtained either bovine lung-lavage derived surfactant Alveofact® (100 mg/kg) or rSP-C-based surfactant Venticute® (100 mg/kg) at volumes of 2 ml/kg each. The animals were then transferred to a 37°C temperature-controlled ventilator-plethysmography system (Boehringer-Ingelheim, Biberach, Germany) using microprocessor-controlled magnet valves for in- and expiration. Constant inspiratory flow was adjusted using an air regulator attached before the inspiration-valve. Volume-controlled, pressure-limited ventilation was adjusted to a tidal volume of 7–8 ml/kg, fraction of inspiratory oxygen (FiO_2) was 1.0 and air humidity 100%. After an initial positive inspiratory pressure (PIP) of 3.5 kPa, ΔP (peak inspiratory pressure [PIP] – PEEP) was limited to 3 kPa for further experimental procedure.

Mechanical ventilation regimes were further stratified using 0.3 kPa or zero PEEP (ZEEP; 0 kPa), respectively. Animals were ventilated at a rate of 30 breaths per minute and an inspiration to expiration rate (I:E) of 1:2 according to Davis et al. [18] for overall 30 min after surfactant administration.

Time course of ΔP, and tidal volumes (ml) were continuously monitored and documented every 5 min during the observation period. Dynamic compliance (C_{dyn}) was calculated per breath according to the following formula:

$$\frac{TV}{\Delta P \times kg}$$

(TV = tidal volume, ΔP = peak inspiratory pressure [PIP] – PEEP). Animals were sacrificed at the end of the protocol with an overdose of ketamine and xylazine. After opening of the thorax at the

end of the experiment animals were included showing a heart rate within the physiologic range and no signs of pneumothorax. Exclusion of animals with no adequate cardiac activity or pneumothorax led to differences in the number of animals within the different study groups. One group of animals served as a non-ventilated control group to define base-line levels of SP expression and animals were therefore sacrificed immediately after cesarean section as described above.

For further evaluation of lung histology and SP expression, lungs were either paraffin-embedded for histological investigation or kept at –80°C for molecular analyses. For histological analyses lungs were perfused with a formaldehyde (4.6%)-glutaraldehyde (0.5%) solution for approximately 10 min under application of 2.0 kPa PIP in a standardized setting. After clamping of the trachea lungs were removed and submersed in the same solution.

Lung Histology

Lung tissue was embedded in paraffin and tissue slides were obtained from dependent and non-dependent parts of the left and right lung. The slides were stained with hematoxylin-eosin (slides of 0.5 μm thickness). Lung histology was evaluated by a pathologist (M.E.), blinded to the animal group assignment. Lung tissue was screened in order to identify areas representative for the alveolar structure of the investigated lung tissue. These representative fields from each ventral and dorsal part of the lungs were studied morphometrically at a 400-fold magnification. Number of alveoli and terminal airways were counted in five representative fields. Septal thickness was measured after photodocumentation of the lung tissue using Axiovision 3.0 for AxioCam MRc (Zeiss, Wetzlar, Germany). Alveolar septa were measured at their narrowest diameter and mean values of ten measurements per lung were calculated excluding not exactly longitudinally truncated septa.

Measurements of SP-B and -C mRNA Expression by Real-Time PCR

Total RNA isolation, random primed reverse transcription and real-time PCR were performed following a standardized protocol as described previously [12]. Lung tissue homogenization was performed in liquid nitrogen and total RNA was then extracted using the aid guanidinium-thiocyanate-phenol-chloroform method (Roti Quick, Roth, Karlsruhe, Germany). Expression of SP-B and -C mRNA was measured in tissue of the left lung lobe using the real-time RT-PCR technique (TaqMan [TM]). Primers and probes for SP-B, SP-C, 18-S and β-Actin were designed using Primer Express software (Applied Biosystems, Foster City, USA) following a fixed set of recommendations to allow the application of standardized cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles for 15 s at 95°C and 1 min at 60°C). Control PCRs showed no signal for pure genomic DNA, proving mRNA-specificity. Primers were purchased from Roth, probes from Applied Biosystems, respectively. Primer and TaqMan probe sequences are depicted below. Relative quantification was performed using the ΔΔCt method, which results in a ratio of target gene expression and the expression of a housekeeping or reference gene. As the validity of this method depends on the constant expression of the housekeeping gene throughout the set of samples studied, we analyzed gene expression ratios of 18S (S) and β-actin (A) as well as the total mRNA content of each sample. Since β-actin showed both no differences between different experimental groups and the lowest variation in all samples as recommended for housekeeping genes, it was chosen as reference gene for further analysis.

Primer and TaqMan™ Probes

β-Actin
Forward primer (FP): 5′-TCA TCA CCA TCG GCA ACG-3′
Reverse primer (RP): 5′-TTC CTG ATG TCC ACG TCG C-3′
Probe: 5′(VIC)-CCT TCC TGG GCA TGG AGT CCT GC-(TAMRA)3′

18S
FP: 5′-GCC GCT AGA GGT GAA ATT CTT G-3′
RP: 5′-CAT TCT TGG CAA ATG CTT TCG-3′
Probe: 5′(VIC)-CCG GCG CAA GAC GGA CCA GA-(TAMRA)3′
SP-B
FP: 5′-TCC GCT GGT CGT TGA TCA C-3′
RP: 5′-GTT TGC ACA GGC CCA AGT G-3′
Probe: 5′(FAM)-CAG AGC CAA ATG AAC CTG AAG GCC ATC-(TAMRA)3′

SP-C
FP: 5′-CAC CTT CTC CAT TGG CTC TAG TG-3′
RP: 5′-ATA CTC TGC GGA GAC ATC TTC ATG-3′
Probe: 5′(FAM)-TGA CTA CCA GCG GCT CCT GAT TGC C-(TAMRA)3′

Quantification of Surfactant Proteins in Lung Homogenates

SP-B was quantified by ELISA methods as described previously with slight modifications [19]. For quantification of SP-B, lung homogenate was diluted 1:1,000 with PBS/1-propanol 1:1, pH 7.4 and transferred to the microtiter plate. Following selective removal of phospholipids by incubation with diisopropylether/butanol, and saturation of free binding sites with BSA buffer (1% BSA in PBS) the plates were probed with a monoclonal anti-SP-B antibody (8B5E, raised against porcine SP-B, 5 μg/ml in BSA buffer, 12 h incubation). Microtiter plate-bound antibody was detected employing a monoclonal, biotinylated anti-mouse antibody (Amersham Biosciences, Freiburg, Germany, 1:1,000 in BSA buffer; 2 h incubation) with subsequent application of AB-complex (diluted in PBS, 2 h incubation) and using ABTS (2,2′-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]) as substrate. Absorbance was measured at 450 nm and SP content was calculated with respect to the dry lung weight employing cubic-spline-interpolated standard curves obtained from isolated human SP-B.

Statistical Analyses and Data Presentation

Data are given as mean and standard deviation. Normal distribution can be assumed. Statistical analyses were performed using two-way ANOVA-test, determining the effects of surfactant administration and application of PEEP (SPSS for Windows version 11.5). For distinct comparison of groups one-way ANOVA with posthoc Scheffé was chosen. SP-B and -C mRNA expression was analyzed for each lung lobe separately. Data of real-time PCR analyses are given as arbitrary units. The level of significance is given in the result section.

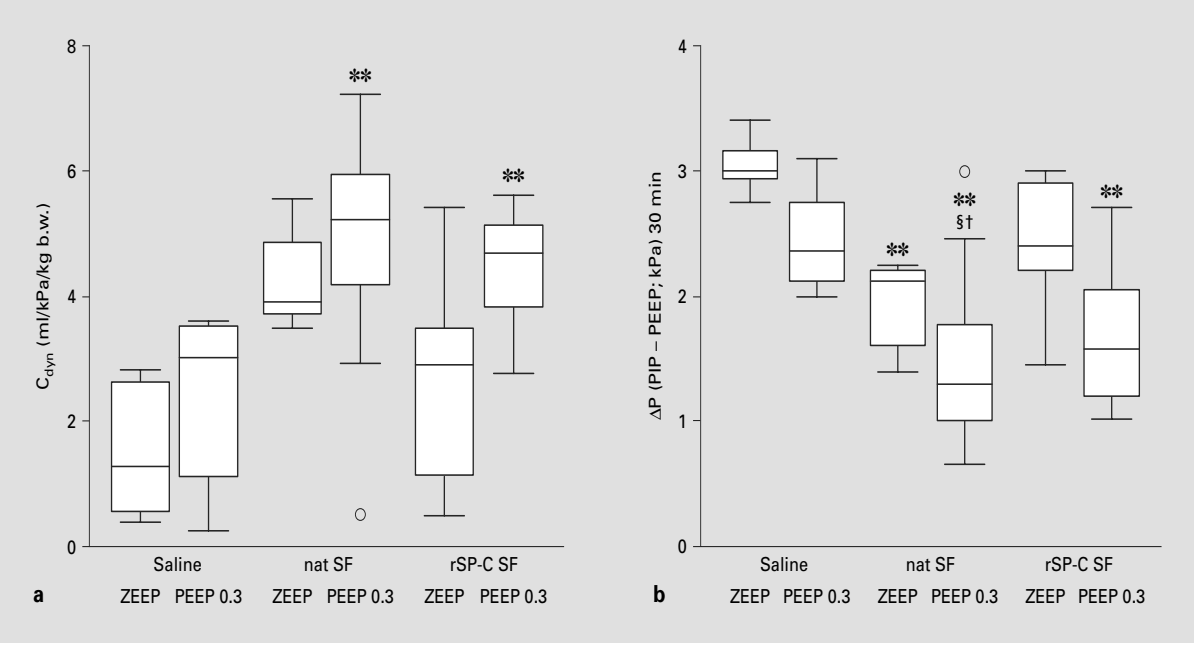

Fig. 1. Dynamic compliance (**a**) and ΔP (PIP – PEEP; **b**) in different treatment groups after ventilation for 30 min. Outlier are indicated (○). ** $p < 0.001$ vs. saline ZEEP (0 kPa); § $p < 0.001$ vs. saline PEEP 0.3; † $p < 0.001$ vs. rSP-C PEEP 0.3.

Table 1. Lung function variables after 30 minutes of ventilation

Group	n (rabbits)	Tidal volume ml/kg b.w.	Dynamic compliance ml/kPa/kg b.w.	Δ P (PIP–PEEP) kPa
Saline (ZEEP)	7	4.57 ± 3.00	1.55 ± 1.11	3.04 ± 0.21
Saline (PEEP 0.3)	6	7.53 ± 2.05	2.41 ± 1.40	2.46 ± 0.44
nat SF (ZEEP)	6	8.00 ± 0.35	4.24 ± 0.80	1.95 ± 0.36[b]
nat SF (PEEP 0.3)	11	7.29 ± 2.02	4.86 ± 1.96[b]	1.49 ± 0.75[b, c, d]
rSP-C SF (ZEEP)	7	5.49 ± 2.63	2.58 ± 1.75	2.44 ± 0.56
rSP-C SF (PEEP 0.3)	14	8.18 ± 1.16[a]	4.45 ± 0.93[b]	1.65 ± 0.53[b]

Mean ± standard deviation; PEEP (positive end-expiratory pressure; kPa); ZEEP (0 kPa PEEP).
[a] $p < 0.02$; [b] $p < 0.001$ vs. saline ZEEP.
[c] $p < 0.001$ vs. saline PEEP 0.3.
[d] $p < 0.001$ vs. rSP-C PEEP 0.3.

Results

Lung Function Variables

The effect of the PEEP level applied varied between both surfactant preparations: In animals ventilated at 0 kPa PEEP (ZEEP), natural surfactant showed a strong tendency towards increased tidal volumes and dynamic compliance; the PIP – PEEP difference (ΔP) accordingly was significantly decreased ($p < 0.001$ vs. controls; table 1; fig. 1). Contrary to this, administration of rSP-C surfactant resulted in less pronounced effects on tidal volumes, dynamic compliance and ΔP compared with the natural surfactant group and controls. Mechanical ventilation at 0.3 kPa PEEP combined with either natural or recombinant surfactant administration both improved dynamic compliance and decreased ΔP significantly

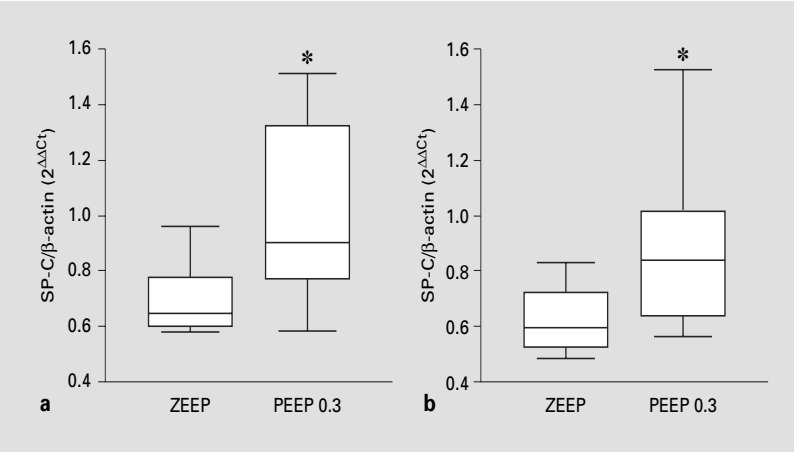

Fig. 2. SP-C mRNA expression in preterm rabbits ventilated with PEEP (0.3 kPa) compared to non-PEEP ventilated animals (ZEEP). SP-C mRNA expression in the upper (**a**) and lower lung lobe (**b**) is given in arbitrary units and normalized to the housekeeping gene β-Actin. * $p < 0.02$.

compared to saline treated controls ($p < 0.001$ vs. controls; table 1; fig. 1). Comparison of both surfactant preparations at PEEP 0.3 revealed no statistically significant differences with respect to improved tidal volumes and dynamic compliance (fig. 1; table 1).

Two-way ANOVA analyses revealed no statistically significant interactions between PEEP strategies and surfactant preparations with respect to dynamic compliance and ΔP ($p = 0.4$; C_{dyn} and $p = 0.7$; ΔP).

Histology

Alveolar count was significantly influenced by application of PEEP as the number of alveoli increased significantly when PEEP at 0.3 kPa was applied (mean 43.8 ± 7.4/field) compared to ZEEP ventilated animals (mean 36.6 ± 5.3/field; $p < 0.02$). This effect was comparable in both surfactant groups and controls, i.e., no statistical significant interaction (two-way ANOVA, $p = 0.2$). Administration of either surfactant preparation had no significant effect on alveolar count (i.e., number of alveoli and terminal airways).

Septal thickness did not differ significantly when comparing both levels of PEEP (0.3 kPa PEEP: mean 7.4 ± 2.6/field vs. ZEEP: mean 6.4 ± 1.1/field) or surfactant and control groups.

SP-B and -C mRNA Expression

Material of two animals (saline, PEEP 0.3; rSP-C SF, ZEEP) had to be excluded because of poor mRNA quality with degradation. Focusing on the effects of different PEEP levels, SP-C expression was found to be significantly up-regulated in upper and lower lung lobes when animals were ventilated with PEEP (0.3 kPa) compared to ZEEP ($p < 0.02$, fig. 2). This effect could further be shown regardless of either surfactant administration, i.e., no statistically significant interaction (two-way ANOVA, $p = 0.5$). SP-B and -C mRNA expression in upper and lower lung lobes was not significantly different between both surfactant groups when compared to each other as well as to ventilated and non-ventilated control animals (table 2). Normalization of mRNA expression to β-Actin and 18S showed comparable results.

Table 2. Pulmonary surfactant protein mRNA expression (upper and lower lung lobe)

Group	n (rabbits)	SP-B/A	SP-C/A
Control (non ventilated)	3	0.92 ± 0.40	0.76 ± 0.19
Saline (ZEEP)	3	0.95 ± 0.36	0.66 ± 0.19
Saline (PEEP 0.3)	2	1.54 ± 1.07	1.21 ± 0.36
nat SF (ZEEP)	3	1.06 ± 0.22	0.61 ± 0.08
nat SF (PEEP 0.3)	6	0.87 ± 0.45	0.97 ± 0.32
rSP-C SF (ZEEP)	2	1.07 ± 0.34	0.74 ± 0.06
rSP-C SF (PEEP 0.3)	7	1.22 ± 0.65	0.85 ± 0.24

Mean ± standard deviation, $2^{\Delta\Delta Ct}$; PEEP (positive end-expiratory pressure; kPa).

p values (two-way ANOVA) are given in the text.

SP-B Protein Content in Lung Homogenates

SP-B concentrations in lung homogenates as determined by ELISA technique were not significantly altered by ventilation strategies (1.25 ± 0.96 (saline; ZEEP); 1.22 ± 0.09 (saline; PEEP 0.3) or administration of different surfactant preparations (1.22 ± 0.09 (saline; PEEP 0.3); 2.72 ± 2.23 (nat SF; PEEP 0.3); 1.07 ± 0.60 (rSP-C SF; PEEP 0.3).

Discussion

Administration of exogenous surfactant and various levels of PEEP have been established as standard treatment regimes in neonatal RDS [17]. In the present animal model of primary surfactant deficiency, administration of the rSP-C-based surfactant could be shown to be effective in improving pulmonary function and was further found to be equivalent to the natural surfactant preparation if PEEP was applied. There was, however, a strong tendency towards a superiority in terms of lung mechanics in favor of the natural surfactant compared to the rSP-C based preparation if low PEEP levels, i.e., 0 kPa were used.

Previous studies have confirmed the efficacy of rSP-C based surfactant preparations in preterm animal models compared to a natural sheep surfactant, although this specific natural surfactant preparation has not accessed clinical routine [18]. We thus aimed at comparing the rSP-C surfactant with a natural surfactant preparation currently in clinical use, and were able to observe effects depending on the surfactant preparation and the level of PEEP applied. In clinical practice, surfactant administration in the preterm infant currently is combined with mechanical ventilation strategies applying different levels of PEEP and thus mean airway pressure, revealing relevant impact not only on pulmonary functions but also on other organ functions, as, among others, cerebral hemodynamics [15, 16]. For this purpose, surfactant preparations, which exert their effects on lung mechanics and oxygenation also at low PEEP levels seem to be qualified for clinical use in preterm neonates.

The present data on lung mechanics are in line with those, published for the effects of a third generation surfactant, containing a SP-B analog in a primate model of RDS [20]. Thus, with respect to their effects on lung mechanics and hemodynamics at different levels of PEEP further comparative studies of natural second and third generation surfactants are mandatory in order to optimize treatment strategies in preterm infants with RDS.

In order to delineate the effect of exogenous surfactant administration on surfactant homeostasis, SP-B and SP-C mRNA expression as well as SP-B protein concentration were measured in lung homogenates. In the present model of primary surfactant deficiency neither the rSP-C based nor the bovine surfactant preparation significantly affected SP-B or -C mRNA expression or SP-B protein concentrations during the observation period. Our data are in line with studies, comparing a natural bovine and protein-free synthetic lung surfactant preparation with respect to maturation of surfactant synthesis. Whereas incorportation of 3H-choline and 14C-glycerol and overall disaturated phosphatidylcholine synthesis was influenced differentially by natural surfactants compared to the protein-free preparation, pulmonary surfactant mRNA synthesis was not affected by neither surfactant preparation [21]. Nevertheless, the time course of the present study setting has to be taken into account when interpreting the results.

As PEEP is routinely used in ventilation strategies for preterm infants suffering from RDS, its effect on pulmonary histologic patterns and intrapulmonary distribution of exogenously administered surfactant containing a SP-B mimetic protein have been focused in previous studies. Maintaining PEEP during the administration procedure further improved gas exchange and surfactant distribution patterns [13]. In the present study, an increasing number of recruited primitive alveoli was observed when PEEP was applied indicating alveolar recruitment by the ventilation strategy. However, no additional effects of surfactant administration could be found regarding histologic variables, although lung mechanics further improved. Histologic long-term effects potentially resulting from reduced ΔP and improved compliance could not be addressed due to the time course of the present experimental setting. Furthermore, we could demonstrate alterations of SP-C mRNA expression following administration of PEEP at a level of 0.3 kPa regardless of the type of surfactant exogenously administered. Increased SP-C mRNA expression may reflect improved alveolar type II cell integrity, preserved by PEEP administration. In accordance with this, previous studies have found ventilation with PEEP to minimize loss of total alveolar surfactant and large surfactant aggregates [22]. Furthermore, stabilization of alveoli by PEEP has been found to reduce ventilator-induced lung injury, which is known to further affect surfactant function [23]. As the effect of PEEP on SP-C mRNA expression could be shown to be most pronounced in controls, this finding may further reflect protective effects of PEEP ventilation on pulmonary epithelial structures. In accordance with this increasing alveolar counts following the application of PEEP compared to ZEEP ventilated animals indicates recruitment of alveoli by unfolding. Formation of new alveoli seems unlikely in the study period.

Conclusions

In conclusion, we were able to demonstrate natural and recombinant surfactant preparations to exert differential effects on lung mechanics depending on the PEEP level applied in a neonatal model of RDS. As recently published clinical trials failed to establish clear-cut superiority of a third generation surfactant compared to standard natural surfactant preparations in terms of relevant neonatal outcome variables using conventional ventilation strategies [6, 7], the present data suggest further studies to be necessary in order to optimize surfactant replacement strategies in preterms with neonatal RDS. Thus, special attention should be paid to the level of PEEP, either by nasal CPAP or mechanical ventilation as this might be a critical modifier of the response to surfactant treatment as well as its complications in neonatal RDS in preterm neonates [24].

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chapter

16

Surfactant replacement or open lung concept? Comparison of two treatment strategies in an experimental model of neonatal ARDS

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Submitted

ABSTRACT

Objective
Several concepts of treatment in neonatal ARDS have been proposed in the last years. The present study compared the effects of an open lung concept positive pressure ventilation (PPVOLC) with a conventional ventilation strategy combined with administration of two different surfactant preparations on lung function and surfactant homoeostasis.

Design and subjects
After repeated whole-lung saline lavage, 16 newborn piglets were assigned to either PPVOLC (n = 5) or surfactant treatment under conventional PPV using a natural bovine (n = 5) or a monomeric protein B based surfactant (n = 6).

Measurements and results
Comprehensive monitoring showed each treatment strategy to improve gas exchange and lung function, although the effect on Pa_{O2} and pulmonary compliance declined over the study period in the surfactant groups. The overall improvement of the VEI was significantly greater in the PPVOLC group. Phospholipid and protein analyses of the bronchoalveolar lavage fluid showed significant alterations to surfactant homoeostasis in the PPVOLC group, whereas IL-10 and SP-C mRNA expression was tendentially increased in the surfactant groups.

Conclusions
The different treatment strategies applied could be shown to improve gas exchange and lung function in neonatal ARDS. To which extent differences in maintenance of lung function and surfactant homeostasis may lead to long-term consequences needs to be studied further.

INTRODUCTION

In neonatal acute respiratory distress syndrome (ARDS)-like lung disorders different mechanical ventilation strategies as well as exogenous surfactant administration have been suggested by various animal studies and introduced into clinical practice although large clinical trials have not been performed yet.[1,2] As shown in experimental studies, the open lung concept (OLC) as an alternative ventilation strategy, improves gas exchange and reduces ventilator-induced lung injury in models of secondary surfactant deficiency.[3,4] These effects were seen while applying the OLC during high-frequency oscillatory ventilation or positive pressure ventilation.[3] Furthermore, histology and biochemical analyses of bronchoalveolar lavage specimen showed reduced signs of lung injury and pulmonary inflammation in OLC ventilated animals.[4] However, respiratory failure in term neonates is often accompanied by secondary surfactant deficiency, contributing to impairment of lung function in these infants. Thus, exogenous surfactant administration in neonatal ARDS-like lung injury is a clinically well established treatment option.[1,5] Even in meconium aspiration syndrome, leading to severe inflammatory-induced lung failure, exogenous surfactant administration is part of therapeutic concepts.[6] Experimentally, the restoration of pulmonary function and gas exchange as well as the amelioration of pulmonary inflammatory processes has been shown.[7] Nevertheless, it has not been extensively investigated whether surfactant therapy in neonatal ARDS attains different effects on gas exchange, lung function, surfactant homoeostasis or pulmonary inflammatory processes compared to the OLC without surfactant replacement, although these effects may lead to differences in short and long term pulmonary outcome following neonatal ARDS. Furthermore, there is still no consensus on ventilation strategy in these infants in combination or without surfactant administration until now.[8]

Concerning the choice of the surfactant preparation applied, surfactant preparations with altered protein and phospholipid contents compared to natural surfactant products have gained increasing interest in treatment of ARDS-like lung disorders[9,10] due to their potential resistance towards surfactant inactivation.[11] Thus, two different surfactant preparations have been chosen for surfactant replacement therapy in the present study: A natural bovine surfactant, frequently used in clinical treatment regimes for neonatal ARDS[12,13] and a modified monomeric SP-B based bovine surfactant, that had recently been demonstrated to improve biological activity of the surfactant preparation compared to standard preparations in a neonatal lung lavage model.[14]

Aim of the present study was to compare a treatment strategy applying the OLC without surfactant replacement with a treatment regime using exogenous surfactant administration under conventional ventilation for their effects on gas exchange and lung function variables in a piglet model of neonatal ARDS. Furthermore, different variables of the surfactant system have been assessed using molecular and

biochemical analyses of surfactant proteins (SP) and phospholipid composition in order to obtain sensitive markers for lung injury processes. Regarding the impact of different treatment strategies on pulmonary inflammatory processes secondary to lavage-induced lung failure, histologic changes and pulmonary interleukin mRNA expression have been analyzed.

MATERIALS AND METHODS

Animal preparation

The experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by local authorities of the animal investigation committee.

Anesthesia was performed in 16 newborn piglets, aged 6 ± 5 days and at a weight of 3.0 ± 0.5 kg (mean ± SD each) with ketamine and midazolam after an intramuscular bolus of ketamine as published previously.[7] The piglets were tracheotomized, a cuffed endotracheal tube (3.0 mm outer diameter; Rüsch, Kernen-Rommelshausen, Germany) was introduced into the trachea and connected to the Evita XL (Dräger, Lübeck, Germany). Animals were ventilated using a pressure-controlled mode with a peak inspiratory pressure (PIP) of 9 - 12 cm H_2O, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, and a respiratory rate of 25 - 30 cycles/min, a rate of inspiration to expiration of 1:2 at 100% oxygen (FiO_2 1.0). Under these conditions, normocapnia was observed. A neuromuscular block was induced with pancuromium bromide (0.5 mg/kg i.v.), followed by a continuous infusion with fentanyl (20 µg/kg/h), midazolam (0.3 mg/kg/h) and pancuromium bromide (0.3 mg/kg/h) to provide sedation, analgesia and muscular relaxation. The right common carotid artery was cannulated (20 G, Arrow, Erding, Germany) for continuous blood gas and blood pressure monitoring (Paratrend/Trendcare, Philips Medical, Böblingen, Germany). A double-lumen central venous line (4.0 Fr, Arrow) was placed in the right femoral vein for infusion of fluids and medication. A continuous infusion of 5% dextrose was started (100 mL/kg/d) and all animals received one dose of cefotaxime (100 mg/kg). Body temperature was measured rectally and kept between 38 °C and 39 °C.

Lavage Procedure

Respiratory failure was induced by repeated saline lavage (50mL/kg; 37 °C) as described previously.[15] Lavage procedures were repeated at 3-min interval until partial arterial oxygen pressure (Pa_{O2}) was below 10.7 kPa at the following ventilator settings: PIP/PEEP 25/5 cm H_2O; rate 40 breaths/min; I:E ratio 1:2 and FiO_2 1.0.

Experimental Protocol

Within 10 min after the final lavage, animals were randomly allocated (T = 0 h) to one of the three study groups and ventilated for a period of 5 h. FiO_2 was kept at 1.0 during the whole experimental procedure. Animals that showed no recovery from lung lavage procedures in the different treatment groups, indicated by a Pa_{O2} < 60 kPa, were excluded from further analyses.

PPV_{OLC} group

In this group (n = 5), animals with an Pa_{O2} below 13.3 kPa one hour after the whole-lung lavage procedure, were applied to pulmonary recruitment, that was attained according to the protocol previously published by van Kaam *et al*.[4] The ventilatory rate was increased to 80 breaths per minute with an I:E ratio of 1:1. These settings remained unchanged during the experiment. PEEP was increased up to 15 cm H_2O and PIP was stepwise increased (5 cm H_2O each step) to open up the lung. Recruitment of previously collapsed alveoli during this procedure decreased intrapulmonary shunt and thus increase oxygenation.[3] Optimal alveolar recruitment was defined as Pa_{O2} levels ≥ 60 kPa.[3] The level of PIP needed to recruit the lung was accordingly defined the opening pressure (PIP_O).

After this recruitment procedure, PIP and PEEP were simultaneously decreased in equal steps every 2 to 3 min until Pa_{O2} dropped below 60 kPa, indicating increased intrapulmonary shunting. The level of PEEP at this stage of alveolar collapse was called the closing pressure ($PEEP_C$). PEEP was then raised to a level of 2 cm H_2O above $PEEP_C$ and PIP was momentarily raised to PIP_O (about 19 s) to fully recruit the lung. Thereafter, the pressure amplitude was set to keep the partial arterial carbon dioxide pressure (Pa_{CO2}) within the target range (4 - 6 kPa). PEEP only was decreased if there were signs of alveolar overdistension such as increasing Pa_{CO2}, decreasing Pa_{O2} or decreasing blood pressure.

Surfactant group

In this group, animals were ventilated in the pressure-controlled mode, applying a conventional ventilation strategy, *i.e.* PIP/PEEP 25/5 cm H_2O; rate 40 breaths/min; I:E ratio 1:2 and FiO_2 1.0.

Ten minutes after surfactant depletion with whole-lung lavage animals received either natural bovine surfactant (SF-RI1; Alveofact®; n = 5) or a modified surfactant with a monomeric protein B (mon SP-B; n = 6) at a dosage of 100 mg/kg each. Details on surfactant preparations are given below. Surfactant preparations were administered as an intratracheal bolus under continuous chest movements and maintenance of PEEP. Again the pressure amplitude was set to keep the Pa_{CO2} within the target range (4 - 6 kPa) and PEEP was only decreased if there were signs of alveolar overdistension such as increasing Pa_{CO2}, decreasing Pa_{O2} or decreasing blood pressure.

Mean arterial blood pressure, heart rate, ventilator settings, and lung function variables were recorded at the end of the instrumentation period, at the end of the lavage procedure and every 30 minutes thereafter. Although blood gas monitoring was available continuously to provide surveillance of ventilation, data were recorded at these same time points.

Therapeutic surfactant preparations

Alveofact® (SF-RI1) is a chloroform/methanol extract of bovine lungs containing phospholipids, neutral lipids and the hydrophobic surfactant apoproteins SP-B and SP-C as described previously.[16] The monomeric SP-B surfactant was prepared from SF-RI1 by selective reduction of dimeric SP-B and -C by addition of mercaptoethanol (ME; 50 mg per 2 mL vial) at room temperature for 12 hours and subsequent removement of ME by vacuum extraction. Except for SP-B content subsequent analyses of the obtained surfactant preparations showed no significant differences regarding their phospholipid and apo-protein profile. Both surfactant preparations were provided as lyophilized powder and resuspended in sterile saline 0.9% (Braun) to a final concentration of 60 mg/mL.

Lung function variables

Tidal volumes, resistance and dynamic compliance were measured by the Evita XL (Dräger, Lübeck, Germany) and related to body weight in order to compensate for different lung volumes. Ventilation efficiency index (VEI[17]) and PIP-PEEP difference (ΔP) were further calculated.

Bronchoalveolar lavage

At the end of the experiment (T = 5h) piglets were sacrificed by an overdose of phenobarbitone and bronchoalveolar lavage (BAL) was performed with physiological saline (3 x 50 mL/kg). The percentage of lung lavage fluid recovered was calculated and recovery of BAL fluid was comparable in all animals studied. Samples were centrifuged for 10 min at 300 x *g* at 5 °C to remove cells and membranous debris and the supernatant was processed for analyses of surfactant protein and phospholipid concentrations.

Surfactant protein and phospholipid analyses

Protein analysis

Total BAL proteins were calculated using a commercial assay (BCA assay, Pierce, Rockford, IL, USA). SP-B and SP-C were analyzed using ELISA techniques as described previously.[18,19]

Lipid analysis

Lipids were extracted from BALF with chloroform/methanol,[20] and the phospholipid content was determined by spectrophotometric measurement of phosphorus according to the method of Rouser *et al*.[21] Individual phospholipid classes were separated by high performance thin-layer chromatography and quantified using

scanning densitometry as previously described.[22] Total fatty acids were analyzed by gas-liquid chromatography (Chrompack CP 9001, Varian, Darmstadt, Germany) following acid-catalyled transmethylation into fatty acid methyl ester (FAME) as previously described.[23] For characterization of relative content of large surfactant aggregates (LSA), BALF was centrifuged at 48,000 x g (1 h, 4°C), the pellet was resuspended in 0.9% NaCl and assessed for the PL-content. Recovery of PL in the pellet was used to calculate relative LSA content.

Histologic processing

After the end of the experiment following the lung lavage procedure, the right lung was perfused with 300 mL of a formaldehyd (4.6%)-glutaraldehyd (0.5%)-solution for approximately 10 minutes. Finally, the trachea was clamped at a PEEP of 10 cmH_2O, right lungs were removed under maintenance of PEEP and submersed in the above mentioned solution for histomorphologic analyses. Tissue slides were obtained from dependent and non-dependent parts of the right lung. Four tissue slides were analysed from both the upper and lower lung lobe and two slides from the middle lung lobe, respectively. The slides were stained with hematoxylin-eosin (slides of 0.5 µm thickness). Lung histology was evaluated by a pathologist (M.E.), blinded to the animal´s group assignment, according to a previously described histologic score.[24] Variables scored for histologic evaluation were atelectasis, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis and overdistension. The variables were scored using a four-point scale with no injury corresponding to 0 points and 4 points indicating maximum injury.

Measurements of interleukin and surfactant protein (SP) -B and -C mRNA expression by realtime PCR

Messenger RNA (mRNA) expression of interleukin (IL) -1β, IL-6, IL-8, IL-10 and SP-B and SP-C was measured in tissue of the left lung lobe using the real-time RT-PCR technique (TaqMan™). Samples were obtained from representative parts of the upper and lower lung lobe.

Primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, USA) following a fixed set of recommendations as described previously.[7] Control PCRs showed no signal for genomic DNA, proving mRNA-specifity. Primers were purchased from Roth (Roth, Karlsruhe Germany), probes from Applied Biosystems, respectively. Lung tissue homogenization was performed in liquid nitrogen and total RNA was then extracted using the acid guanidinium thiocyanate-phenol-chloroform method (Roti Quick, Roth). Total RNA isolation, random primed reverse transcription and real-time PCR was performed following a standardized protocol as described previously.[7] Primer and TaqMan probe sequences are depicted in table 1. Relative quantification was performed using the ΔΔCt method, which results in a ratio of target gene expression and the expression of a housekeeping or reference gene.

Table 1 Primer and TaqManTM probes

Primer and Probe Description	**Primer sequences for PCR (forward (F) and reverse (R))** **Probe sequences for TaqMan™ analysis**
Hypoxanthin-guanine-phosphoribosyl-transferase	F: 5′-TGGAAAGAATGTCTTGATTGTTGAAG-3′ R: 5′-ATCTTTGGATTATGCTGCTTGACC-3′ Probe: 5′(VIC)-ACACTGGCAAAAVAATGCAAACCTTGCT-(TAMRA)3′
β-Actin	F: 5′-TCATCACCATCGGCAACG-3′ R: 5′-TTCCTGATGTCCACGTCGC-3′ Probe: 5′(VIC)-CCTTCCTGGGCATGGAGTCCTGC-(TAMRA)3′
Interleukin 1 beta (IL-1ß)	F: 5′-GGTTTCTGAAGCAGCCATGG-3′ R: 5′-GATTTGCAGCTGGATGCTCC-3′ Probe: 5′(FAM)-AAAGAGATGAAGTGCTGCACCCAAAACCTG-(TAMRA)3′
Interleukin 6 (IL-6)	F: 5′-GGGTAGGGAAGGCAGTAGCC-3′ R: 5′-GAATCCCTCTCCACAAGCG-3′ Probe: 5′(FAM)-CTTCAGTGGAGTCGCCTTCTCCCTAA-(TAMRA)3′
Interleukin 8 (IL-8)	F: 5′-TTCTGCAGCTCTCTGTGAGGC-3′ R: 5′-GGTGGAAAGGTGTGGAAGTC-3′ Probe: 5′(FAM)-TTCTGGCAAGAGTAAGTGCAGAACTTCGATG-(TAMRA)3′
Interleukin 10 (IL-10)	F: 5′-TTGGAGCTTGCTAAAGGCACT-3′ R: 5′-CGGCGCTGTCATCAATTTCT-3′ Probe: 5′(FAM)-CACCTCCTCCACGGCCTTGCTCTT-(TAMRA)3′
Surfactant protein B (SP-B)	F: 5'- TCC GCT GGT CGT TGA TCA C -3' R: 5'- GTT TGC ACA GGC CCA AGT G -3' Probe: 5'(FAM)- CAG AGC CAA ATG AAC CTG AAG GCC ATC -(TAMRA)3'
Surfactant protein C (SP-C)	F: 5'- CAC CTT CTC CAT TGG CTC TAG TG -3' R: 5'- ATA CTC TGC GGA GAC ATC TTC ATG -3' Probe: 5'(FAM)- TGA CTA CCA GCG GCT CCT GAT TGC C -(TAMRA)3'

For housekeeping genes β-Actin (A) and hypoxanthin-guanine-phosphoribosyl-transferase (HPRT) were chosen and validated by variation analyses in all samples.

As β-actin showed both no differences between different experimental groups and the lowest variation in all samples, it was chosen as reference gene for further analysis.

Statistical analyses and data presentation
Results are given as mean and standard deviation (SD). Results of real-time PCR analyses are given in arbitrary units and were normalized to housekeeping gene expression. Data analysis was performed using SPSS for Windows Version 6.1.3. As normal distribution has been shown, two-way ANOVA with repeated measurements was performed for analysis of lung function variables over the time course. In order to compare outcome levels between the groups differences from T = 5 – T = 0 were calculated and one-way ANOVA was performed with posthoc Scheffé. Results of the histologic evaluation as well as from determination of lavage specimen and real-time PCR were analyzed using one-way ANOVA with posthoc Scheffé. Calculated *P*-values are given in the result section.

RESULTS

All animals survived during the study period. There were no statistical significant intergroup differences in age, weight or number of lavages needed to induce the lung injury. No air leaks were observed within the study period. Blood gas values and lung function variables before and immediately after lavage were comparable in the three treatment groups (Table 2a). One animal of the natural surfactant group and 2 animals of the mon SP-B surfactant group did not recover after surfactant administration and required intensified ventilation (OLC) in order to reach Pa_{O2} levels $\geq$ 60 kPa. As this alteration in treatment strategy was not comparable with the other study groups, these animals were therefore excluded from further analysis. Thus, 13 animals were available for analyses.

No animal included for further analyses showed signs of hemodynamic compromise. Data on hemodynamics were not significantly different between the groups.

Gas exchange and lung function
After induction of lung injury, Pa_{O2}, dynamic compliance and VEI were significantly reduced in all animals (see figure 1), whereas the resistance increased significantly. Pa_{CO2} remained largely unchanged.

Mean opening pressure was 35 $\pm$ 4 cm H_2O, mean closing pressure 8 $\pm$ 3 cm H_2O in the PPV_{OLC} group.

Table 2a Gas exchange and lung function variables at different time points

	Pa_{o2}	Pa_{CO2}	C_{dyn}/kg	VT/kg	Res	VEI	ΔP
Healthy (H)							
PPV_{OLC}	74.21 ± 16.75	5.67 ± 2.05	1.94 ± 0.89	16.0 ± 4.8	37.62 ± 8.09	0.29 ± 0.17	10.20± 0.45
mon SP-B	64.11 ± 34.10	3.75 ± 0.75	2.56 ± 0.50	20.4 ± 2.7	40.50 ± 8.63	0.35 ± 0.08	10.00 ± 0.00
nat SF	78.44 ± 15.09	5.29 ± 1.95	2.12 ± 0.87	19.2 ± 7.0	37.88 ± 7.43	0.29 ± 0.14	10.25 ± 0.50
Lavaged (T = 0)							
PPV_{OLC}	6.70 ± 3.04 **	6.95 ± 2.84	0.56 ± 0.23 *	11.3 ± 2.6	52.43 ± 13.53 *	0.10 ± 0.06 *	20.00 ± 0.00
mon SP-B	9.24 ± 0.79 *	3.86 ± 1.64	1.21 ± 0.42 *	21.0 ± 5.0	63.28 ± 15.30 *	0.11 ± 0.02 **	20.00 ± 0.00
nat SF	8.16 ± 1.26 **	5.57 ± 1.35	0.88 ± 0.35 *	16.3 ± 5.4	50.25 ± 11.95 *	0.10 ± 0.04 *	20.00 ± 0.00
1 h							
PPV_{OLC}	9.68 ± 2.26	6.91 ± 2.07	0.57 ± 0.20	11.3 ± 4.5	47.50 ± 12.90	0.09 ± 0.04	20.00 ± 0.00
mon SP-B	38.60 ± 24.14	3.83 ± 2.84	1.52 ± 0.66	22.9 ± 8.4	58.55 ± 14.04	0.16 ± 0.09	19.50 ± 1.00
nat SF	56.86 ± 14.62	3.75 ± 1.13	1.50 ± 0.43	23.1 ± 6.2	53.98 ± 14.52	0.14 ± 0.05	20.00 ± 0.00
2 h							
PPV_{OLC}	71.45 ± 20.29	4.49 ± 1.97	1.03 ± 0.38	10.2 ± 2.2	34.34 ± 8.13	0.33 ± 0.20	12.60 ± 1.82
mon SP-B	64.87 ± 29.48	3.51 ± 0.91	1.53 ± 0.10	16.1 ± 7.0	47.80 ± 20.69	0.33 ± 0.27	13.75 ± 3.86
nat SF	75.18 ± 19.67	4.12 ± 0.41	1.39 ± 0.31	18.6 ± 4.0	45.20 ± 7.52	0.16 ± 0.02	17.75 ± 1.71
3 h							
PPV_{OLC}	74.08 ± 12.99	5.19 ± 2.35	1.13 ± 0.75	9.9 ± 3.0	34.76 ± 10.65	0.30 ± 0.19	12.40 ± 2.07
mon SP-B	59.05 ± 33.81	4.77 ± 1.11	1.23 ± 0.14	14.7 ± 4.1	50.63 ± 14.95	0.19 ± 0.12	14.00 ± 3.74
nat SF	72.32 ± 17.01	4.81 ± 0.55	1.15 ± 0.30	17.1 ± 5.0	43.85 ± 9.29	0.15 ± 0.04	17.00 ± 0.82
4 h							
PPV_{OLC}	70.09 ± 12.32	5.54 ± 1.67	1.11 ± 0.73	9.7 ± 2.4	35.46 ± 7.11	0.26 ± 0.15	12.60 ± 3.21
mon SP-B	60.05 ± 33.26	4.73 ± 1.28	1.21 ± 0.15	14.8 ± 3.4	53.70 ± 19.89	0.18 ± 0.11	14.50 ± 3.87
nat SF	56.53 ± 11.97	5.37 ± 0.53	1.05 ± 0.27	15.5 ± 2.8	44.18 ± 11.66	0.14 ± 0.04	16.50 ± 0.58
5 h							
PPV_{OLC}	71.61 ± 9.23	5.25 ± 1.01	0.97 ± 0.51	9.6 ± 3.2	35.78 ± 8.46	0.25 ± 0.14	12.80 ± 3.27
mon SP-B	60.38 ± 33.21	5.49 ± 1.17	1.04 ± 0.22	13.1 ± 3.7	51.93 ± 14.55	0.16 ± 0.08	14.0 ± 4.69
nat SF	48.94 ± 20.65	5.20 ± 0.52	1.01 ± 0.25	15.0 ± 2.2	50.55 ± 13.11	0.12 ± 0.04	17.00 ± 0.82

*mean ± SD; open lung concept positive pressure ventilation (PPV_{OLC}; n = 5); surfactant treatment under conventional positive pressure ventilation: natural bovine surfactant (Alveofact®; nat SF; n=4), monomeric surfactant protein B based surfactant (mon SP-B; n = 4). * P < 0.05; ** p < 0.01 vs initial values (healthy; H). Further levels of significance (two-way ANOVA) are given in detail in the text.*

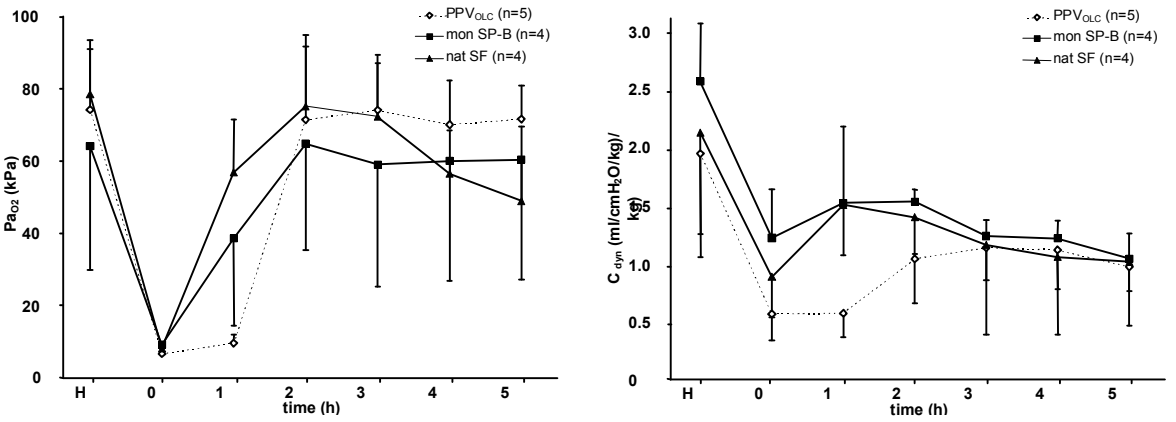

Figure 1 *Gas exchange (Pa_{o2}) and lung function (C_{dyn})*
Time course of Pa_{O2} (left panel) and dynamic compliance (C_{dyn}; right panel) over the observation period. Induction of lung injury by repetitive lavage procedures (T = 0) is followed by open lung concept positive pressure ventilation (PPVOLC; n = 5) or surfactant treatment under conventional positive pressure ventilation with a modified monomeric surfactant protein B surfactant (mon SP-B; n = 4) or a natural bovine surfactant (Alveofact®; nat SF; n = 4). Results are given as mean and standard deviation.

After experimental intervention (OLC, surfactant administration), Pa_{O2}, tidal volumes, difference of PIP-PEEP (∆P), resistance, VEI ($P < 0.001$ each) and dynamic compliance ($P = 0.01$) significantly improved over the time course in each treatment group (Table 2a).

Regarding differences between the treatment groups, two-way ANOVA testing revealed a significant difference with respect to Pa_{O2} ($P < 0.001$; figure 1) and Pa_{CO2} ($P < 0.05$) levels over the time course with declining effects in the nat SF group 3 hours after intervention. Comparing the relative change of the variables over the study period (T = 5 – T = 0), gas exchange variables showed a response to each experimental strategy and the overall change did not differ between the groups (Table 2b).

Comparable results were found for the development of the dynamic compliance and the ∆P over the study period with a significant difference between the study groups: Dynamic compliance could be shown to increase up to two-fold, with declining effects in the surfactant groups after 3 hours (figure 1), whereas ∆P could be decreased in all experimental groups during the observation period with most pronounced effects in the PPV_{OLC} and the mon SP-B group ($P = 0.001$). Nevertheless, changes in dynamic compliance or ∆P over the study period (T = 5 – T = 0) were not statistically significantly different between the groups. Mean airway pressure was found to be significantly higher in the PPV_{OLC} group compared to both surfactant treated groups ($P = 0.015$; data not shown).

The tidal volumes achieved under different ventilation strategies were significantly lower in the PPV_{OLC} group over the whole study period compared to both surfactant

groups (P = 0.002). Relative changes of tidal volumes (T = 5 – T = 0) showed a significant decline in the mon SP-B group when compared to the nat SF group (P < 0.05 *vs* nat SF).

Improved VEI were found in all treatment groups but differed in its time course between the groups (P = 0.015; figure 2). Most pronounced effects were seen in the PPV_{OLC} and the mon SP-B group, although effects decreased in the mon SP-B group towards the end of the observation period (figure 2). The relative change of the VEI over the observation period (T = 5 – T = 0) was found to be significantly greater in the PPV_{OLC} group compared to the mon SP-B and nat SF treated group (P < 0.05; figure 2).

Lung histology

Lung histology was examined in tissue slices of the right upper, middle and lower lung lobes. All study groups revealed significantly higher histologic scores in the upper and lower lung lobe compared to the middle lung lobe (P < 0.05, data not given in detail). Figure 3 shows the histological sum score of the different treatment groups. Although there was a tendency towards lower sum scores in the mon SP-B group (27 ± 20) compared to the PPV_{OLC} group (46 ± 17) and the nat SF group (41 ± 23) there was no significant difference.

Surfactant protein (SP) and phospholipid analyses

Total phospholipid and total protein content, phospholipid class profiles, total lipids fatty acid profiles, recovery of large surfactant aggregates and surfactant proteins (SP)-B and –C, were determined in cell depleted BAL specimen and are displayed in table 3. Total PL concentrations were elevated up to 3-fold in both surfactant groups with significant differences between the nat SF group compared to the PPV_{OLC} group (P = 0.012; figure 4). Total protein content was not different between the study groups, resulting in an increase of the phospholipid-protein ratio in both surfactant groups compared to the PPV_{OLC} group (nat SF P = 0.05; mon SP-B P = 0.09). Regarding phospholipid composition, the relative content of phosphatidylcholine and phosphatidylglycerol was found to be significantly increased in both surfactant groups (P < 0.05 *vs* PPV_{OLC}) and reached near normal levels. Accordingly, the relative content of phosphatidylserine, phosphatidylinositol, phosphatidyl-ethanolamine and sphingomyelin decreased in the mon SP-B and nat SF groups with significant effects for phosphatidylserine (P < 0.001 *vs* PPV_{OLC}). Analyses of the total fatty acids revealed a significantly increased relative content of palmitic acid in both surfactant groups (P < 0.01 *vs* PPV_{OLC}; figure 4) whereas levels of polyunsaturated fatty acids and all unsaturated fatty acids decreased significantly (P < 0.01 *vs* PPV_{OLC}). Levels of arachidonic acid decreased in both surfactant groups compared to the PPV_{OLC} group, eicospentaenoic acid increased in the nat SF treated group (P < 0.01 *vs* PPV_{OLC}).

Table 2b Overall differences in gas exchange and lung function variables (T5 - T0)

	Pa_{o2}	Pa_{CO2}	C_{dyn} / kg	VT/kg	Res	VEI	ΔP
T5 – T0							
PPV_{OLC}	64.90 ± 9.22	-1.71 ± 2.57	0.42 ± 0.40	-2.75 ± 0.96	-17.2 ± 5.43	0.19 ± 0.078 ***/§**	-7.2 ± 3.27
mon SP-B	51.14 ± 33.47	1.63 ± 1.27	-0.18 ± 0.42	-8.0 ± 4.24 *****	-11.35 ± 13.10	0.043 ± 0.07	-6.0 ± 4.69
nat SF	40.78 ± 20.49	-0.38 ± 1.71	0.13 ± 0.27	-1.25 ± 3.59	0.3 ± 5.91	0.02 ± 0.06	-3 ± 0.82

** P < 0.05 vs nat SF, § P < 0.05 vs mon SP-B*

Table 3 Phospholipid and surfactant protein analyses of lavage specimen

group	n (piglets)	PL [µg/ml]	PPQ	LSA [% PL]	SP-B/PL [% (w/w)]	SP-C/PL [% (w/w)]	PC [%]	PG [%]	PS [%]	PI [%]
PPV_{OLC}	5	48.65 ± 7.99	0.08 ± 0.01	47.7 ± 4.4	13.71 ± 3.08	3.06 ± 0.28	73.84 ± 5.57	3.59 ± 1.82	3.97 ± 2.44	6.87 ± 2.37
mon SP-B	4	131.10 ± 59.69	0.33 ± 0.16	62.7 ± 5.3	4.78 ± 2.01**	1.23 ± 0.45**	81.87 ± 1.38*	7.27 ± 2.25*	0.00 ± 0.00**	3.87 ± 1.94
nat SF	4	176.03 ± 68.91**	0.37 ± 0.21*	63.6 ± 6.2*	6.19 ± 1.73**	1.06 ± 0.62***	81.19 ± 1.43*	8.44 ± 1.10**	0.00 ± 0.00**	3.52 ± 0.79

group	n *(piglets)*	PE [%]	SPH [%]	PUFA [%]	Usat FA [%]	TL 16:0 [%]	Tlfa 20:4 (AA) [%]	Tlfa 20:5(EPA) [%]
PPV_{OLC}	5	7.94 ± 3.63	3.80 ± 1.42	19.94 ± 1.39	40.34 ± 1.41	44.78 ± 1.73	5.54 ± 0.55	0.16 ± 0.03
mon SP-B	4	4.99 ± 1.21	2.02 ± 0.85	12.16 ± 3.76**	33.19 ± 3.27**	52.97 ± 3.78**	3.03 ± 0.71**	0.15 ± 0.02
nat SF	4	0.47 ± 0.18	2.11 ± 0.81	11.52 ± 3.09**	35.76 ± 1.78*	50.24 ± 2.17**	2.58 ± 0.82***	0.29 ± 0.06**

PPQ (phospholipid-to-protein ratio); Phospholipid classes are given as percent (w/w) of all phospholipids. LSA = large surfactant aggregates, given as percent (w/w) of all BALF phospholipids. PC (phosphatidylcholine); PG (phosphatidylglycerol); PS (phosphatidylserine); PI (phosphatidylinositol); PE (phosphatidylethanolamine); SPH (sphingomyelin); Fatty acids were dermined in the BAL total lipid fraction and are given as percent (w/w) of all fatty acids. PUFA (polyunsaturated fatty acids in the total lipid fraction) ; TL 16:0 (palmitic acid in the total lipid fraction). AA = Arachidonic acid, EPA = Eicospentaenoic acid
** P < 0.05 vs PPV_{OLC} ; ** P < 0.01 vs PPV_{OLC}; *** P < 0.001 vs PPV_{OLC}*
§ P < 0.05 vs mon SP-B; §§ P < 0.01 vs mon SP-B

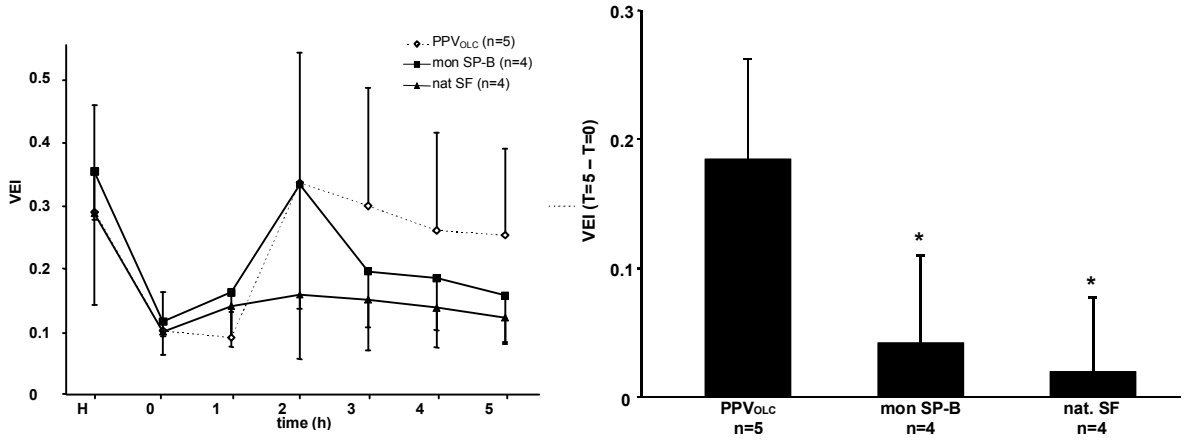

Figure 2 *Ventilation efficiency index (VEI)*
*Left panel: Time course of ventilation efficiency index (VEI) over the observation period. Induction of lung injury by repetitive lavage procedures (T = 0) is followed by open lung concept positive pressure ventilation (PPV_{OLC}; n = 5) or surfactant treatment under conventional positive pressure ventilation with a nat SF (n = 4) or a mon SP-B (n = 4). Right panel: Difference of VEI over the study period (T5 - T0). Results are given as mean and standard deviation. * P < 0.05 vs PPV_{OLC}.*

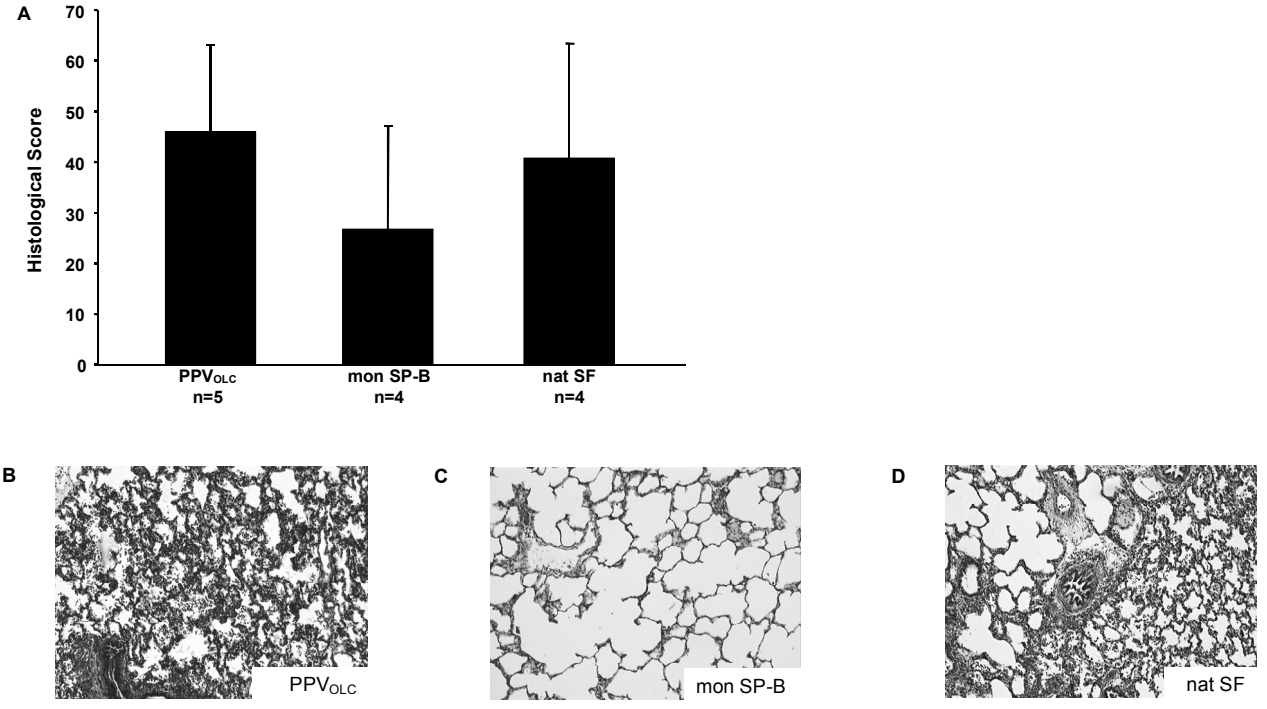

Figure 3 *Histological sum scores (upper, middle and lower right lung lobe)*
*Histologic sum scores (atelectasis, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis and overdistension) of upper, middle and lower right lung lobe using a four-point scale with no injury corresponding to 0 points and 4 points indicating maximum injury (**A**). Hematoxylin-eosin staining of exemplary histologic slides showing dystelectasis and neutrophiles in nearly all alveoli in the PPV_{OLC} group (**B**), regularly ventilated lung parenchyma in the monomeric SP-B group (**C**) and dystelectasis and neutrophiles in some of the alveoli in the natural SF group (**D**; hematoxylin-eosin, magnification 100x). Study groups: PPV_{OLC} (n = 5); surfactant treatment under conventional PPV with mon SP-B (n = 4) or a nat SF (n = 4). Results are given as mean and standard deviation.*

The relative content of large surfactant aggregates was increased in the surfactant-treated groups compared to PPV_{OLC} (nat SF $P = 0.06$; mon SP-B $P = 0.048$). Both, total SP-B and SP-C concentrations were not statistically different between the experimental groups. Nevertheless, levels were significantly lower in the surfactant groups compared to the PPV_{OLC} group ($P < 0.001$) when normalized to the total PL content of each sample.

Interleukin and surfactant protein B and C mRNA expression analysis.
Interleukin and SF mRNA expression was determined in the upper and lower left lung lobes. As there were no significant differences between the results obtained from the upper and lower left lung lobes, means were calculated and used for further analyses. Results normalized to β-Actin are represented in table 4 and in figure 5. Normalization to the housekeeping gene HPRT confirmed the results.

Analyses of the pulmonary interleukin mRNA expression in the left lung revealed no significant differences between the study groups. Nevertheless, there was a tendency towards increased mRNA expression levels of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 in the nat SF group compared to mon SP-B and the PPV_{OLC} group ($P = 0.1$; figure 5; table 4). Furthermore, mRNA expression of the anti-inflammatory cytokine IL-10 showed a tendency towards increased levels in both surfactant groups compared to the PPV_{OLC} group ($P = 0.1$; figure 5). Furthermore, SP-B and -C mRNA expression were found to be not significantly different. However, SP-C mRNA expression was shown to be tendentially increased in the mon SP-B and nat SF groups compared to the PPV_{OLC} group (table 4).

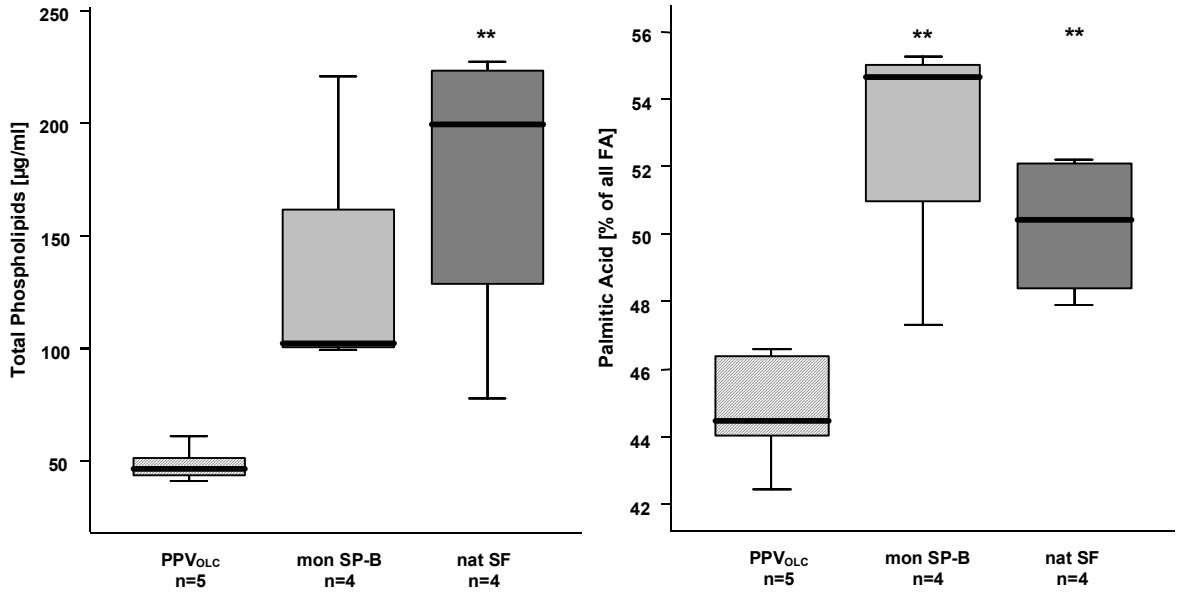

Figure 4 *Total Phospholipids and Palmitic Acid in BAL fluid*
*Total Phospholipids and palmitic acid (in percent (weight/weight; w/w) of all BAL fatty acids) in cell depleted BALF in the three different study groups. Study groups: PPV_{OLC} (n = 5); surfactant treatment under conventional PPV with mon SP-B (n = 4) or a nat SF (n = 4). Median, 95% confidence interval and range are indicated in the figure. ** P < 0.01 vs PPV_{OLC}*

Table 4 Pulmonary cytokine and surfactant protein mRNA expression (left lung lobe)

group	n *(piglets)*	IL-1β/A	IL-6/A	IL-8/A	IL-10/A	SP-B/A	SP-C/A
PPV_{OLC}	5	0.73 ± 0.51	1.27 ± 1.21	0.10 ± 1.3	0.34 ± 0.16	2.84 ± 2.17	0.82 ± 0.65
mon SP-B	4	0.35 ± 0.38	0.59 ± 0.78	0.03 ± 0.04	0.62 ± 0.32	4.05 ± 2.31	1.67 ± 0.87
nat SF	4	2.10 ± 1.77	3.96 ± 3.82	0.23 ± 0.22	0.67 ± 0.2	2.95 ± 1.27	1.09 ± 0.35

Normalization to β-Actin (A). Values are given as fold change ($2^{-\Delta\Delta Ct}$). Means were calculated from values of upper and lower lung lobe.

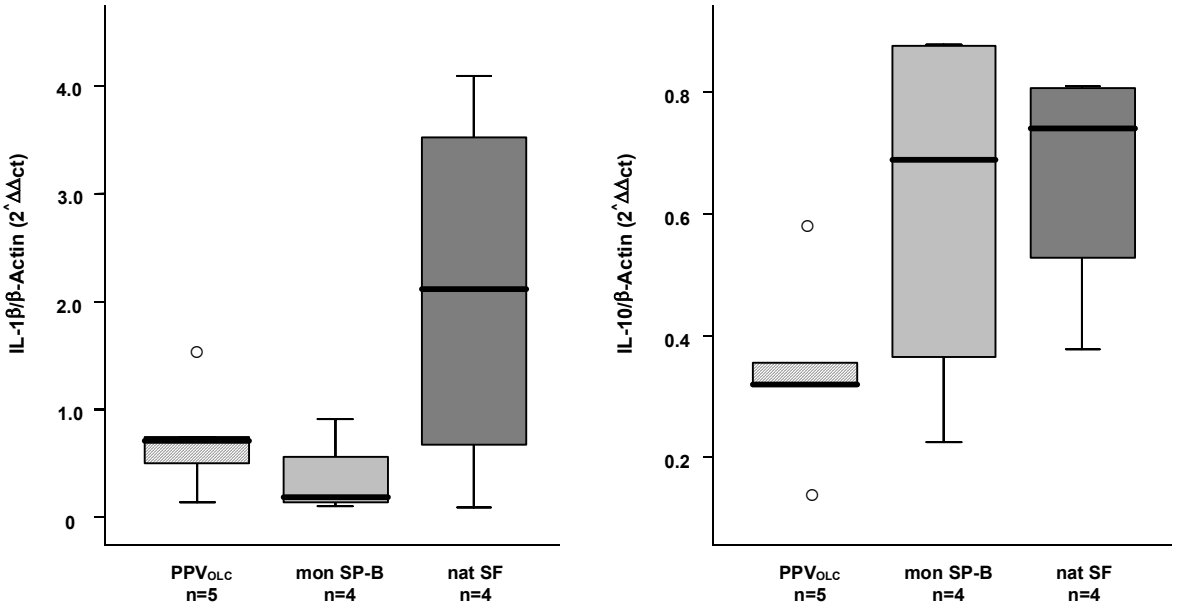

Figure 5 *Pulmonary interleukin mRNA expression (upper and lower left lung lobe) Pulmonary interleukin (IL) -1β (left panel) and IL-10 (right panel) mRNA expression normalized to β-Actin in piglets with experimentally induced neonatal ARDS –like lung failure. Study groups: PPV_{OLC} (n = 5); surfactant treatment under conventional PPV with mon SP-B (n = 4) or a nat SF (n = 4). Median, 95% confidence interval, range, and extreme values (○) are indicated in the figure. Means were calculated from values of upper and lower lung lobe.*

DISCUSSION

Respiratory failure often accompanies critical illness and determines morbidity in term neonates. Thus, evaluation of therapeutic strategies in neonatal ARDS-like lung disorders remains an important issue as demonstrated by a multitude of experimental and clinical studies.[13,25] Several concepts of treatment have been established in the last years. Under these, surfactant treatment is an established therapeutic option, as secondary surfactant deficiency may be causally related to the clinical picture and a potential cause for the impairment in lung function. Nevertheless, surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects or the need for repetitive doses. Thus, conventional ventilation is often used as first-line management of the disease. If

conventional ventilation strategies fail, further treatment regimes are searched for in order to minimize ventilator-induced lung injury. Nevertheless, there is still no consensus on ventilation strategy in these infants in combination or without surfactant administration until now.[8] Furthermore, different treatment regimes as exogenous surfactant administration under conventional ventilation or application of open lung ventilation strategies without surfactant replacement may result in differing effects on gas exchange, lung function, surfactant homoeostasis or pulmonary inflammatory processes and thus short and long term pulmonary outcome following neonatal ARDS.

In the present study, both treatment strategies investigated, administration of exogenous surfactant under conventional ventilation as well as application of the OLC, were found to be efficient in improving gas exchange and lung function in neonatal ARDS. The effects on lung function and gas exchange have been confirmed for each treatment strategy in previous experimental studies.[3,12,26]

To experimentally assess the effects of exogenous surfactant administration in neonatal ARDS compared to the OLC, a standard treatment regime using natural bovine surfactant has been chosen. However, surfactant preparations with varying protein and phospholipid contents have gained increasing interest in treatment of ARDS-like lung failure.[9,10] As native SP-B in humans is secreted in the alveoli predominantly in its dimeric form, modification of the dimeric structure leads to differences in the function of SP-B and is currently under further investigation.[27,28] Recently, modification of a natural surfactant preparation (SF-RI1) into a monomeric SP-B content has been demonstrated to improve biological activity compared to standard preparations *in vivo*.[14] Thus, the monomeric SP-B surfactant has been chosen as an alternative surfactant treatment in the present model.

Regarding the effects of the different treatment regimes applied on lung function and gas exchange in detail, the decrease of Pa_{O2} levels in the natural surfactant group at the end of the study period might reflect the need for repetitive doses of surfactant to achieve sustained treatment effects and may be a consequence of surfactant inactivation in the alveolar compartment.[29] Furthermore, levels of dynamic compliance did not reach initial values during the observation period and treatment effects declined to the end of the study period in the surfactant treated groups. In line with our findings, van Kaam and colleagues have shown a dose-dependency of the surfactant effect whereas a time-dependency with declining effects over time could be shown in the present study.[30] Besides the the need for repetitive or increased doses of exogenous surfactant the indicated effects may be due to ventilator-induced lung injury under the conventional ventilation regime in the surfactant treated animals. Improvement of VEI over the study period was found to be most pronounced in the PPV_{OLC} group, which may reflect the potential of this ventilation strategy to more equal recruitment of different parts of the lung. In contrast, surfactant is known to preferably reach lower lung lobes and its

administration therefore often leads to inhomogenous recruitment and holds the risk of partial overdistension. However, ΔP values could be reduced in all study groups possibly leading to a reduction of shear stress and therefore traumatic lesions in the lung in the experimental ventilatory setting.

In order to define the impact of the treatment strategies applied on lavage-induced lung injury, surfactant homoeostasis and inflammatory processes, histologic patterns, cytokine and surfactant mRNA expression as well as phosholipid profiles of BALF were investigated.

Regarding histologic patterns, the evaluated score comprising different variables indicating the degree of lung injury showed no significant differences between the groups. Nevertheless, analyses showed alterations to pulmonary tissue, reflecting the delay of structural recovery compared to reconstitution of lung function and gas exchange after indcution of neontatal ARDS-like lung injury. Interestingly, the monomeric SP-B preparation revealed distinct improvement in lung structure, indicating the altered surfactant preparation to be effective in influencing surfactant homeostasis and thus the recovery in physiologic lung function.

Alterations to the surfactant system have been shown to closely reflect lung injury processes, especially alveolar type-II cell integrity and metabolism.[19,22,31] Furthermore, reconstitution of the surfactant system has been discussed as an important measure assessing different treatment regimes in ARDS-like lung disorders in children and infants. As expected, the phospholipid profile in BAL specimen showed a normalization of the phospholipid and fatty acid profile in both surfactant groups with an increase in concentrations of phosphatidylcholine and phosphatidylglycerol as well as the palmitic acid concentration in the total lipid fraction. In contrast, the phospholipid analyses of the PPV_{OLC} group showed the typical profile for ARDS-like lung failure with a reduced recovery of phosphatidylcholine and phosphatidylglycerol, an increased relative content of polyunsaturated fatty acids, *i.e.* arachidonic acid and a decreased relative content of palmitic acid.[22,31] Furthermore, the relative content of large surfactant aggregates and the phospholipid-to-protein ratio in the BALF were significantly reduced in the PPV_{OLC} group. These findings reflect a significantly reduced recovery of the surfactant system in the PPV_{OLC} ventilated animals compared to the surfactant treated groups, potentially leading to short and long term consequences regarding lung injury processes and pulmonary function. Regarding the surfactant protein concentrations in the BALF, levels were comparabel in all study groups. In the case of SP-C, this may suggest a stabilization of type-II cell integrity and surfactant homeostasis under the indicated treatment regimes as previous studies showed significantly reduced SP-C levels in BAL specimen from conventionally ventilated ARDS patients.[19] An early recovery of alveolar type-II cell integrity and metabolism after lavage-induced lung injury may be further indicated by tendentially increased SPC mRNA expression in the in the surfactant treated groups.

Nevertheless, the combination of both treatment strategies will gain increasing interest as shown by latest studies.[2,32] Here, open lung ventilations strategies after exogenous surfactant administrations allowed reduction of both the opening and the closing pressures after some hours, which may be explained by a higher alveolar stabilization after recruitment maneuvers in combination with the effect of surfactant administration. Futhermore, the present study showed the relative SP-B and SP-C concentration after normalization to the total PL content of each sample, to be lower in the surfactant-treated groups. This may be assigned to a higher rate of ventilator-induced lung injury in the conventionally ventilated, surfactant treated animals, where higher ΔP values and tidal volumes compared to the PPV_{OLC} group may led to an increased rate of shear stress to the lung. Furthermore, limits of surfactant treatment in combination with a conventional ventilation strategy are demonstrated by cases with an absent response to exogenous surfactant administration as one animal in the natural surfactant and two animals in the monomeric SP-B group did not recover from lung failure and required an intensified ventilation regime.

In terms of pulmonary inflammatory processes, previous studies have been shown for the OLC ventilation to reduce signs of inflammation in BALF when compared to conventional ventilation strategies.[3] In the present study analyses of pulmonary pro-inflammatory cytokine mRNA expression showed no significant differences between the OLC and the surfactant treated groups. However, mRNA expression of the anti-inflammatory cytokine IL-10 showed a tendency towards increased levels in the surfactant groups compared to the PPV_{OLC} group, possibly indicating anti-inflammatory or lung protective effects of exogenous surfactant. The tendency towards increased IL-1β, IL-6 and IL-8 mRNA expression in the natural surfactant group has also previously been shown *in vivo* for natural surfactant treated animals in a model of experimental meconium aspiration syndrome[7] and for endothelial cell activation *in vitro*[33] and may be explained by a higher concentration of arachidonic acid in the natural surfactant preparation.

In conclusion, we could show in a descriptive manner both treatment strategies, the administration of exogenous surfactant under conventional ventilation as well as the application of an intensified mechanical ventilation concept following the OLC to be efficient in improving lung function and gas exchange in neonatal ARDS. Nevertheless, administration of surfactant led to a more pronounced effect on gas exchange and compliance in the first hours, although improvement in oxygenation declined after 3 hours in the natural surfactant group. In contrast, improvement in VEI was found to be more evident in the PPV_{OLC} group. Thus, differences between the surfactant and the PPV_{OLC} groups regarding maintenance of the effects on lung function as well as surfactant homoeostasis and the pulmonary inflammatory balance may lead to pulmonary long-term consequences which should be addressed in further studies. Limitations of the study that need to be addressed are the potential induction of lung injury processes by the initial conventional ventilation as

well as the application of relatively high tidal volumes in the PPV_{OLC} group compared to previous OLC ventilation regimes. Changing ventilation strategies and modifying existing concepts reflect everyday clinical practice, but further studies are needed to address the impact of these variables on pulmonary outcome. As well clinical studies are needed in order to verify findings from animal studies. As the results indicate differing surfactant preparations to reveal distinct effects on lung function variables, further studies are needed to define an optimized surfactant composition.

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SUPPLEMENTAL METHODS

Animal preparation

The piglets were tracheotomized, a cuffed endotracheal tube (3.0 mm outer diameter; Rüsch, Kernen-Rommelshausen, Germany) was introduced into the trachea and connected to the Evita XL (Dräger, Lübeck, Germany). Animals were ventilated using a pressure-controlled mode with a peak inspiratory pressure (PIP) of 9 - 12 cm H_2O, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, and a respiratory rate of 25 - 30 cycles/min, a rate of inspiration to expiration of 1:2 at 100 % oxygen (FiO_2 1.0). Under these conditions, normocapnia was observed. A neuromuscular block was induced with pancuromium bromide (0.5 mg/kg i.v.), followed by a continuous infusion with fentanyl (20 µg/kg/h), midazolam (0.3 mg/kg/h) and pancuromium bromide (0.3 mg/kg/h) to provide sedation, analgesia and muscular relaxation. The right common carotid artery was cannulated (20 G, Arrow, Erding, Germany) for continuous blood gas and blood pressure monitoring (Paratrend/Trendcare, Philips Medical, Böblingen, Germany). A double-lumen central venous line (4.0 Fr, Arrow) was placed in the right femoral vein for infusion of fluids and medication. A continuous infusion of 5% dextrose was started (100 mL/kg/d) and all animals received one dose of cefotaxime (100 mg/kg). Body temperature was measured rectally and kept between 38 °C and 39 °C.

Experimental Protocol

PPV_{OLC} group

The ventilatory rate was increased to 80 breaths per minute with an I:E ratio of 1:1. These settings remained unchanged during the experiment. PEEP was increased up to 15 cm H_2O and PIP was stepwise increased (5 cm H_2O each step) to open up the lung. Recruitment of previously collapsed alveoli during this procedure decreased intrapulmonary shunt and thus increased oxygenation and optimal alveolar recruitment was defined as PaO_2 levels $\geq$ 60 kPa.[3] The level of PIP needed to recruit the lung was accordingly defined the opening pressure (PIP_O).

After this recruitment procedure, PIP and PEEP were simultaneously decreased in equal steps every 2 to 3 min until Pa_{O2} dropped below 60 kPa, indicating increased intrapulmonary shunting. The level of PEEP at this stage of alveolar collapse was called the closing pressure ($PEEP_C$). PEEP was then raised to a level of 2 cm H_2O above $PEEP_C$ and PIP was momentarily raised to PIP_O (about 19 s) to fully recruit the lung. Thereafter, the pressure amplitude was set to keep the partial arterial carbon dioxide pressure (Pa_{CO2}) within the target range (4 - 6 kPa). PEEP only was decreased if there were signs of alveolar overdistension such as increasing Pa_{CO2}, decreasing Pa_{O2} or decreasing blood pressure.

PART VI

GENERAL DISCUSSION

chapter 17

Disturbance of late lung development

Many lung diseases in children - and even sometimes in adults too - are characterized by failure of development and/or destruction of the alveoli. Maturation of the human lung continues well after the newborn period through a spectrum of lung developmental stages ranging from neonatal to infant and adult phases of lung development.[1,2] Infant and adult lungs is a differ importantly in alveolar structure, matrix composition and angiogenesis, especially when new alveoli are formed and septum differentiation takes place.[3-5] Injury to the developing lung often leads to impaired alveolarization and vascularization, and thus in poor lung function.[6-11] Ventilator-induced lung injury (VILI) may be associated with alveolar structural damage, pulmonary edema, inflammation and fibrosis.[12] Oxygen toxicity and barotraumas - but also volutrauma - induce an inflammatory response in the developing lung, which persists in infants who develop bronchopulmonary dysplasia (BPD).[13] Disturbance of late lung development resulting from ventilation and oxygen toxicity is not only found in premature lungs. Infants born with compromised respiratory status, either due to immaturity or conditions such as congenital lung hypoplasia, congenital diaphragmatic hernia (CDH) or space-occupying processes including lobar emphysema, often require mechanical ventilation and supplemental oxygen. Individually and in combination, these measures will predispose the newborn to VILI. In CDH, BPD occurs in around 33% of survivors.[14]

Septum differentiation and Extracellular matrix

Two developmental phases are to be distinguished in the formation of the respiratory system: (1) the initial creation of an air-conducting system and (2) the later development of the respiratory alveoli.[15] Septum differentiation (secondary crest outgrowth) is the achievement of the final mature morphology of the lung. During this process, the airways grow into the surrounding mesenchyme while alveolar septa undergo branching into 2nd and 3rd generations and invaginate inwards into the airspace.[15] Alveolar septation is a necessary step to increase the blood-gas interface. Among the variety of factors that participate in the control of budding of secondary septa, elastin deposition in the thickness of primary septa appears to have a spatially instructive role.[16,17] The specific sites of elastic fiber formation correspond precisely to the location of future buds. During alveolarization, alveolar type (ATII) cells proliferate and differentiate into type I cells. Alveolar septa divide the terminal respiratory saccules, increasing the number of alveoli.[15] The septa are supported by the extracellular matrix (ECM), composed of a collagen scaffold, in which glycoproteins and elastin are involved.[17]

The onset of septa formation is heralded by the initial deposition of elastin at specific sites in the walls of the developing saccules. In the normal lung, deposition and arrangement of elastin fibers is particularly important in the formation and maintenance of alveolar structures. Various abnormalities of lung elastin have been documented in infants with Bronchopulmonary Dysplasia (BPD).[18] Bruce and coworkers showed an increase of urinary desmosine excretion, which is a biomarker

of elastin degradation in mechanically ventilated infants developing BPD during the first week.[19] Breakdown of lung elastin in BPD has been attributed to inflammatory processes associated with infection and/or hyperoxia.[20,21] Non-survivors of severe BPD showed increased accumulation of distributed elastic fibers in the distal lung parenchyma. This accumulation was associated with reduced septation and fewer alveoli which are typical pathological feature of the "new-type" of BPD.[22]

In animal studies, Wendel et al. described the importance of elastin in the formation of the alveoli.[23] Deletion of the elastin gene in mice led to neonatal death from respiratory failure associated with reduced terminal airway branching and defective vasculogenesis in the lung. These mice showed loss of septa formation and emphysema.

The mechanism by which abnormal elastin synthesis contributes to failed alveolar and lung capillary formation in BPD is unclear. Several studies have shown that cyclic stretch, such as that induced by mechanical ventilation of the developing lung, may increase elastin deposition associated with increased tropelastin gene expression.[24,25]

Alveolarization is mediated by paracrine, autocrine, and juxtacrine communication between the epithelium and endothelium, and their associated fibroblasts, modulated by signaling molecules including members of the TGF-β and BMP pathways. Schwartz et al have shown that increased mechanical forces activate epithelial cells to produce signals and inflammatory proteins that stimulate the interstitial fibroblasts to produce collagen, metalloproteins, and other inflammatory products.[26] This process causes remodelling and continued tissue repair as long as higher airway pressure persists. One might speculate that by removing the pressure would halt the remodelling and cause regression of the excessive interstitial collagen.[18] Oki et al showed elevated levels of collagen IV, which normally lines the subepithelial basement membrane of all of the distal airways and saccules, in the bronchoalveolar lavage fluid of infants who had respiratory distress syndrome in the first two weeks of life and developed BPD.[27] This finding underlines the role of collagen fibers in the development of BPD.

As mentioned before, elastin deposition and alveolar formation can also be disrupted by increased elastase activity as seen in lung inflammation during acute and chronic neonatal respiratory insufficiency.[28] Members of the TGF signaling pathway are known to play a role in inflammatory processes. Bland et al. observed high expression of TGF-α and TGF-β in the lung of preterm lambs after one day of mechanical ventilation.[28] This would suggest that these mitogens are involved in modifying elastin assembly during mechanical ventilation of the developing lung, as they both induce tropoelastin secretion in lung myofibroblasts in the septal crest. Thus, increased TGF-α causes severe remodeling in the premature lung.[29,30] Elevated expression of TGF-α during the saccular phase disrupted lung morphogenesis, caused mesenchymal and vascular remodeling, and led to neonatal

mortality within the first week of life in mice. Interestingly, induction of TGF-α during the saccular stage of lung development caused a different phenotype and gene expression profile than did an increase in TGF-α during the alveolar phase and in the adult lung. TGF-β will raise, and fibroblast growth factor-β and interleukin-1β, by transcriptional and post-transcriptional processes, will lower elastin mRNA levels.[31]

The sustained integrity of alveolar structures requires the maintenance of alveolar cells and ECM, but the repair capacity of these components in response to injury is unclear.[32,33] Coordinated deposition of new matrix elements such as collagen and elastin has been proven to be involved in the repair mechanism after alveolar destruction. Excess deposition of collagen in the lung leads to fibrosis and dysfunction as seen in infants within the "old-type" of BPD with fibroproliferation.[34]

During septation, myofibroblasts appear at the edge of the developing septa and these cells are essential for normal alveoli formation.[35,36] They show alpha-smooth muscle actin (alpha-SMA) expression and synthesize abundant elastin and collagen.[37] Depletion of myofibroblasts from the alveolar wall, as seen in PDGF-A null mice, results in emphysema secondary to failure of septation. This would seem to indicate that myofibroblasts are crucial for septation.[35] Bland and colleagues found reduced expression of PDGF-A and its receptor in the lungs of preterm lambs developing BPD, and suggested a possible role for this growth factor in the abnormal distribution of elastin.[28]

Chung Ming and colleagues found that increased levels of lung connective tissue growth factor (CTGF) mRNA and protein expression were associated with hyperoxia-induced lung fibrosis in neonatal rats, and that upregulation of CTGF expression preceded the increase in collagen levels. This suggests that CTGF is involved in the pathogenesis of hyperoxia-induced lung fibrosis.[38]

Injury to the developing lung will disrupt critical signaling pathways that regulate alveolarization, and these pathways are indeed defective in premature infants developing BPD.[10] Elevated levels of TGF-β ligand have been detected in animal models of BPD, but also in infants with BPD undergoing mechanical ventilation.[39] Furthermore, exposure of alveolar ATII cells to hyperoxia promotes association of Smad2/3/4, TGF-β regulated transcription factors, with DNA, indicating that hyperoxia upregulates the TGF-β signaling pathways. Under hyperoxia conditions we demonstrated temporal changes in the expression and localization of key signaling components of the TGF-β/BMP system.[39]

Proliferation and differentiation of ATII cells are key steps in alveolarization. It is known that TGF-β can arrest proliferation of ATII cells and prevent keratinocyte growth factor-stimulated ATII cell proliferation. Hyperoxia-induced upregulation of TGF-β signaling in the alveolar epithelium is conductive to apoptosis of ATII cells, thereby contributing to alveolar hypoplasia in BPD.[40]

Deregulation of TGF-β signaling also impacts ECM deposition and remodeling, other key processes in alveolarization. TGF-β controls the secretion of some matrix proteases as well as their inhibitors. Three families of proteases and their corresponding antiproteases play a crucial role in lung destruction and the development of BPD.[41,42] These are the serine proteases (e.g. neutrophil proteases and trypsin), matrix metalloproteinases (MMPs) and their inhibitors tissue inhibitors of metalloproteinases (TIMPs), and the papain family (cathepsin B, H, K, L, and S). Altiok et al. found increase levels of all cathepsins in a baboon model of BPD, whereas levels of cystatin B and C mRNA were unchanged.[42] Cathepsin functionality in bronchoalveolar fluid and lung tissue homogenate had correspondingly increased. Levels of cathepsin B, H, S, and K decreased with advancing gestational age.

Recent work implicating a protease-antiprotease imbalance in BPD studied the role of serine protease inhibitor B1 (SERPINB1), which is a monocyte neutrophil elastase inhibitor, expressed in neutrophils and macrophages.[41] Transcriptional factors that are prominent in the inflammatory response, such as nuclear factor-kappaB and PU.1/Spi-1, have been identified as regulatory elements for the SERPINB1 gene.[43,44] SERPINB1 protected surfactant protein A and D from degradation by neutrophil elastase. The findings from Yasumatu et al. suggest that SERPINB1 upregulation in "new-type" BPD is a part of protection mechanism and that SERPINB1 contributes to the regulation of neutrophil elastase activity along with other well-known elastase inhibitors.[41]

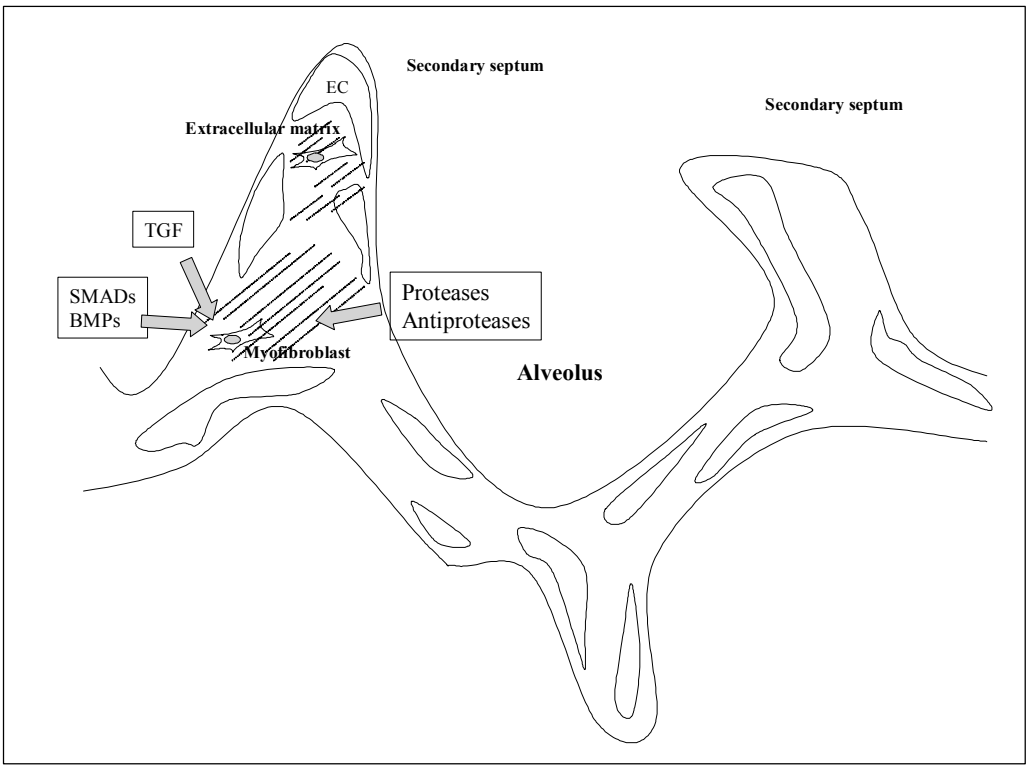

Figure 1 *Pathways described in this thesis that are involved in secondary septum formation and that are deregulated in disturbances of late lung development, which can lead to BPD. EC: endothelial cells, TGF: Transforming Growth Factor, BMPs: Bone Morphogenetic Proteins.*

Throughout lung development and alveolarization, MMP1, MMP2, MMP9, and TIMP-2 (tissue inhibitor of metalloprotease-2) are strongly expressed in humans and mice.[45,46] TGF-β stimulation increased TIMP-1 mRNA expression in fibroblast in hyperoxia conditions, compared to fibroblast in normoxic conditions.

Vascular Development

Various animal models and autopsy studies of humans who died from BPD have consistently shown fewer small arteries and abnormal distribution of vessels within the distal lung suggestive of defective vascular development.[47,48] Two processes are involved in vascular development, i.e. vasculogenesis and angiogenesis. The latter process, by differentiation of angioblasts in the mesoderm, generates new vessels from pre-existing ones, not only during development but also during tumor growth. Angiogenesis is distinct from vasculogenesis, which process is prominent in embryogenesis. Vasculogenesis involves de novo formation of blood vessels from angioblast or endothelial precursor cells. These cells migrate and differentiate to form vascular tubes in response to local transcription and growth factors as well as ECM. The relative contributions of vasculogenesis and angiogenesis to lung vascular growth at each stage of lung development are being debated and further studies are needed to better define the underlying mechanisms. In human fetal lungs, the airways would seem to act as a template for pulmonary artery development, with endothelial tubes forming around the terminal buds of distal airspaces, suggesting an inductive influence of the epithelium.[49] A study by Parera and colleagues from our own institution suggested distal angiogenesis as a new mechanism for pulmonary vascular morphogenesis.[50]

The bronchial vessels develop with the preacinar airways and are complete by approximately 16-weeks gestation with further growth in size to match lung growth. Preacinar pulmonary arteries, supplied by the right heart, grow with the airways into the intra-acinar region and fuse with peripheral microvasculature that has arisen from mesenchyme by vasculogenesis.[49] The importance of vascular supply for alveolarization was demonstrated by several studies using antiangiogenic agents such as a VEGF receptor inhibitor. This agents disrupts vascular development, leading to reduced pulmonary arterial density and inhibition of alveolar growth. The VEGF signaling pathway has been shown to play a crucial role in embryonic vasculogenesis.[51]

The hypoxia-inducible factors (HIFs) are highly regulated by oxygen tension.[52] Molecular oxygen directly regulates HIF-dependent transcription and angiogenic growth factor expression within the lung. When a fetus is exposed to oxygen at birth, when breathing starts, steady-state levels of HIF expression within the lung will rapidly and dramatically decrease. This downregulation is important to control the production of several growth factors, angiogenic factors, including VEGF and its receptors, and NO.[53,54] Suppression of VEGF and NO production during critical stages of lung vasculogenesis and alveolar development may lead to long-term impairment of lung growth.[55-57] Nitric oxide as a factor enhancing HIF, and

subsequently VEGF expression, is inhibited by endogenous arginine metabolites such as asymmetric dimethylarginine (ADMA). Dimethylarginine dimethylaminohydrolase (DDAH) selectively degrades ADMA and thus eliminates an inhibitor for NOS.[58-63] DDAH activity in the whole lung substantially increases for 24 hours after birth, and this would be expected to reduce tissue concentrations of NOS inhibitors and thereby to stimulate NO generation.[64] Experimental animals exposed to hypobaric hypoxia showed markedly suppressed DDAH activity. These data suggest that DDAH isoforms are developmentally regulated in the lung and could contribute to pulmonary vascular adaptation and to the dysfunction in vascular reactivity dysfunction seen in persistent pulmonary hypertension of the newborn.[64]

The hypoxic environment of the developing lung favors HIF-1α-dependent gene expression. HIF-2α controls expression of the VEGF isoforms in the developing lung, thereby preventing disturbed development. HIF-1α and HIF-2α levels were found to decrease following preterm birth of lambs with respiratory distress syndrome (RDS). This is likely due to suddenly heightened activities of all prolyhydroxylases (PHDs) caused by the rapid rise in molecular oxygen that is followed by higher prolylhydroxylase domain containing protein (PHD)-2 expression. With HIF expression, VEGF mRNA expression declined as well, which therefore may be one of the factors contributing to the pathophysiologic changes seen in neonatal respiratory failure.[52,65-67]

Recent evidence suggests that blood vessels in the lung actively promote normal alveolar development and thus help maintain alveolar structures throughout life. Consequently, modulation of angiogenic growth factors and vascular precursor cells may have therapeutic potential for lung diseases characterized by alveolar damage.[67]

The role of the endothelium-dependent relaxing factor NO in the regulation of the pulmonary vascular tone in the perinatal period has been well established. Recent studies suggest that inhaled NO may decrease the incidences of death and BPD in premature infants. Ventilated primates given low-dose inhaled NO have fewer myofibroblasts and less elastin deposition in alveoli than control animals.[68] Endothelial nitric oxide synthetase (eNOS) transgenic animals are less susceptible to ventilator-induced lung injury and have less fibrosis following injury with bleomycin.[69,70] Patients with interstitial lung diseases, such as idiopathic pulmonary fibrosis and BPD, suffer from lung fibrosis secondary to myofibroblast-mediated excessive ECM deposition and destruction of lung architecture.[71] TGF-β1 induces epithelial-mesenchymal transition of alveolar epithelial cells to myofibroblasts.[72,73] Read and colleagues suggest a potential mechanism by which NO impacts alveolar fate and protects against fibrosis. Their findings indicate that NO is important in preserving an epithelial phenotype and in decreasing epithelial-mesenchymal transition (EMT) in alveolar epithelial cells.[74]

Endothelial progenitor cells (EPCs) have been isolated from the peripheral blood of humans. *Ex vivo*, CD34+ EPCs differentiate to an endothelial phenotype and incorporate into neovessels at sites of ischemia. EPCs can migrate from the bone marrow to the peripheral circulation, where they contribute to repair of injured endothelium and the formation of new blood vessels. Lung injury in mice rapidly released EPCs into the circulation to help repair the lung, together with other bone marrow derived progenitor cells. In elastase-induced emphysema, cells derived from the bone marrow develop characteristics of endothelial cells and contribute to repair of the alveolar capillary wall. A biological role of EPC in lung repair is suggested by significantly higher numbers of circulating EPCs in patients with pneumonia, and the fact that patients with low EPC counts tend to have persistent fibrotic changes in their lungs even after recovery from pneumonia.[75,76] Findings from Balasubramaniam and colleagues in an experimental model of BPD suggest lowered circulating, lung, and bone marrow EPC levels in BPD.[77] Those observations taken together, one might speculate, that EPCs migrate from the bone marrow to the peripheral circulation and the lung, to aid in the repair of injured endothelium and to help restore lung integrity.

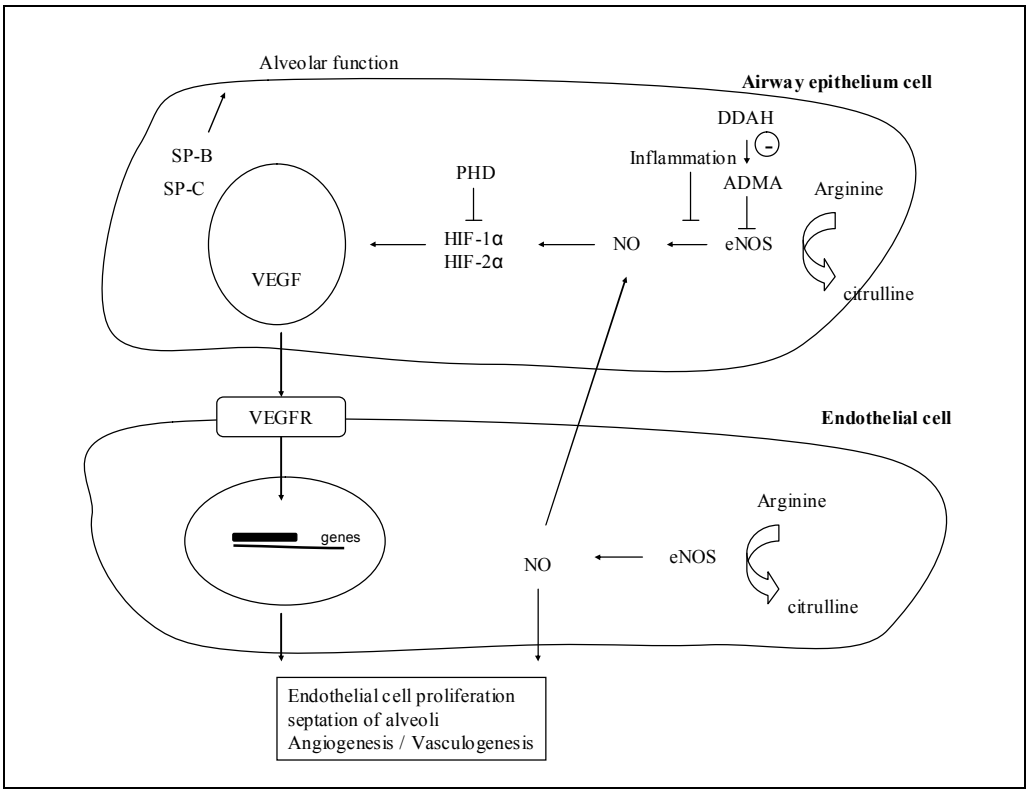

Figure 2 *Epithelial-endothelial interactions in pulmonary vascular development showing pathways involving NO, VEGF and HIF, described in this thesis. See text for details.*

Lung development: Bronchopulmonary dysplasia and chronic obstructive pulmonary disease

As originally described, BPD is a specific form of chronic lung disease of infancy with varying degrees of alveolar and vascular growth arrest, airway branching abnormalities, and peribronchiolar fibrosis.[34] Occasionally there may be relentless progression towards pulmonary fibrosis, with progressive scarring of the fine structures of the lung parenchyma. Chronic obstructive pulmonary disease (COPD) is a devastating disorder that causes much human suffering. It is currently the fourth leading cause of death in the United States.[78] Pulmonary emphysema is classified as a major component of COPD. The pathology of emphysema has been characterized as loss of respiratory surface area, as evidenced by abnormal, permanent enlargement of airspaces distal to the terminal bronchioles.[71] Both BPD and COPD lungs show markedly enlarged distal airspaces. There is a difference, however, in that COPD is related to the destruction of established alveoli, whereas BPD represents the disruption of normal lung development. In contrast to many forms of lung disease in adults showing increased angiogenesis is present, COPD is characterized by a reducted numbers of blood vessels, similar to infant with BPD.[79] Liebow as early as 1959 reported, remarkably thin and almost avascular alveolar septa in lung emphysema underlining that vascular development or impairment of vasculogenesis is important in the pathophysiology of the disease.[80] Confirmation came from animal models in which VEGF receptor blockade induced alveolar septal cell apoptosis and airspace enlargement.[57,81] Various animal models of BPD and studies of humans who died from BPD have consistently shown a reduction in the number of fewer small arteries and abnormally distributed vessels within the distal lung as well as enlarged distal airspaces resulting from disrupted lung development.[48,56] The response of the developing lung to injury encompasses a multitude of cellular and molecular interactions. Dependent on these interactions there will be either regeneration and repair or progression to growth arrest, irreversible tissue destruction, and/or fibrosis. Injury to an immature or abnormally developed lung, such as in premature babies or infants with CDH, can eventually stop alveolar septation and alter pulmonary vascularization.[13] Arrest of lung development is the hallmark of chronic lung disease, the most common complication in preterm infants, ventilated newborns with ARDS like-disorders, such as meconium aspiration syndrome or pneumonia, and congenital lung diseases, such as CDH or CCAM.[14]

Genetic abnormalities can impact early fetal lung development, postnatal lung maturation, as well as adult lung injury and repair. Studies suggest that abnormally developed lung structure and function may contribute as a susceptibility factor for several adult lung diseases.[82,83] In mice, many key genes result in common phenotypes comprising either neonatal respiratory distress if the structural effect is severe, or reduced alveolarization and early onset emphysema if the effect is milder.[84,85]

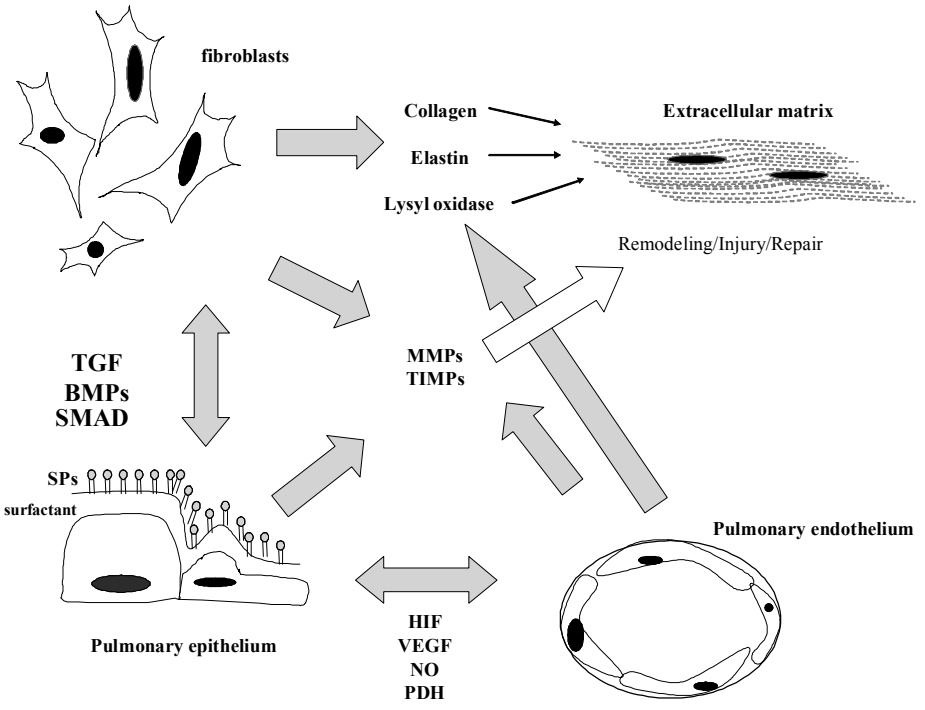

Figure 3 *Cell-cell and cell-matrix interactions during disturbance of late lung development and COPD, described in this thesis. See text for details.*

Null mutation of either Fgf10 as well as Fgfr2b completely abrogates lung branching morphogenesis distal to the carina, whereas hypomorphic or ectopic FGF signaling results in neonatal lethal alveolar dysplasia.[86-88]

Null mutation of Smad3, which is a key receptor activated Smad in the TGF-β signaling pathway, results initially in a rather subtle failure of correct organization of the matrix, which in turn is an antecedent of subsequent, early onset but still relatively mild pulmonary emphysema.[89] This early onset emphysema in Smad3 null mutants is also associated with activation of excessive matrix metalloproteinase (MMP) activity.

Null mutation of lysyl oxidase prevents correct elastin cross-linking, and hence alveolarization is also incomplete.[33,90] Disordered extracellular matrix deposition and remodeling are key features of arrested alveolarization associated with BPD. The causes underlying this perturbed ECM remodeling are unknown. Lysyl oxidases, which catalyse the cross-linking of collagen and elastin fibers, are an important group of ECM-remodeling enzymes. We observed elevated mRNA and protein expression of three lysyl oxidases, Lox, LoxL1 and LoxL2, in the lungs of neonatal mice exposed to chronic hyperoxia, as well as in human patients with BPD (unpublished data, see Appendix B). Both lysyl oxidase catalytic activity in lung protein extracts, and staining intensity for lysyl oxidases in the septae and smooth muscle layers of the developing lung from hyperoxia-exposed mice were dramatically increased. Lysyl oxidase gene expression and secretion induced by

transforming growth factor (TGF)-β was also increased in embryonic fibroblast-like NIH/3T3 cells and human fibroblasts exposed to chronic hyperoxia (unpublished data). Administration of TGF-β neutralizing antibodies to hyperoxia-exposed neonatal mice improved alveolarization and restored Lox expression. Our unpublished data suggest that elevated lysyl oxidase activity in the lungs of mice exposed to chronic hyperoxia are responsible, at least in part, for the aberrant elastin and collagen metabolism observed in hyperoxia-induced BPD. This idea is reinforced by our observation that lungs from mice exposed to chronic hyperoxia exhibit elevated levels of desmosine, a marker of elastin cross-linking, and that infants with BPD have elevated desmosine levels in their lung tissue and urine.[19,91]

Similarly, in mice with the Pdgf-a null mutation, alveolar myofibroblasts fail to differentiate and produce elastin; hence, alveolar crests also fail to form.[35] Failure to protect elastin from proteolytic degradation in α1-antitrypsin deficiency, or from excessive destruction mediated by neutrophil elastase results in emphysema, a disease characterized by destruction of the alveolar wall.[92] Nevertheless, destruction of elastin through neutrophil elastase is also seen in ventilated preterm infants developing bronchopulmonary dysplasia.[93,94] Elastic complexity of the lung is important in respiratory physiology. Thus, absence of correctly cross-linked and oriented elastin containing matrix predisposes to failure of correct establishment of elastic linkage and alveolarization. Deregulation at several critical points in different pathways involved in ECM remodeling, angiogenesis and alveolarization in the injured lung may result in alveolar hypoplasia and fibrosis, and then lead to BPD or COPD.

Lung hypoplasia due to intrauterine growth impairment

Intrauterine growth retardation (IUGR) is a relatively common complication during pregnancy.[95] It occurs when greater placental resistance restrict fetal perfusion or when specific intrinsic fetal factors are in place, such as chromosomal anomalies. Decreased tissue perfusion will cause hypoxemia, limit the delivery of nutrients and raise cortisol concentration, all of which can affect lung development.[96-98] High fetal cortisol levels can cause lung development in preterm fetuses with IUGR to accelerate, as high cortisol levels induce pneumocytes to secrete more surfactant. We found higher incidence of BPD, however in premature infants with IUGR as compared with prematures without IUGR, as compared with prmature without IUGR, although the rate of RDS did not differ between these groups.[99] It would seem, therefore, that mechanisms other than the pulmonary surfactant system underlie the development of BPD. On the other hand, long-term prenatal administration of corticosteroids has been found to decelerate rather than accelerate of pulmonary development.[100] Animal studies have documented that reduced nutrient availability may impede lung growth.[101] Prenatal nutrient and oxygen restriction in IUGR infants could affect ECM components such as elastin, which in turn could alter alveolar properties. Epidemiologic studies indicate that exposure to a suboptimal intrauterine environment that results in IUGR increases the risk of respiratory illness in infants, children and adults.[96,102,103] It is likely that

undernutrition contributes to neonatal chronic lung disease in preterm infants. With lung development still ongoing undernutrition, or lack of essential minerals or micronutrients, could have detrimental effects on lung anti-oxidant and defense mechanisms and surfactant production, as well as on structural maturation of alveoli and small airways.[104,105] Fetal breathing movements are important for maintaining the appropriate degree of lung expansion and hence normal lung growth. Humans fetuses with IUGR, however, show less breathing movements, perhaps because circulating and/or cerebral adenosine concentrations have increased.[106,107] Experimental studies have clearly shown that nicotine exposure during gestation interferes with the gas exchanging region of the lungs.[108] The observed effects include decreased pulmonary elastin content, fewer alveoli and greater alveolar volumes, more alveolar wall fenestrations, and less surface area available for gas exchange.

Pulmonary Surfactant

Pulmonary surfactant found at the air-liquid interface of the lung functions to reduce surface tension. It is synthesized by type II pneumocytes, stored in lamellar bodies, and secreted into the airspace as tubular myelin, from which it is then absorbed into the air-water interface to form a surfactant monolayer. Pulmonary surfactant is composed of 80% phospholipids, 8% neutral lipids, and 12% proteins. The phospholipid components of natural surfactant, especially dipalmitoylphosphatidylcholine (DPPC), are primarily responsible for lowering surface tension. Surfactant related proteins, on the other hand, are critical for surfactant behavior, immune defense, and particle clearance.[109,110,111] Members of the collectin family of host defense proteins, surfactant protein A (SP-A) and SP-D are hydrophilic oligomers that, play a role in the immune response to microbial challenge by binding microorganisms and modulating leukocyte function such as chemotaxis, cytokine function, and phagocytosis.[112] SP-D is thought to help reduce inflammation in the lung. This was postulated from the finding of delayed removal of apoptoticSP-D deficient mice have delayed removal of apoptotic cells, such as alveolar macrophages, in SP-D deficient mice, an important step in the resolution of the inflammation that may contribute to chronic lung disease.[113,114] Recombinant SP-D binds preferentially to these apoptotic cells and may have a role in reducing inflammation in synthetic surfactant preparations. SP-B and SP-C are critically important small hydrophobic apoproteins that promote absorption and dynamic spreading of surfactant to form a phospholipid monolayer that lines the alveoli. Polymorphisms in the SP-B gene and mutations of SP-C have been associated with BPD and other interstitial lung diseases. Congenital SP-B deficiency is a lethal cause of respiratory distress.[115, 116]

Surfactant replacement therapy for the premature infant with respiratory distress has now become standard therapy.[117] A significant body of evidence supports the view that surfactant deficiency in neonates with severe respiratory failure either represents a primary deficiency such as in RDS, or results from surfactant inhibition

or inactivation, as in pneumonia or meconium aspiration syndrome.[118] Endogenous and exogenous pulmonary surfactant may be inhibited by albumin together with proteases and coagulation factors present in the alveolar compartment during the acute phase of respiratory failure. Adding dextran or polymyxin to surfactant preparations, will counteract this inhibition.[119,120] Numerous *in vivo* studies have demonstrated that mechanical ventilation of the lung alters the composition and homeostasis of surfactant.[12] Decreased large aggregate surfactant after mechanical ventilation was found to result in impaired lung function.[121] Both rodent and primate animal studies have reported altered production of surfactant proteins after the animals' exposure to high oxygen concentrations.[122,123] In a rat model of bleomycin-induced lung injury, respiratory distress was associated with a deficiency of SP-B and SP-C. Addition of exogenous SP-B *in vitro* reconstituted the minimum surface tension to a level seen in the control animals. Bronchial lavage specimens from human infants with severe BPD have revealed abnormal surface tension, elevated to the level seen in specimens from infants with inherited SP-B deficiency.[124,125,126]

Further studies have shown that the gene regulation and release of surfactant protein B and C are mechanosensitive, suggesting that mechanical ventilation and stretch may influence the pulmonary surfactant system.[127]

Additional studies will need to address the question of the usefulness of surfactant treatment in preterm infants who still show respiratory failure requiring mechanical ventilation beyond the first week of life. Surfactant therapy ameliorates respiratory failure in neonatal lung diseases showing surfactant inhibition and dysfunction, so that less rescue cardiopulmonary bypass are needed and the incidence of chronic lung disease is brought down. As recombinant SP-C surfactant was found efficient in premature lambs and rabbits suffering from respiratory distress syndrome as well as in different models of acute lung injury, Hilgendorff and colleagues addressed its potencies in a model of lung injury mainly characterized by severe inflammatory changes.[128,129] As recent studies revealed that synthetic SP-C surfactant preparation shows resistance to inactivation by meconium and that recombinant SP-C possesses anti-inflammatory potencies of recombinant SP-C, it might well be that synthetic surfactant exerts a regulatory or protective effect on type II cells or different parts of the alveolar-capillary compartment.[128,129] The future of neonatal medicine requires designer surfactants that mimic natural surfactant, thereby increasing resistance to inhibition and improving the clinical response.

In conclusion, many different processes are involved in late lung development, such as vasculogenesis, septation and alveolarization. Disturbances of these processes have major impacts on the structure of the developing lung. These processes nevertheless also offer good starting points for therapeutic interventions. Further research will have to shed light on the details of these processes and the most effective ways to implement therapies. The similarities between BPD and COPD may provide possibilities for combined research.

Bronchopulmonary dysplasia in CDH

Congenital diaphragmatic hernia (CDH) is a rare congenital anomaly with an incidence of around 1 in 3,000 live births.[130] Over the past 20 years, pulmonary hypoplasia and pulmonary hypertension haveemerged as the two cornerstones of the pathophysiology of CDH. More recently, ventilator induced lung injury (VILI) was recognized as a contributing iatrogenic factor for chronic lung disease in CDH.[131,132] Lung injury secondary to mechanical ventilation certainly is one of the major contributors to mortality and is a major risk factor for severe morbidity following initial admission for CDH. Consequently, infants with CDH have a high mortality risk of up to 30%, depending on case selection. In addition, long-term pulmonary morbidity is high and is mostly bronchopulmonary dysplasia (BPD), which develops in 33% of survivors.[14] This may extend into infancy.[133] The etiology of BPD in CDH is unknown in a large majority of cases and is thought to be multifactorial.

BPD is a chronic lung disease characterized by persistent respiratory signs, prolonged need for mechanical ventilation or oxygen dependency for a longer period of time. Many infants with BPD have pulmonary hypertension. BPD mostly occurs in premature infants who have needed mechanical ventilation and oxygen therapy for acute respiratory distress (proportions are reported to be up to 20%).[34] Histologically, BPD is characterized by abnormal lung development with simplified acinar structures, poorly formed secondary crests, dysmorphic alveolar capillarie, and expression of angiogenic factors and their receptors. This results in an arrest of lung and pulmonary vascular development, the so called "new-type" BPD.[34]

"Gentle mechanical ventilation" with permissive hypercapnia is the initial and only accepted therapy for infants with respiratory failure due to CDH[5]. Individually and in combination, oxygen therapy and mechanical ventilation predispose the newborn to ventilator-induced lung injury, which can lead to BPD.[134] Secondary to mechanical ventilation, infants can develop lung injury characterized by hyaline membrane formation, parenchymal hemorrhage, pneumothorax and pulmonary interstitial emphysema.[134] Inflammatory processes as well as endothelial dysfunction play a role in the development of BPD, as they lead to vascular injury and disturbance of normal vascular integrity.[135] Crucial in this process is, the release of vasoconstrictors/vasodilators (such as endothelin, nitric oxide and its inhibitors), adhesion molecules (such as ICAM, VCAM and selectins, e.g.sP-selectin and sE-selectin), angiogenic factors (such as VEGF) and procoagulants (such as Von Willebrand factor, thrombomodulin and activated factor VIII:C) play a crucial role.[136] A few studies which suggest that a ventilation strategy using smaller tidal volumes (high frequency oscillation (HFO)) may reduce the incidence of BPD, seeing that this strategy will bring down ventilator induced lung injury.[137] Thus, HFO would fit well in the management scheme for neonates with CDH, the more so as it could prevent the need for ECMO. Clinical studies of HFO and mean airway pressure in lung injury found that HFO provides better oxygenation and raises this pressure

without increasing the incidence of barotrauma.[138] On the other hand, low mean airway pressure settings do not allow the alveoli to open in the low-compliance lung, which causes atelectasis and higher amplitude of swing pressure, and consequently excessive expression of cytokines in the airway. Studies of this kind are mainly observational, however, no randomized controlled trials have been published.[139] Having seen that HFO reduces ventilator-induced lung injury, we might further speculate that HFO gives less damage to the pulmonary vascular endothelium than does conventional mechanical ventilation. This may also have a positively influence the development of BPD due to the resolution of the pulmonary hypertension.

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PART VII

CONCLUSION AND FUTURE PERSPECTIVES

chapter 18

Conclusion

- Components of the TGF-β signaling pathways are dynamically regulated during late lung development in the mouse, rat and human. Pronounced changes in the expression and localization of the TGF-β receptors AcvrII, Tgfbr1, Tgfbr2, Tgfbr3, and endoglin, and the intracellular messengers Smad2, Smad3, Smad4, Smad6 and Smad7 were noted through the canalicular, saccular and alveolar stages of development. TGF-β signaling was detected in the vascular and airway smooth muscle cells, as well as in the alveolar and airway epithelium throughout late lung development demonstrating that active TGF-β signaling is required for normal late lung development

- Hyperoxia exposure of neonatal C57BL/6J mice illustrates that hyperoxia dysregulates the expression of components of the TGF-β/BMP signaling pathways. This dysregulated signaling has at least two functional consequences relevant to hyperoxia-induced arrest of alveolarization and BPD: 1) hyperoxia increases the sensitivity of alveolar type II cells to TGF-β-induced apoptosis, and 2) hyperoxia modulates the expression of ECM and ECM-remodeling components induced by TGF-β in fibroblasts. Abnormal upregulation of the TGF-β system in the lung upon exposure to hyperoxia swings the balance in favor of interstitial ECM deposition and prevents turnover or breakdown of ECM components.

- Chronic hyperoxia leads to restricted lung development togehther with inflammatory modifications in a critical period of alveolarization.

- SGA neonates below 32 weeks gestation are a high-risk group regarding neonatal mortality and neonatal pulmonary morbidity.

- Growth restriction leads to reduced expression of surfactant protein mRNA and could induce increased inflammatory activation.

- Prolonged mechanical ventilation contributes to the development of chronic lung disease by the induction of lung inflammation without adequate stimulation of the counter-regulatory cytokine interleukin 10 in preterm infants with respiratory distress syndrome.

- Surfactant therapy in severe ARDS improves oxygenation immediately after intratracheal administration. Improvement of oxygenation is sustained only in the subgroup of patients with mild or moderate ARDS. Ventilator induced lung injury in children with ARDS can probably be ameliorated with surfactant treatment.

- *In vivo* studies concerning the impact of pulmonary surfactant on inflammatory conditions involving the adhesion of neutrophils to endothelial cells reveal that

natural surfactant but not synthetic surfactant has pro-inflammatory effects when higher amounts of the exogenous dose reach the circulation.

- After investigating the impact of a recombinant surfactant protein (SP)-C based surfactant in a model of meconium aspiration syndrome (MAS), we conclude that impairment of lung function in MAS, associated with marked changes in surfactant associated protein messenger RNA, can be sufficiently treated using recombinant surfactant protein-C or natural surfactant. Surfactant administration improved pulmonary gas exchange as well as pulmonary inflammatory cytokine transcription.

- The response to recombinant SP-C surfactant depends on the level of positive end-expiratory pressure (PEEP) used in ventilated immature rabbits suffering from respiratory distress syndrome.

- Comparing the effects of an open lung concept positive pressure ventilation with a conventional ventilation strategy combined with administration of two different surfactant preparations on lung function and surfactant homeostasis, we conclude, that both treatment strategies improved gas exchange and lung function in neonatal ARDS.

chapter 19

Future perspectives

INJURY TO THE DEVELOPING LUNG

Most lung diseases are complex and no single gene or protein alone will either help us to identify the pathogenesis or result in new approaches of treatment and/or prevention. The clinical features of acquired lung diseases such as bronchopulmonary dysplasia, chronic obstructive pulmonary disease, interstitial pulmonary fibrosis and others are heterogeneous. Understanding the complex interactions among many genes that result in disease will require a systematic approach aimed at identifying not only single genes or pathways but also the networks of genes that interact to produce the disease. It is important to coordinate research involving different disciplines to begin to "translate" the knowledge of genomics and proteomics into our understanding of developmental aspects of the normal human lung, its diseases and its response to injury. Targeted proteomics will likely assist us in identifying specific genotypes and in understanding phenotypes of these multifactorial and complex disorders. Both BPD and COPD have impaired septation as a prominent morphological feature. The similar pathophysiological features of BPD and COPD may prompt basic scientists and clinicians (both in adult and pediatric medicine) to combine their research efforts and focus on the underlying molecular mechanisms. This will lead to a better understanding of mechanisms of injury, repair and regeneration of the lung. As COPD is one of the major causes of death in adulthood and as the increasing number of infants with BPD might form a higher risk group for developing COPD, research into these underlying processes could have a major impact, not only at an individual level, but also on public health.[1]

The data presented in this thesis implicate that the TGF-β/BMP signaling pathway is relevant in the pathogenesis of BPD. Given that the TGF-β/BMP pathways are also amenable to pharmacological and genetic manipulation in the lung, they provide possible avenues for the management of this disease

Today, the importance of ventilator-induced lung injury to the developing lung is commonly accepted. Mechanotransduction, mechanical force-induced signaling cascades, has been the focus of several studies.[2-5] In addition, research into mechanosensation, physico-chemical conversion mediated by specific protein-protein interactions, has provided new insights into cellular and molecular mechanisms of ventilator-induced lung injury of the developing lung. By knowing the mechanosensory mechanisms, mechanotransduction pathways and genes activated by mechanical ventilation and involved in VILI, one might be able to selectively prevent physical force-induced cell damage. One might speculate that interfering with these pathways by genetic manipulation, e.g.by small inhibitor molecules (siRNA's), or by pharmacological interventions may have beneficial effects on the course of the injury and may enhance recovery. In clinical practice, reduction of physical force-induced injury to immature as well as hypoplastic lungs might be achieved by low tidal volume strategies.[6-8] However, thus far, current

treatment strategies have failed to reduce the incidence of BPD.[7] In part, this might be due to the fact that, as a consequence of improved perinatal care, more premature newborns, who are at high risk of developing BPD, are treated and survive. Large multicenter studies will have to be conducted to identify which ventilation strategies lead to the best outcomes in these extremely premature patients by studying ventilation modalities, such as HFO, as well as additional therapies such as surfactant, inhaled NO, liquid ventilation, or combinations of these modalities. A similar approach should be used to study the optimal treatment modality in patients with hypoplastic lungs, such as in CDH.

In the last 10 years, a number of key control factors have already been identified determining impaired septation as a crucial feature of BPD. Nevertheless, analyses of lung transcriptome or proteome offer new prospects in the identification of candidate genes, pathway and networks and add to a more comprehensive view of this process. Restoring balance through supply of insufficient factors and inhibition of excessive factors appears a promising clinical approach for prevention or treatment of this disease.

Mature differentiated lung epithelial cells, endogenous progenitors, and putative stem cell niches all appear likely to participate in the response of the lung to injury. As has recently been suggested for the gut, identification of endogenous lung progenitors and delivery of exogenous cells are potential therapeutic approaches that are currently being explored in animal models of lung injury.[9] As a detailed understanding of stem cell biology, lung development, lineage commitment, and epithelial differentiation emerges, the ability to modulate lung injury and repair will likely follow. Protecting the alveolar progenitor cell population with small, soluble molecules or providing the correct exogenous signals can guide alveolar progenitor cells to maintain or repair the alveolar gas diffusion surface.

Clinically, ventilator-induced lung injury is one of the major contributors to mortality and morbidity in patients with congenital lung anomalies. The clinical picture has changed following the application of a lung protective strategy consisting of preservation of spontaneous ventilation, permissive levels of hypercapnia and avoidance of high inspiratory airway pressure, increasingly known as the gentle ventilation concept.[10-12] In this concept, high frequency oscillation has been shown to prevent ventilator-induced lung injury at least in premature infants. There is a continuing need for international multi-center randomized clinical trials (RCTs) with enough power to determine the role of HFO compared with conventional ventilation under a variety of conditions, especially in CDH.

VASCULOGENESIS DURING LUNG DEVELOPMENT

Lung vasculogenesis, under the influence of the secretion of angiogenic growth factors, such as VEGF and NO, contributes to normal alveolar development. Impaired alveolar development in BPD is associated with arrested and dysmorphic vascular growth and impaired lung angiogenic growth factor expression. Exogenous VEGF or NO may provide new therapeutic avenues for preserving or enhancing alveolar structure. Hypoxia-inducible factor (HIF) is a master transcription factor modulating O_2-sensitive gene expression and vessel growth. HIF is activated by hypoxia and inhibited by increased O_2 levels. HIF activation via inhibition of prolyl hydroxylase domain-containing proteins prevents lung damage, supporting a potential role in promoting alveolar development. It will be crucial to determine the appropriate dosing and pre- or postnatal timing of angiogenesis stimulating factors to avoid abnormal pulmonary vascular proliferation, pulmonary hemorrhage or lung edema and other problems.

The regenerative potential of stem cells has recently been under intense investigation.[13] However, it is far from clinical application at this moment. In vitro, stem and progenitor cells have the ability for self-renewal and differentiation into organ specific cell types. In vivo, transplantation of these cells may reconstitute organ systems, as shown in animal models of diseases such as ischemic heart disease.[14] Endothelial progenitor cells (EPCs) have been isolated from the peripheral blood, expanded in vitro and committed to an endothelial lineage in culture. Cell therapy using EPCs or transfected EPCs that modify their microenvironment to restore damaged organ integrity might be a future therapeutic option.

As mentioned above, impaired alveolar development in chronic lung disease is associated with abnormal vascular growth and decreased expression of lung angiogenic growth factors. Nanotechnology for improving drug delivery may become of increasing importance in the next decade. A variety of mechanisms contribute to the potential of nanotechnology. The availability of a number of different nanoparticle structures, including solid particles, nanoshells, polymeric micelles and liposomes, provides a flexible range of opportunities that can be tailored to meet different therapeutic needs. Targeting of nanoparticles with angiogenic growth factors or HIF stabilizers to specific cell-surface receptors, can enhance drug delivery to the intended target, reducing systemic load at the same time. Inhalational delivery offers additional opportunities for nanotechnology to impact pulmonary disease.

PULMONARY SURFACTANT

Extensive research has provided increased understanding of molecular mechanisms involved in the formation and preservation of the surfactant film of the alveolar lining. These efforts will help to develop stable surfactant associated peptides which mimic the function of SP-B and -C. Besides treatment of premature infants with RDS, surfactant might be effective in newborns with inactivated surfactant, as is the case in inflammatory lung disorders such as meconium aspiration syndrome or in case of insufficient pool size and inactivation in hypoplastic lungs.[15,16] Surfactant may also be used as a carrier for antibiotics, specific antibodies, immunosuppressants, vasodilators and other drugs and for treatment of various lung diseases not primarily related to the surfactant system, such as BPD, lung fibrosis and COPD.

GENETIC FACTORS OF ADULT-ONSET LUNG DISEASE

In the future, we have to characterize and appreciate genetic and environmental susceptibility factors that predispose children with impairment of lung development to apparent adult onset lung disease in more detail. It is equally important to identify factors that protect against it. Many lung diseases in children and adults are characterized either by a failure of development and/or by destruction of the alveoli. In premature neonates, alveolarization may be impaired, resulting in poor lung function. In contrast, emphysema in adults is characterized by destruction of the mature alveoli. Understanding the mechanisms that regulate the formation of the gas exchange surface will enhance development of therapies capable of promoting alveolarization. Genes that regulate alveolar morphogenesis are differentially expressed in periods of alveolar formation compared to periods in which no alveoli are formed. Abnormalities of lung development may contribute to the development of COPD. The factors that affect pulmonary development in utero and postnatally are not well understood. Lung morphogenesis is a highly regulated process that can be impaired in utero by both genetic and environmental factors. One of the best recognised causes is maternal cigarette smoking, but more work is needed to identify other causes, both environmental and genetic. This work should involve the collection of large prospective cohorts of newborn babies, possibly in multicenter studies, to highlight factors with a role in etiology, using multivariate analysis. In addition, gene-environment and fetal-maternal interactions have been a focus of recent research.[17] The main targets of identifying gene-environment interactions are to provide insight into mechanisms of disease development and to identify patients with an inherent vulnerability to certain conditions based on disease susceptibility genes. This in turn may allow patients with conditions such as lung immaturity or hypoplasia or even patients at risk of developing a chronic lung disease such as BPD or COPD to be treated with targeted and individualised therapies.

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chapter 20

Summary
Samenvatting

SUMMARY

Respiratory insufficiency and failure are common problems for many infants in the neonatal and pediatric intensive care unit. Lung development begins very early in gestation and continues during pregnancy and after birth into young adulthood. Injury to the developing lung or disturbance of normal lung development in different stages may lead to chronic lung disease which can persist into adulthood.

Bronchopulmonary dysplasia (BPD) is a chronic lung disease, characterized by an arrest of development of the lung and the pulmonary vascular system. It is seen in around 20% of all ventilated newborns. It often occurs in prematurely born infants, but it can also be found in term or near-term babies with congenital lung malformations. Mechanical ventilation, oxygen therapy and inflammation are the main risk factors for the development of BPD. However, other environmental factors as well as genetic factors are currently being investigated.

The exact pathogenetic mechanisms of chronic lung disease in general and of BPD in particular are not fully understood. Identifying and describing pathways that lead to BPD may provide important clues about the pathophysiology of this condition and may lead to the development of preventive and therapeutic strategies that may have implications for long-term outcome.

In **Part I** of this thesis, an overview of the subject is given. **Chapter 1** gives an introduction to normal and abnormal lung development and possible mechanisms leading to acute and chronic lung disease in newborns.

Infants born with compromised respiratory status, whether due to immaturity or other conditions, such as congenital lung hypoplasia, often require mechanical ventilation and supplemental oxygen to maintain adequate oxygenation and ventilation. Individually and in combination, these therapies predispose the newborn to ventilator-induced lung injury. **In chapter 2**, mechanical ventilation strategies for congenital lung malformations are discussed.

Part II describes experimental aspects of lung development and BPD. Late lung development is characterized by progressive subdivision of developing airspaces into alveoli. An important family of growth factors known to play a role in early lung development is the TGF-β/BMP (transforming growth factor- β/bone morphogenetic protein) family. It is unknown if this pathway also has a role in late lung development. In **chapter 3**, the expression, tissue localization and activity of components of the TGF-β signaling pathway during late lung development are described. We found that these components are dynamically regulated in mouse and rat, as well as in human lung development. The results of this study suggest that the TGF-β signaling pathway plays a role in normal late lung development in rodents and humans.

Hyperoxic injury is believed to disrupt critical signaling pathways that direct lung development, which may lead to bronchopulmonary dysplasia. In **chapter 4**, the effects of 21% or 85% O_2 on TGF-β/BMP superfamily signaling in the lung of neonatal C57BL/6J mice were investigated. Growth and lung function were significantly impaired in pups exposed to 85% oxygen. An arrest of lung development was observed accompanied by a dysregulated expression and localization of TGF-receptors and their downstream Smad proteins. TGF-β signaling was potentiated, whereas BMP signaling was impaired, both in the lungs of pups exposed to hyperoxia and in isolated mouse alveolar type II cells and human primary lung fibroblasts. After exposure to 85% O_2, primary lung alveolar type II cells were more susceptible to TGF-β-induced apoptosis, whereas primary pulmonary artery smooth muscle cell were unaffected. In primary lung fibroblasts exposed to hyperoxia, TGF-β-stimulated production of the α1-subunit of type I collagen, tissue inhibitor of metalloproteinase-1, tropoelastin, and tenascin-C is enhanced. Thus, hyperoxia significantly affects TGF-β/BMP signaling in the lung, including processes of septation and alveolarization which are disturbed in BPD.

Angiogenic factors, inflammatory cytokines and the nitric oxide system have also been implicated in the pathogenesis of BPD and play important and interrelated roles in late lung development. In **chapter 5**, we demonstrated a pronounced dysregulation of expression of oxygen-related factors (HIF1-α and HIF2-α), angiogenic factors (VEGF and its receptor Flt-1) and components of the NO-biosynthetic pathway (NOS-2, NOS-3, DDAH) in a rodent model of hyperoxia-induced BPD. Furthermore, expression of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 was upregulated, and surfactant proteins (SP)-B and –C were downregulated. Unravelling the interaction of these differentially regulated factors may contribute to the knowledge of the pathogenesis of BPD and help to identify new therapeutic targets.

Preterm infants with intrauterine growth retardation (IUGR) have an increased risk for the development of acute and chronic pulmonary disorders as compared to preterm infants who are appropriate for gestational age. In **chapter 6**, the effect of immaturity and IUGR on pulmonary development was investigated in a hypoxia-induced intrauterine growth retardation model in mouse pups. Pups born after hypoxic intrauterine conditions had significantly ($P < 0.001$) lower birth weights and body lengths compared to controls born after normoxic intrauterine conditions. mRNA expression of surfactant protein (SP) -A, -B and C, but not SP-D, could be shown to be significantly reduced in hypoxic fetuses compared to normoxic controls. This implicated that low expression of surfactant associated proteins might be a causal factor for the increased risk of acute and chronic pulmonary disorders in preterm infants with IUGR.

The clinical aspects of BPD are discussed in **Part III**. We studied the association of IUGR with increased neonatal mortality as well as neonatal pulmonary morbidity in a large population-based group of infants born below 32 weeks of gestation. The results of this study are presented and discussed in **chapter 7** and **chapter 8**. We found significantly increased rates of neonatal mortality [OR 4.54, 95% CI 2.56, 8.0] and BPD [OR 3.80, 95% CI 2.11, 6.84] in those children born below 32 weeks of gestation that were small for gestational age compared to those who were appropriate for gestational age.

As mentioned above, inflammatory processes as well as mechanical ventilation play important roles in the pathogenesis of chronic lung disease in preterm infants. In **chapter 9**, we report on a study performed in ventilated preterm infants, who suffered from respiratory distress syndrome. We analyzed pro- (IL-8, elastase and epithelial neutrophil-activating peptide 78) and anti-inflammatory (IL-10) mediators in bronchoalveolar lavage fluid sampled at different time points during ventilatory treatment. There was a strong correlation between the duration of mechanical ventilation and the level of the proinflammatory mediators studied. However, levels of the anti-inflammatory mediator IL-10 did not change during the observation period. These data support the hypothesis that prolonged mechanical ventilation contributes to the development of chronic lung disease by the induction of lung inflammation without adequate stimulation of the counter regulatory cytokine IL-10.

Clinically, BPD can also be associated with increased pulmonary arterial pressure. One of the factors influencing the pressure in the pulmonary vasculature is the nitric oxide (NO) pathway. L-Arginine (ARG) is the physiological substrate of the enzyme-catalyzed synthesis of NO by various NO synthase isoforms. The aim of the study described in **chapter 10** was to determine whether preterm infants with BPD and increased pulmonary artery pressure had a deficiency of plasma ARG and systemic NO synthesis. We found no significant differences in total amino acid concentrations and plasma ARG concentrations between those infants with BPD and increased pulmonary vascular pressure and those without BPD. Urinary nitrate and nitrite concentrations, the excreted end-products of the oxidative metabolic pathway of NO, were not different between groups at day 28, but they were found to be significantly increased in infants with BPD at 36 weeks. It was concluded that there was no deficiency of ARG concentrations and systemic NO synthesis in our cases with BPD.

Surfactant therapy has become the standard therapy for respiratory distress syndrome in premature infants. Nowadays, there is a significant amount of evidence that near-term or term infants, as well as children, with acute respiratory failure, such as in meconium aspiration syndrome or pneumonia, have a profound functional surfactant deficiency. In these infants, inflammatory processes in the lungs may lead to inactivation of surfactant. To overcome this, treatment with exogenous surfactant which is more resistant to inactivation has to be established.

In **Part IV**, **chapter 11**, a randomized multicenter study is described which was conducted to determine whether tracheal application of bovine surfactant improved oxygenation in children with acute respiratory distress syndrome. Improved oxygenation was found in the group that was treated with surfactant, compared to a control group treated with standard therapy. A sustained improvement was only found in the subgroup of patients without pneumonia and in that with an initial PaO_2/FiO_2 ratio above 65.

Both synthetic and natural surfactants have been well documented to improve oxygenation in preterm infants with RDS and acute lung injury in adults. A small proportion of surfactant also enters the circulation in cases of increased permeability of the alveolar-capillary membrane. In **Part V**, **chapter 12**, we investigate the impact of natural and synthetic surfactant (recombinant SP-C surfactant) on inflammatory conditions involving the adhesion of neutrophils to tumor necrosis factor-alpha-activated human umbilical cord vein endothelial cells (HUVECs) in a flow chamber. Using natural pulmonary surfactant in the preincubation media, the number of adherent neutrophils increased by 10-20% at high concentrations of the bovine surfactant compared to controls. This increased neutrophil adhesion was associated with a significant up-regulation of mRNA levels for E-selectin and VCAM-1. Interestingly, synthetic surfactant in the same concentration had no effect on neutrophil adhesion to activated endothelial cells. One might speculate, that natural surfactant but not synthetic surfactant might have pro-inflammatory effects when higher amounts of the exogenous dose reach the circulation.

In **chapter 13** and **chapter 14**, we investigate the effects of the two different surfactant preparations - recombinant SP-C surfactant and natural bovine surfactant - on pulmonary gas exchange, inflammatory response and the pulmonary surfactant system in a piglet model of meconium aspiration syndrome (MAS). Compared to untreated controls with MAS, improvement of gas exchange and lung function was observed after surfactant administration in both surfactant treated groups. mRNA expression of the pro-inflammatory cytokine interleukin (IL)-1β was significantly lower after administration of recombinant SP-C surfactant than in natural surfactant and controls whereas IL-10 mRNA expression was significantly induced in both treatment groups. The exact sequence of the reduced inflammatory response following surfactant is unclear and further studies are mandatory to address potential underlying mechanisms.

Compared with healthy (non-MAS) controls, SP-B mRNA expression was significantly increased in MAS, whereas SP-C mRNA expression was found to be significantly reduced. Surfactant protein concentrations, however, were not significantly different between groups. Although a significant improvement of gas exchange and lung function was observed after surfactant administration in both

surfactant-treated groups, surfactant mRNA expression and protein concentration were not significantly altered.

In **chapter 15**, we describe the effect of positive end expiratory pressure (PEEP) on lung function in an immature newborn rabbit model for respiratory distress syndrome. PEEP largely influenced lung function after administration of recombinant SP-C surfactant, whereas natural surfactant improved lung compliance regardless of the PEEP applied. Thus, in this model, response to recombinant SP-C surfactant depends on the PEEP level applied. This observation has to be considered in treatment strategies with recombinant SP-C surfactants in patients with respiratory distress syndrome. The study presented in **chapter 16** compares the effect of an open lung concept positive pressure ventilation with a conventional ventilation strategy combined with administration of two different surfactant preparations on lung function and surfactant homeostasis in a whole-lung saline-lavaged model. Each treatment strategy showed improvement of gas exchange and lung function, although the improvement of PaO2 and pulmonary compliance declined over the study period in the surfactant groups. Phospholipid and surfactant protein analyses of the bronchoalveolar lavage fluids showed significant alterations in surfactant homeostasis in the open lung concept positive pressure ventilation group as compared to the surfactant groups, whereas IL-10 and SP-C mRNA expression was tendentially increased in the surfactant groups. Long-term consequences of these differences need to be further studied.

In **Part VI**, **chapter 17**, we discuss different aspects of disturbance of late lung development as well as their short and long term consequences. We also focus on aspects of the role of the extracellular matrix in the development of bronchopulmonary dysplasia and chronic obstructive pulmonary diseases and discuss similarities between both diseases, which could have a large impact on future research and treatment strategies.

SAMENVATTING

Respiratoire insufficiëntie is een veel voorkomende probleem bij pasgeborenen en kinderen op neonatale en pediatrische intensive care afdelingen. Longontwikkeling is een proces dat vroeg in de zwangerschap begint en dat tijdens de hele zwangerschap en zelfs na de geboorte doorgaat tot op de jong-volwassen leeftijd. Schade aan de zich ontwikkelende longen of verstoringen van de verschillende stadia van de normale longontwikkeling kunnen leiden tot chronische longziekten die zelfs op de volwassen leeftijd een rol kunnen spelen.

Bronchopulmonale dysplasie (BPD) is een chronische longziekte, waarbij de ontwikkeling van de longen en de longvaten te vroeg stopt. Het komt voor bij ongeveer 20% van alle pasgeborenen die beademd moeten worden. Vaak treedt het op bij prematuur geboren kinderen, maar het kan ook voorkomen bij (bijna) à terme geboren baby's met aangeboren longafwijkingen. De belangrijkste risicofactoren voor BPD zijn kustmatige beademing, zuurstoftoediening en ontstekingsprocessen in de longen, maar ook andere omgevingsfactoren en genetische factoren zouden een rol kunnen spelen. Op dit moment wordt onderzoek gedaan naar deze factoren.

De exacte pathogenese van chronische longziekten in het algemeen en van BPD in het bijzonder is niet bekend. Het identificeren en beschrijven van de mechanismen die tot BPD leiden kan meer inzicht geven in de pathofysiologie van deze aandoening en kan leiden tot de ontwikkeling van preventieve en therapeutische maatregelen, die grote implicaties kunnen hebben voor de lange-termijn prognose.

In **Deel I** van dit proefschrift wordt een overzicht gegeven van het onderwerp. **Hoofdstuk 1** is een introductie en beschrijft de normale en abnormale longontwikkeling en mechanismen die kunnen leiden tot acute en chronische longziekten bij pasgeborenen.

Kinderen die na de geboorte respiratoire insufficiëntie ontwikkelen, of dit nu komt door immaturiteit of door andere aandoeningen, zoals congenitale longhypoplasie, hebben vaak kunstmatige beademing en extra zuurstof nodig om de oxygenatie en ventilatie op peil te houden. Individueel en samen geven deze behandelvormen een verhoogd risico op "ventilator-induced lung injury". In **hoofdstuk 2** worden beademingsstrategieën besproken voor kinderen met congenitale longafwijkingen.

Deel II beschrijft experimentele aspecten van longontwikkeling en BPD. Tijdens de late longontwikkeling verdelen de zich ontwikkelende luchtwegen zich verder in alveoli. Een belangrijke familie van groeifactoren die een rol spelen tijdens de vroege longontwikkeling is de TGF-β/BMP (transforming growth factor-β/bone morphogenetic protein) familie. Of deze familie ook een rol speelt tijdens de late longontwikkeling is onbekend. In **hoofdstuk 3** worden de expressie, weefsellocatie

en activiteit van een aantal onderdelen van de TGF-β pathway tijdens de late longontwikkeling besproken. Wij hebben gevonden dat deze onderdelen dynamisch gereguleerd worden, zowel in muizen en ratten als bij de humane longontwikkeling. Onze resultaten laten zien dat de TGF-β pathway ook een rol speelt tijdens de normale late longontwikkeling in knaagdieren en mensen.

Men neemt aan dat schade door hyperoxie belangrijke pathways in de longontwikkeling verstoort, wat kan leiden tot BPD. In **hoofdstuk 4** worden de effecten van 21% of 85% O_2 op de signaling van de TGF-β/BMP superfamilie in de longen van neonatale C57BL/6J muizen beschreven. In pups die blootgesteld waren aan 85% zuurstof waren de lichaamsgroei en de longfunctie significant verminderd. We zagen dat de longontwikkeling stopte en dat er een dysregulatie was van de expressie en de lokalisatie van TGF-receptoren en hun downstream Smad-proteïnen. De TGF-β signaling was versterkt en de BMP signaling was verminderd, zowel in de longen van pups blootgesteld aan hyperoxie als in geïsoleerde alveolaire type II cellen van muizen en in humane primaire longfibroblasten. Na blootstelling aan 85% O_2 vertoonden de alveolaire type-II-cellen meer TGF-β-geïnduceerde apoptose, terwijl primaire gladde spiercellen uit de longarteriën geen verandering lieten zien. In primaire longfibroblasten die blootgesteld waren aan hyperoxie was de TGF-β-gestimuleerde aanmaak van de α1-subunit van type I collageen, tissue inhibitor of metalloproteïnase-1, tropoelastine en tenascin-C versterkt. Dit betekent dat hyperoxie significante veranderingen veroorzaakt in de TGF-β/BMP signaling in de long. Hierbij kan ook de late longontwikkeling beïnvloed worden, die verstoord is bij BPD.

Angiogene factoren, cytokinen en het NO (stikstofoxide)-systeem hebben mogelijk ook een functie in de pathogenese van BPD en spelen een belangrijke rol tijdens de late longontwikkeling. In **hoofdstuk 5** laten we zien dat er een duidelijke dysregulatie is van de expressie van zuurstofgerelateerde factoren (HIF1-α en HIF2-α), angiogene factoren (VEGF en zijn receptor Flt-1) en van componenten van de NO-biosynthese pathway (NOS-2, NOS-3, DDAH) in een muismodel van hyperoxie-geïnduceerde BPD. Daarnaast laten we zien dat de expressie van de pro-inflammatoire cytokines tumor necrosis factor-α (TNF-α) en interleukine (IL)-6 verhoogd is en dat de expressie van de surfactant proteïnen (SP)-B en -C verlaagd is. Het bestuderen van de interacties tussen deze differentieel-gereguleerde factoren kan bijdragen aan de kennis over de pathogenese van BPD en kan helpen bij het vinden van nieuwe therapeutische mogelijkheden en aangrijpingspunten.

Prematuur geboren kinderen met intra-uteriene groeiretardatie (IUGR) hebben een verhoogd risico op acute en chronische longziekten vergeleken met premature kinderen die geen groeivertraging hebben. In **hoofdstuk 6** hebben we het effect onderzocht van immaturiteit en IUGR op de longontwikkeling in een muismodel van hypoxie-geïnduceerde IUGR. Pups die geboren werden na intra-uteriene hypoxie hadden een significant lager geboortegewicht waren beduidend kleiner dan

controledieren geboren na intra-uteriene normoxie. De mRNA expressie van SP-A, -B en -C, maar niet van SP-D, was significant verlaagd in hypoxische foetussen vergeleken met normoxische controles. Dit betekent dat een lage expressie van surfactant-geassocieerde proteïnen mogelijk een oorzaak is voor het verhoogde risico op acute en chronische longziekten bij premature kinderen met IUGR.

De klinische aspecten van BPD worden besproken in **Deel III**. Wij hebben de relatie onderzocht tussen IUGR en verhoogde neonatale sterfte en neonatale longaandoeningen in een grote groep pasgeborenen, geboren na minder dan 32 weken zwangerschapsduur. De resultaten hiervan worden gepresenteerd en besproken in **hoofdstuk 7** en **8**. We hebben gevonden dat kinderen geboren na minder dan 32 weken zwangerschapsduur die small for gestational age (klein voor de zwangerschapsduur) waren een significant verhoogde neonatale sterfte hadden [OR 4.54, 95% CI 2.56, 8.0] en significant meer BPD [OR 3.80, 95% CI 2.11, 6.84] dan kinderen van minder dan 32 weken die een normaal gewicht voor de zwangerschapsduur hadden.

Zoals hierboven al genoemd is, spelen zowel ontstekingsprocessen als kunstmatige beademing een belangrijke rol in het ontstaan van chronische longziekten bij prematuur geboren kinderen. In **hoofdstuk 9** beschrijven we een studie uitgevoerd bij beademde prematuren, die respiratory distress syndrome (RDS) hadden. We hebben pro- (IL-8, elastase en epithelial neutrophil-activating peptide 78) en anti-inflammatoire (IL-10) factoren geanalyseerd in vloeistof van bronchoalveolaire lavages, afgenomen op verschillende momenten tijdens de beademingsperiode. Er was een sterke correlatie tussen de duur van de beademing en de hoeveelheid pro-inflammatoire factoren. De hoeveelheid anti-inflammatoire factor (IL-10) veranderde echter niet tijdens de periode van observatie. Onze data ondersteunen de hypothese dat langdurige kunstmatige beademing bijdraagt aan de ontwikkeling van chronische longziekten doordat het ontstekingsprocessen in de long induceert zonder dat er een adequate stimulatie optreedt van de anti-inflammatoire tegenhanger IL-10.

BPD kan klinisch samengaan met een verhoogde druk in de longarteriën. Een van de factoren die de druk in de longvaten beïnvloeden is de NO (stikstofoxide) pathway. L-Arginine (ARG) is het fysiologische substraat voor de enzym-gekatalyseerde synthese van NO door verschillende NO-synthase isovormen. Het doel van de studie die in **hoofdstuk 10** beschreven wordt was te bepalen of prematuur geboren kinderen met BPD en een verhoogde pulmonale arteriële druk een deficiëntie hadden van plasma ARG en van de systemische NO-synthese. We hebben geen significante verschillen gevonden in de totale aminozuurconcentratie en in plasma ARG concentratie tussen kinderen met BPD en een verhoogde longvaatdruk en kinderen zonder BPD. De urineconcentraties van nitraat en nitriet, de eindproducten van de oxidatieve metabole pathway van NO, verschilden niet tussen de groepen op dag 28, maar waren wel significant verhoogd bij kinderen met

BPD op de leeftijd van 36 weken postconceptioneel. Uit deze resultaten concluderen wij dat er geen deficiëntie was van ARG en systemische NO synthese in onze patiënten met BPD.

Behandeling met surfactant is nu standaard therapie voor RDS bij prematuur geboren kinderen. Er is veel bewijs dat (bijna-) à terme pasgeborenen en ook andere kinderen met acuut respiratoir falen, bijvoorbeeld bij meconiumaspiratie-syndroom of pneumonie, een ernstige functionele surfactantdeficiëntie hebben. Ontstekingsprocessen in de long kunnen bij deze kinderen tot surfactantinactivatie leiden. Om dit te behandelen moet therapie met exogeen surfactant dat minder geïnactiveerd wordt gegeven worden.

In **Deel IV**, **hoofdstuk 11**, beschrijven wij een gerandomiseerde multicenter studie die gedaan is om te bepalen of behandeling met bovien surfactant intratracheaal de oxygenatie bij kinderen met acuut RDS verbetert. We zagen een verbeterde oxygenatie in de groep die behandeld was met surfactant vergeleken met een controlegroep, behandeld met standaardtherapie. Een blijvende verbetering trad alleen op in de subgroep van patiënten zonder pneumonie en in de subgroep met een initiële PaO_2/FiO_2 ratio boven de 65.

Zowel synthetisch als natuurlijk surfactant verbeteren de oxygenatie bij prematuren met RDS en bij volwassenen met acute longschade. Een klein deel van het surfactant kan in de circulatie terecht komen als de permeabiliteit van het alveolo-capillaire membraan verhoogd is. In **Deel V**, **hoofdstuk 12**, hebben we het effect onderzocht van natuurlijk en synthetisch surfactant (recombinant SP-C surfactant) op ontstekingsreacties waarbij er adhesie van neutrofielen optreedt aan tumor necrosis factor-α–geactiveerde humane endotheelcellen uit navelstrengvenen (HUVECs) in een flow chamber. Na toevoeging van hoge concentraties bovien surfactant aan de preïncubatie media waren er 10 - 20% meer adherente neutrofielen vergeleken met controles. Deze versterkte neutrofiel-adhesie ging gepaard met een significante verhoging van het mRNA van E-selectine en VCAM-1. Synthetisch surfactant in dezelfde concentratie had geen effect op de adhesie van neutrofielen aan geactiveerde endotheelcellen. Het zou kunnen dat natuurlijk surfactant, in tegenstelling tot synthetisch surfactant, pro-inflammatoire effecten heeft wanneer grotere hoeveelheden van de exogene dosis de circulatie bereiken.

In **hoofdstuk 13** en **hoofdstuk 14** onderzoeken we de effecten van twee verschillende surfactants – recombinant SP-C surfactant en natuurlijk bovien surfactant – op de gaswisseling in de long, de inflammatoire respons en het pulmonale surfactant systeem in een model van meconiumaspiratiesyndroom (MAS) in biggen. We zagen een verbetering van de gaswisseling en de longfunctie na toediening van surfactant in beide met surfactant behandelde groepen vergeleken met onbehandelde controles met MAS. De mRNA expressie van het pro-inflammatoire cytokine interleukine (IL)-1β was significant lager na toediening van

recombinant SP-C surfactant dan bij natuurlijk surfactant en controles, terwijl de mRNA expressie van IL-10 significant verhoogd was in beide behandelde groepen. Het exacte mechanisme van de verminderde inflammatoire respons na toediening van surfactant is nog onduidelijk.

De SP-B mRNA expressie was significant verhoogd in de MAS-groep vergeleken met gezonde controles zonder MAS, terwijl de SP-C mRNA expressie significant verlaagd was. Er was geen significant verschil tussen de groepen in surfactant proteïne-concentraties. Hoewel er een significante verbetering van de gaswisseling en de longfunctie na toediening van surfactant in beide behandelde groepen was, waren er geen significante veranderingen in surfactant mRNA expressie en proteïneconcentratie.

In **hoofdstuk 15** beschrijven wij het effect van positieve end expiratory pressure (PEEP) op de longfunctie in een model van RDS in immature konijnen. PEEP had een grote invloed op de longfunctie na toediening van recombinant SP-C surfactant, terwijl natuurlijk surfactant onafhankelijk van de gegeven PEEP de longcompliance verbeterde. In dit model hangt de respons op recombinant SP-C surfactant dus af van de PEEP die gegeven wordt. Hiermee moet rekening worden gehouden bij de behandeling van patiënten met RDS met recombinante SP-C surfactant. De studie die in **hoofdstuk 16** beschreven wordt, vergelijkt het effect van een "open lung concept" met dat van conventionele beademing gecombineerd met de toediening van twee verschillende surfactants op de longfunctie en de surfactant homeostase in een diermodel waarin surfactantdeficiëntie geïnduceerd werd door longspoelingen met zout. Alle behandelmethoden gaven een verbetering van de gaswisseling en de longfunctie, hoewel de verbetering van PaO_2 en longcompliance in de surfactant groepen afnamen over de studieperiode. Analyse van de fosfolipiden en de surfactant proteïnen in de bronchoalveolaire lavagevloeistof liet significante veranderingen zien in de surfactant homeostase in de "open lung concept" groep vergeleken met de surfactant groepen, terwijl IL-10 en SP-C mRNA een tendens naar verhoging lieten zien in de surfactant groepen. De lange termijn consequenties van de gevonden verschillen moeten nog verder bestudeerd worden.

In **Deel VI**, **hoofdstuk 17**, bespreken we verschillende aspecten van verstoring van de late longontwikkeling en de gevolgen hiervan op de korte en lange termijn. We richten ons ook op aspecten van de rol van de extracellulaire matrix in de ontwikkeling van bronchopulmonale dysplasie en COPD (chronic obstructive pulmonary disease) en we bespreken overeenkomsten tussen de beide ziektebeelden, die van grote invloed zouden kunnen zijn op toekomstig onderzoek en behandelingsstrategieën.

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Dani, thank you for our two beautiful children and for your support in Lübeck and Giessen. I wish you all the best for the future.

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Hannah and Joshi, I know things are not always easy for all of us. You give me strength and I promise I will always be there for you. I love you.

Curriculum vitae

Irwin Reiss was born on the 19th of October, 1963, in Lübeck, Germany. He studied medicine at the Medical-University of Lübeck from 1983 until 1989 and spent one year doing research in the department of internal medicine at the Ludwig-Maximilian-University of Munich, which led to his German thesis (Doktorarbeit). He followed his pediatric training at the university of Lübeck, Germany, from 1991 to 1997 and finished his specialization in neonatology and pediatric intensive care in 1997. He received his doctorate magna cum laude from the medical university of Lübeck in 1994. In 1998 he became a senior physician (Oberarzt) in the department of pediatrics and neonatology of the Justus-Liebig-University of Giessen, Germany, where he worked until 2005. During this period he was doing research in the field of bronchopulmonary dysplasia and surfactant treatment of acute respiratory failure in neonates. In January 2005 he received his postdoctoral lecture qualification (Habilitation) from this university. From November 2005, he has been working as a member of staff and senior investigator at the pediatric surgical intensive care unit of the Erasmus MC – Sophia Children's Hospital in Rotterdam. The work presented in this thesis is part of his ongoing research.
He has two children, Hannah and Joshua, and lives together with Janine Felix.

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Appendices

APPENDIX A

Different therapeutic perspectives for novel exogenous surfactant preparations*

Surfactant replacement therapy has been established for years as one of the most important therapeutic interventions in the management of premature infants with respiratory distress syndrome. Recently, researchers in the field have focused on evaluating and comparing not only different exogenous surfactant compositions but also their application in disease states other than the respiratory distress syndrome, which could involve a secondary deficiency of surfactant (1). Moreover, controversy over the advantages of natural vs. synthetic surfactants has led to the synthesis of newer surfactants both synthetic and human recombinant. Although not yet disseminated, the emergence of new recombinant formulations of surfactant is promising and could provide benefits over traditional synthetic surfactants and natural surfactants.

Investigators have evaluated different situations in which surfactant therapy can be beneficial, and several trials have evaluated the use of exogenous surfactant in meconium aspiration syndrome (MAS). MAS is the most common cause of hypoxemic respiratory failure in full-term or near-term newborn infants and together with persistent pulmonary hypertension has been the most frequent indication for extracorporeal membrane oxygenation.

Various pathophysiolologic mechanisms are involved in the development of MAS, including mechanical airway obstruction, release of vasoconstrictive and inflammatory mediators, inflammatory cell infiltration, proteinaceous exudates, and surfactant dysfunction and inactivation (2). The most severe cases of MAS are usually part of a multiple-system disorder including myocardial involvement, hemodynamic failure, and severe pulmonary hypertension.

Studies in animal models of MAS have pointed toward an improvement in mechanical properties of the respiratory system, gaseous exchange, and lung histology, after surfactant (3, 4). Additionally, randomized controlled trials have also shown improvement in oxygenation and respiratory morbidity (5) and a decreased need for extracorporeal membrane oxygenation (6) after surfactant replacement therapy. However, results are still inconclusive, and there is much more to learn regarding this newer indication for exogenous surfactant. Besides the need for stronger evidence on efficacy and safety, information on other aspects such as dosage, response to different surfactant preparations, and ways of administration is also missing.

In this issue of *Critical Care Medicine*, Dr. Hilgendorff and colleagues (7) present an animal trial focused on the effect of exogenous surfactant on an experimental model of MAS in piglets. The authors not only look for the efficacy of this strategy but also compare two different surfactant preparations, namely Venticute®, a recombinant human surfactant protein C (rSP-C)-based surfactant, and Alveofac®, a calf lung lavage natural surfactant containing both SP-C and SP-B. Previous evidence from this group and other researchers suggested an enhanced resistance to inactivation by meconium with the inclusion of hydrophobic surfactant proteins (4, 8). In this study, investigators looked into the possible mechanisms of action of these compounds by evaluating both surfactant protein synthesis and morphologic differences on electron microscopy.

Twenty-three newborn piglets were randomized after intubation and induction of MAS to one of three groups receiving intratracheal saline, rSP-C surfactant, or natural surfactant. Surfactant-treated animals showed a significant improvement over saline controls in measurements of gaseous exchange and lung function. Despite a trend in favor of rSP-C surfactant, the small number of animals included precludes any conclusion about potential differences between both surfactant preparations.

Electron microscopy showed differences between the MAS control group and rSP-C surfactant group in regard to neutrophil infiltration and alveolar septa thickening as well as cytoplasmic changes in alveolar epithelial cells that were prominent in the former and absent in the latter. No reference is apparent in the article about the evaluation of ultrastructural differences between both surfactant groups. To evaluate treatment effects on surfactant protein synthesis, both SP-B and SP-C messenger RNA and protein content in lung homogenate were measured by polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

To evaluate the effects of MAS over protein expression, results from the three experimental groups were referenced to another group of healthy term piglets provided by one of the authors. MAS plus mechanical ventilation significantly increased SP-B messenger RNA expression and decreased SP-C m-RNA compared with the reference healthy term piglets. No significant differences were seen in any of the treatment groups compared with the MAS controls. As for SP-B and SP-C content in lung homogenate, no significant difference could be found both after the induction of lung injury or after the treatment with any of the surfactant preparations. The relevance and potential implications of these findings remain unclear.

In summary, the authors have established an animal model of MAS to evaluate the response to two different surfactant preparations and attempt, at least partially, to explain its effects by investigating both surfactant protein synthesis and ultrastructural morphology. The negative results regarding SP-B and SP-C synthesis could be attributed to a failure

of the model or experimental setting or simply to a lack of that specific action. Other ways of reversing surfactant inactivation by meconium have been proposed (9, 10). Hypothetically surfactant proteins could act by a similar mechanistic effect.

It is worth noting that the investigators delivered surfactant by bolus administration as opposed to the strategy of surfactant lavage advocated by some investigators for MAS and recently questioned (11, 12). Although this is well substantiated, other investigators have found better results with administration by continuous infusion (3).

As stated, MAS is, especially in its most severe presentations, a multiple-system disease, and practitioners should remain cautious when interpreting animal model studies. The search for a synthetic surfactant that can compare with the natural surfactants available is ongoing. This happens mainly because of the concern about the potential antigenic and infectious complications that could be associated with animal products and because of the potential to reduce manufacturing costs and availability. These newer synthetic preparations attempt to overcome the superiority shown by natural surfactants by incorporating functional synthetic apoproteins, both recombinant, as in the preparation used in this study as totally synthetic as sinapultide, a polypeptide mimicking SP-B.

This article provides new evidence on the effect of a novel surfactant formulation on a neonatal animal model of MAS. It also could motivate the realization of adequately powered experiments to evaluate differential effects on lung function and gas exchange and either confirm or refute the trends reported in this setting. More questions also remain on the differential effects of the absence of SP-B and the different concentrations of SP-C between different preparations of surfactant both in MAS and in other pathologic entities.

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APPENDIX B

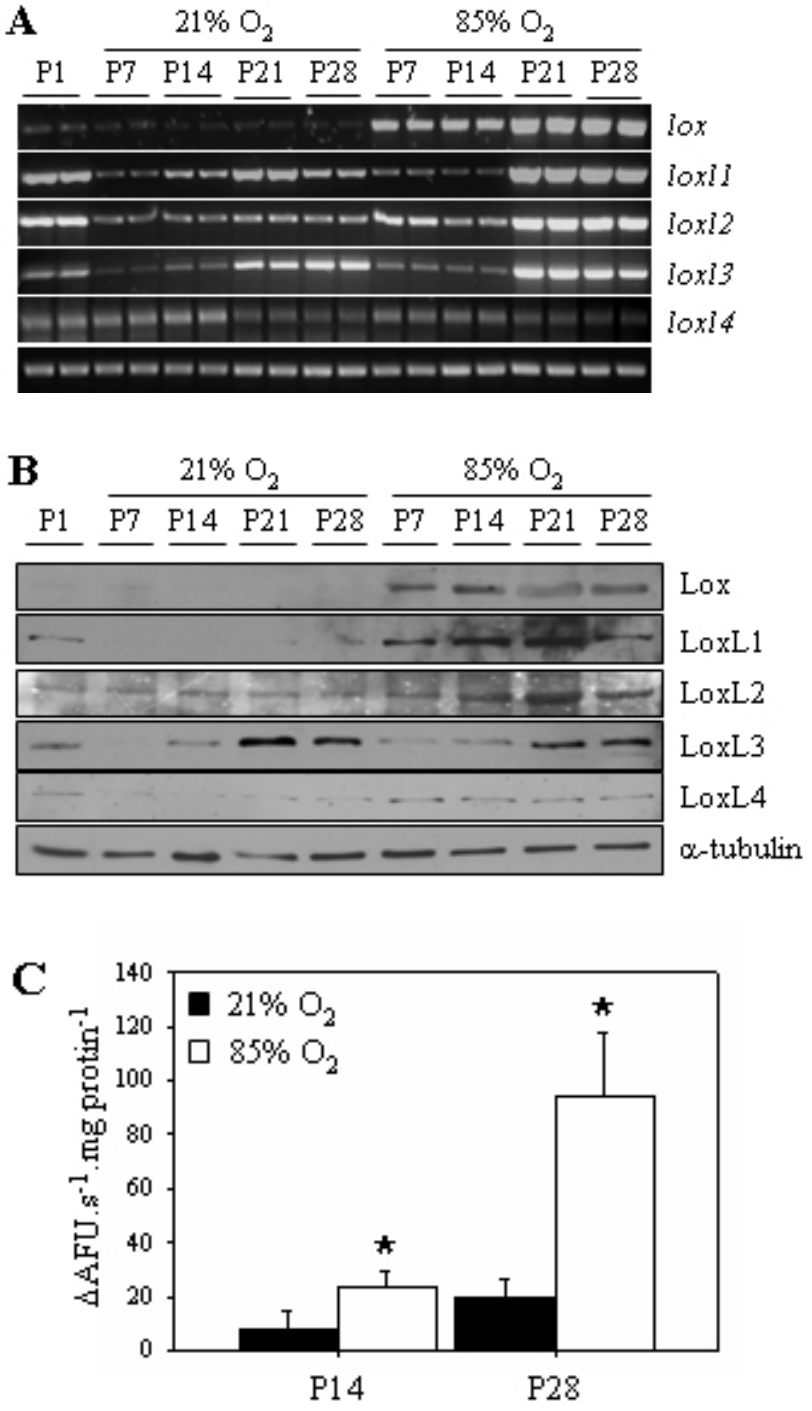

Figure 1 *Assessment of lysyl oxidase expression and activity in the lungs of mice exposed to normoxia (21% O2) or chronic hyperoxia (85% O2).* ***A.*** *Lysyl oxidase expression was assessed by reverse-transcription polymerase chain reaction (RT-PCR) in mRNA isolated from the lungs of mice exposed to different gas mixtures from day P1, at post-natal days P1, P7, P14, P21 and P28. Expression of the constitutively-expressed hspa8 gene served as a loading control.* ***B.*** *Lysyl oxidase expression was assessed by immunoblot in protein extracts from lungs from mice exposed to different gas mixtures from day P1, at post-natal days P1, P7, P14, P21 and P28. Expression of the constitutively-expressed α-tubulin served as a loading control.* ***C.*** *Assessment of lung lysyl oxidase activity by the Amplex Red assay, in the lungs of mice at post-natal days P14 and P28. P < 0.05, by ANOVA with Neuman-Keul modification.*

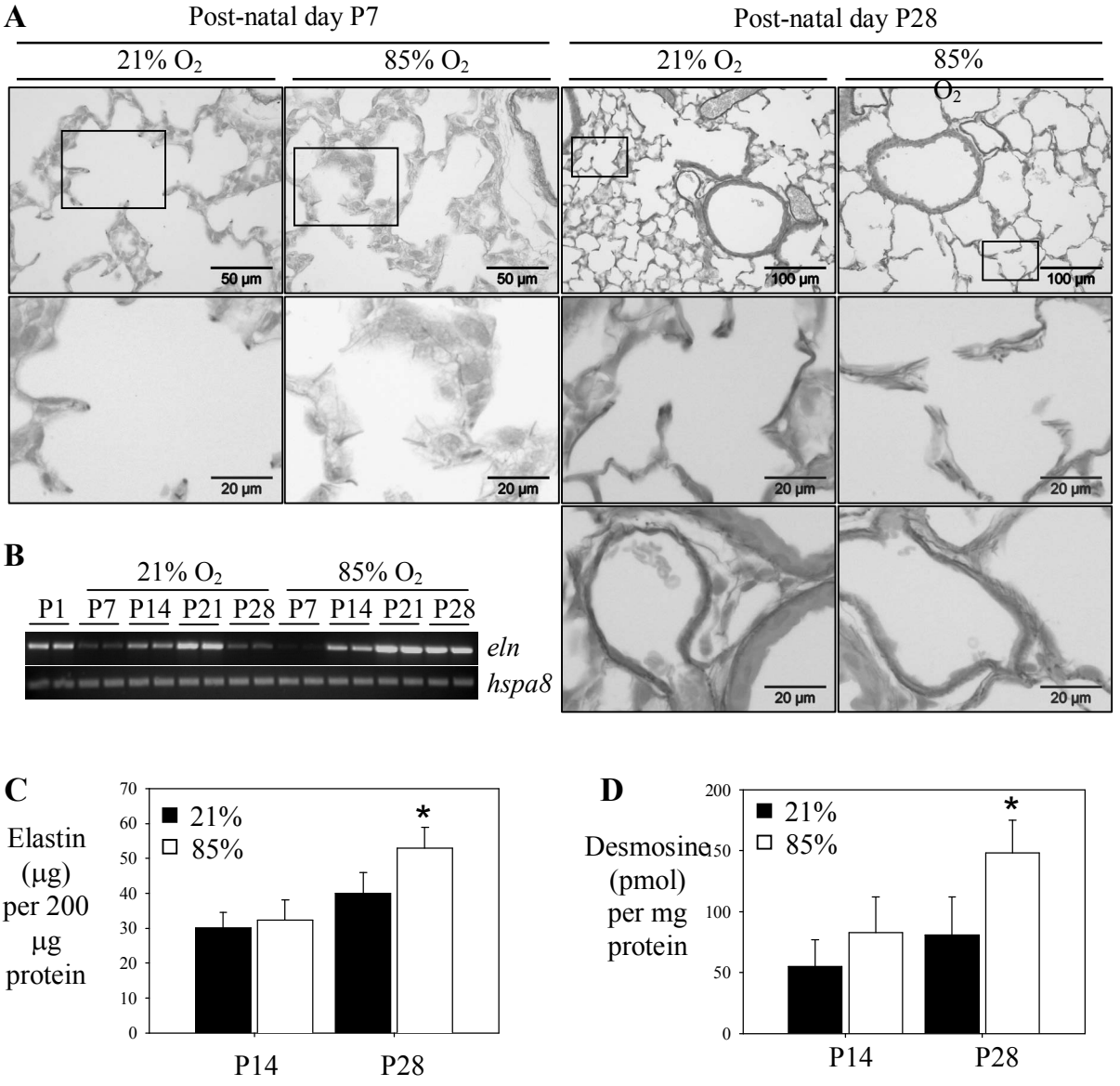

Figure 2 *Assessment of elastin expression in the lungs of mice exposed to normoxia (21% O2) or chronic hyperoxia (85% O2).* ***A.*** *Elastin expression was assessed by immunohistochemical analysis in paraffin-embedded lung sections, by Hart's elastin stain. Neonatal mice were exposed to different gas mixtures from post-natal day P1.* ***B.*** *Assessment of lung elatistin mRNA levels by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) at post-natal days P1, P7, P14, P21 and P28. Expression of the constitutively-expressed hspa8 gene served as a loading control.* ***C.*** *Assessment of lung elastin content by dye-binding assay (FASTINTM elastin assay; BioColor) in mice at post-natal days P14 and P28.* ***D.*** *Assessment of lung desmosine content at post-natal days P14 and P28. P < 0.05, by ANOVA with Neuman-Keul modification.*

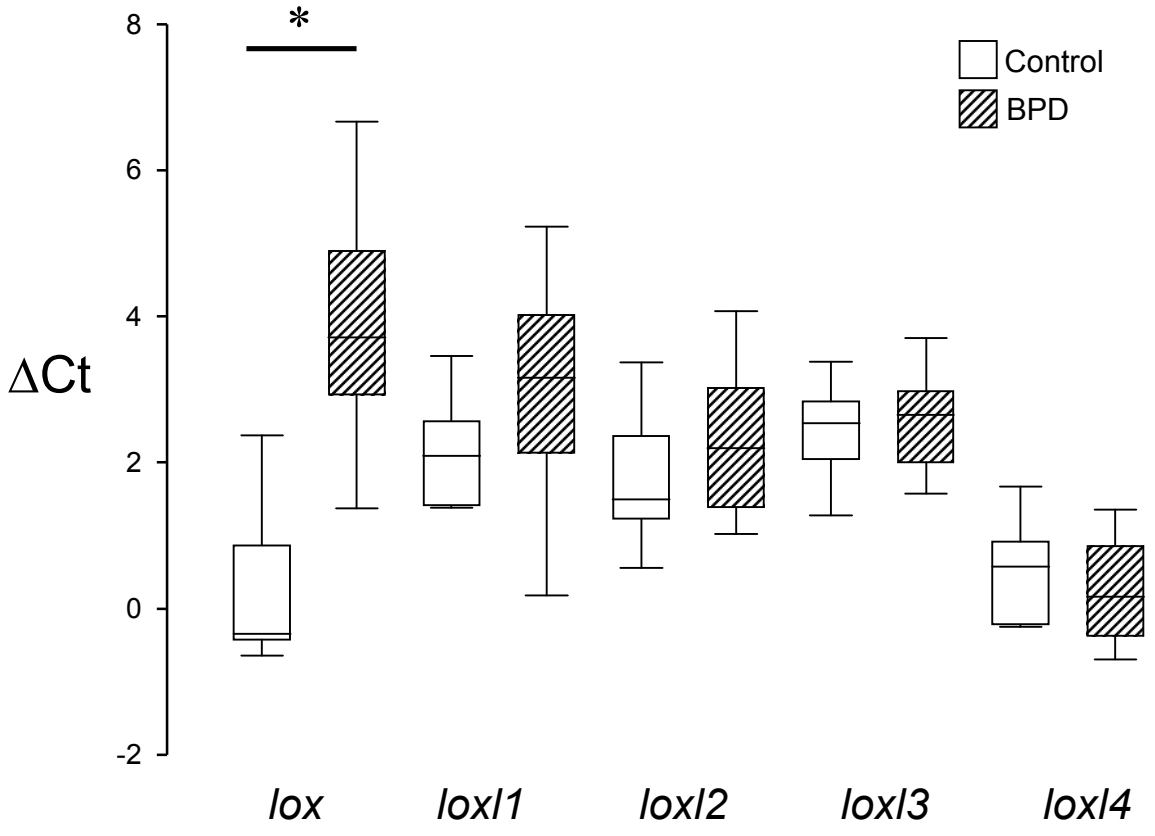

Figure 3 *Assessment of lysyl oxidase gene expression in the lungs of neonates who died from non-pulmonary causes (open bars) or neonates who died with BPD (dashed boxes). The bars represent the data range, while the boxes represent lower and upper quartiles. The line within the quartile box indicates the mean (n = 5, per group). *, P < 0.05, by ANOVA with Neuman-Keul modification.*

APPENDIX C

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Synthetic and natural surfactant differentially modulate inflammation after meconium aspiration

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Due to an unfortunate error, Table 2 was misprinted. The corrected table is printed below.

Table 2 Cytokine mRNA expression in superior and inferior left lung lobes and in inferior left lung lobe; normalization to HPRT (*H*) and β-actin (*A*) and further to mRNA expression in control subjects. Data given as geometric mean and dispersion factor

	Superior, inferior			Inferior		
	Control (*n*=3)	rSP-C Surf (*n*=5)	nat SF (*n*=4)	Control (*n*=3)	rSP-C Surf (*n*=5)	nat SF (*n*=4)
IL-1β/H	1±0.19	0.17±0.30**[b]	0.77±0.05	1±0.41	0.08±0.37*[b]	1.76±0.14
IL-1β/A	1±0.33	0.19±0.27**[a]	0.63±0.1	1±0.75	0.14±0.35[a]	1.28±0.10
IL-6/H	1±0.19	1.92±0.29	4.61±0.09	1±0.35	0.74±0.27[b]	17.3±0.27
IL-6/A	1±0.33	2.11±0.38	3.78±0.2	1±0.51	1.31±0.35[a]	12.6±0.21
IL-8/H	1±0.14	0.35±0.30	1.34±0.07	1±0.41	0.14±0.35	1.49±0.06
IL-8/A	1±0.22	0.38±0.16	1.1±0.11	1±0.70	0.25±0.20	1.08±0.04
IL-10/H	1±0.35	3.19±0.47*	3.61±0.28**	1±0.31	2.23±0.46	3.94±0.46
IL-10/A	1±0.56	3.5±0.54***	2.96±0.8***	1±0.48	3.96±0.48*	2.87±0.88
TGF-β/H	1±0.21	0.88±0.4	2.52±0.23	1±0.28	0.62±0.36	1.75±0.59
TGF-β/A	1±0.42	0.97±0.24	2.06±0.58	1±0.55	1.19±0.41	1.28±0.47

*$p<0.05$; **$p<0.02$; ***$p<0.01$ compared to controls
[a] $p<0.05$; [b] $p<0.02$ compared to natural bovine surfactant (nat SF)