Growth Factors in the Human Preterm Lung

VEGF AND HGF IN PULMONARY DEVELOPMENT AND IN ACUTE AND CHRONIC LUNG INJURY

Patrik Lassus

Hospital for Children and Adolescents
and
Department of Plastic Surgery
Helsinki University Central Hospital
University of Helsinki

Academic Dissertation

To be publicly discussed, with the permission of the Faculty of Medicine
of the University of Helsinki, in the Niilo Hallman Auditorium
of the Hospital for Children and Adolescents,
on 2 December, 2002, at 12 noon.

Helsinki 2002

SUPERVISED BY

Professor Sture Andersson M.D., Ph.D.
Hospital for Children and Adolescents
Helsinki University Central Hospital
Helsinki, Finland

REVIEWED BY

Professor Kari Alitalo M.D. Ph.D.
Molecular/Cancer Biology Laboratory, Haartman Institute
University of Helsinki
Helsinki, Finland

Professor Steven H. Abman M.D. Ph.D.
Department of Pediatrics
University of Colorado School of Medicine
Denver, U.S.A.

OFFICIAL OPPONENT

Professor Peter Carmeliet M.D. Ph.D.
Center for Transgene Technology and Gene Therapy
Catholic University Leuven
Leuven, Belgium

ISBN 952-91-5350-3 (nid.)
ISBN 952-10-9818-0 (PDF)
http://ethesis.helsinki.fi

Helsinki 2002
Yliopistopaino

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List of original publications

This thesis is based on the following original publications which are referred to in the text by their Roman numerals.

I Lassus P, Ristimaki A, Ylikorkala O, Viinikka L, Andersson S. Vascular endothelial growth factor in human preterm lung. Am J Respir Crit Care Med 1999;159:1429-33.

II Lassus P, Turanlahti M, Heikkilä P, Andersson L, Nupponen I, Sarnesto A, Andersson S. Pulmonary Vascular Endothelial Growth Factor and Flt-1 in fetuses, in Acute and Chronic Lung Disease, and in Persistent Pulmonary Hypertension of the Newborn. Am J Respir Crit Care Med 2001;164:1981-7.

III Lassus P, Heikkilä P, Andersson L, von Boguslawski K, Andersson S. Lower pulmonary hepatocyte growth factor is associated with more severe lung disease in preterm infants. Submitted in 2002.

IV Lassus P, Nupponen I, Kari A, Pohjavuori M, Andersson S. Early postnatal dexamethasone decreases hepatocyte growth factor in tracheal aspirate fluid from premature infants. Pediatrics 2002;110:768-771.

Abbreviations

ARDS	Adult respiratory distress syndrome
ANOVA	Analysis of one-way variance
BPD	Bronchopulmonary dysplasia
ELISA	Enzyme-linked immunoassay
Flt-1 / VEGFR1	Fms-like tyrosine kinase-1 / VEGF receptor-1
Flt-4 / VEGFR3	Fms-like tyrosine kinase-4 / VEGF receptor-3
Flk-1 / KDR / VEGFR2	Fetal liver kinase-1 / Kinase domain region / VEGF receptor-2
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible-factor
IgA-SC	Secretory component of immunoglobulin-A
LS-ratio	Lecithin/sphingomyelin ratio
PDA	Patent ductus arteriosus
PlGF	Placental growth factor
PPHN	Persistent pulmonary hypertension of the newborn
RDS	Respiratory distress syndrome
sFlt-1	Soluble fms-like tyrosine kinase-1
TAF	Tracheal aspirate fluid
VEGF	Vascular endothelial growth factor

Abstract

The aims of the present study were to evaluate the roles of two distinct growth factors - vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) - during the perinatal period and in acute and chronic lung injury in preterm infants. In addition, we measured the effect of early postnatal dexamethasone on concentrations of VEGF and HGF in lung lining fluid in preterm infants.

The patient population comprised selected preterm infants treated in the neonatal intensive care unit of the Hospital for Children and Adolescents of Helsinki University Central Hospital in Helsinki, Finland, between August 1993 and July 1999. In Study IV, preterm infants were randomized to receive either dexamethasone or to serve as controls. Tracheal aspirate fluid (TAF) samples were collected from preterm infants during the early postnatal period by standardised tracheal lavage. Concentrations of VEGF and HGF in TAF were analyzed by commercial VEGF and HGF immunoassays, and the results were related to concentrations of the secretory component of immunoglobulin-A. Subjects for immunohistochemistry studies were collected between 1985 and 1999. Immunohistochemistry stainings for VEGF and its receptor Flt-1 were performed on lung samples obtained at autopsy. All studies were performed in the Scientific Laboratory in the Hospital for Children and Adolescents, and in the Haartman Institute Laboratory, University of Helsinki.

For VEGF, we found that, in preterm infants during the early postnatal period, its postnatal concentrations in TAF increased constantly. Preterm infants had higher VEGF in TAF than did term infants. In immunohistochemistry, staining for VEGF appeared in all fetuses and infants in bronchial epithelium and alveolar macrophages, and additionally, in fetuses and preterm infants also in alveolar epithelium. For Flt-1, we found positive staining in endothelial cells lining capillaries, veins, and small arteries, as well as in bronchial epithelial cells. Preterm infants with more severe respiratory distress syndrome (RDS), as well as those subsequently developing bronchopulmonary dysplasia (BPD), had lower VEGF in TAF during the early postnatal period. In BPD infants, additional staining was discovered for VEGF and Flt-1 in type-II cells in alveolar epithelium. For HGF, a negative correlation was evident in preterm infants between gestational age and HGF levels in TAF. Preterm infants with more severe RDS had lower HGF concentrations in TAF. Moreover, those infants who developed BPD had less HGF in TAF than did those who survived without BPD. We detected no differences in VEGF levels in TAF in preterm infants receiving dexamethasone or not. However, infants receiving dexamethasone had lower HGF levels in TAF during the early postnatal period.

We therefore conclude that the consistent perinatal pulmonary expression of VEGF and Flt-1, the higher VEGF in TAF in the more immature infants, and the postnatal increase in VEGF in TAF all indicate a physiological role for VEGF in the developing human lung. Since infants with more severe RDS and those subsequently developing BPD had lower VEGF in TAF, we suggest that in the preterm infant ,VEGF plays a role in protection against or in recovery from acute lung injury and that VEGF may have beneficial effects in preventing development of BPD. Lower HGF in infants with more severe RDS and in those subsequently developing BPD may indicate a protective or regenerative role for HGF. The suppressive effects of glucocorticoids on lung development may in part be mediated by reduction in pulmonary HGF.

Introduction

In neonatology, premature birth presents a continuing challenge. Improvements in treatment, including use of supplemental oxygen, advances in mechanical ventilation, surfactant therapy, and antenatal glucocorticoid treatment, have resulted in dramatic improvement in mortality rates. The more immature infants - even as small as birth weight 280 g - have been able to survive (Muraskas et al 1991). A significant decrease has occurred in the incidence of BPD in infants with birth weight over 1500 g. However, the overall incidence of BPD has increased, due to the increase in survival of extremely low birth-weight infants (Parker et al 1992). At present, the mortality rate for the most immature premature infants of less than 1000 g is 35%, and of the survivors, 30% develop BPD - chronic lung injury. It is now the most immature infants that are developing BPD, infants with birth weights between 500 and 1000 g and who are born at 24 to 28 weeks of gestation (Stevenson et al 1998).

In addition to changes in its epidemiology, there has also been a change in the pathophysiology of BPD. Recent findings in the lungs of very premature infants who develop fatal BPD include less airway epithelial disease, and only varying degrees of interstitial fibrosis. Autopsy findings in infants with fatal BPD include a persistence of simple terminal air spaces, consistent lack of significant alveolarization, and dysmorphic pattern of vascular organization which together result in emphysematous-appearing lungs (Chambers et al 1989, Hislop et al 1990, Van Lierde et al 1991, Margraf et al 1991, Husain et al 1998, Bhatt et al 2001). A newborn of 24 gestational weeks will have severe pulmonary prematurity: no alveoli will yet be present, surfactant production will just be starting, and the capillary bed will be poorly developed. Premature birth interrupts normal alveolar development. Pathogenesis of new BPD in very immature preterm infants may therefore result primarily from arrest in normal lung development; in this developmental disturbance, inhibition of capillary growth and defects in alveolarization may also play essential roles (Jobe AH 1999, Abman 2001).

VEGF and HGF are each known to play a significant fetal and postnatal developmental role, VEGF in vascular and HGF in epithelial development. In addition, both are believed to participate in repair of lung injury in neonatal animals. Because development of BPD may result from developmental arrest, we chose to evaluate the roles of VEGF and HGF during the perinatal period and in lung injury in preterm infants.

Review of the literature

1. Normal lung development (Fig. 1)

Development of the human lung starts as the appearance of the tracheal bud in the developing embryo and ends during early childhood. Histologically, lung development has been divided into five distinct overlapping stages, based primarily on epithelial processes. The lung vasculature develops parallel to the respiratory tract development. In a prematurely born infant, the early start of respiration may speed functional maturation of the lung; however, the effect on the lung tissue framework of replacement of lung fluid by air has still not been studied in detail (reviewed in McDonald 1997, in Bland et al 2000, and in Haddad et al 2002).

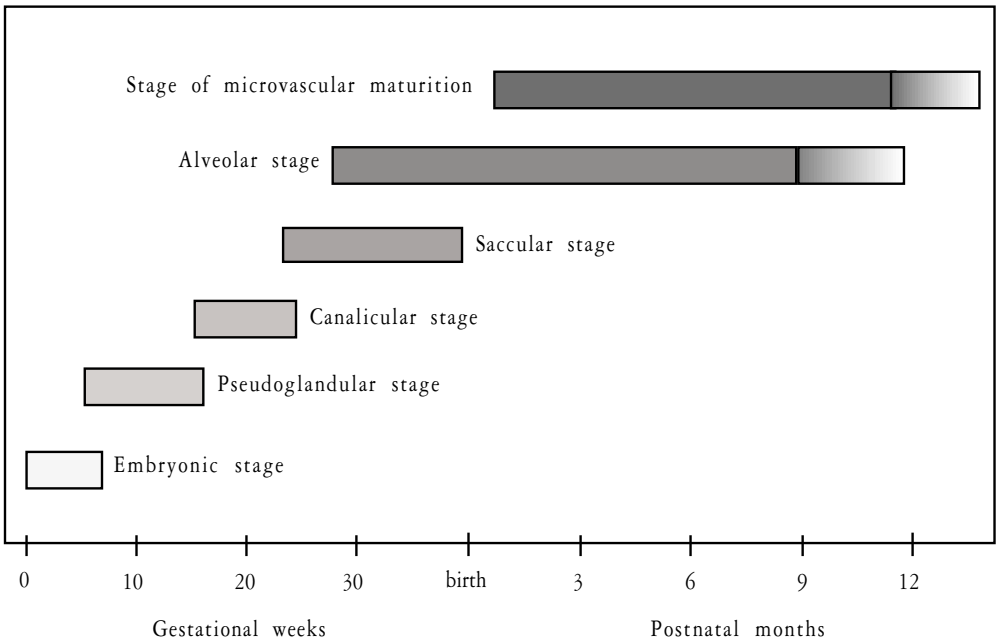
Embryonic stage (1-7 weeks). Organogenesis is the early phase of development during which most organs are laid down. The trachea and lungs develop as a ventral outpouching of the foregut at gestation day 26. This ventral outpouch consists of two parts, the future trachea and two primordial lung buds. At the gestational age of 4.5 weeks, the precedors of five lung lobes are formed as five tiny saccules. Branching of the airway tree increases rapidly by dichotomous divisions, and by the end of the 7th week the branching has progressed to subsegmental branches. The pulmonary arteries and the pulmonary vein are established at this stage.

Pseudoglandular stage (5-17 weeks). By the end of this stage, the complete set of generations of gas-conducting airways of the future lung are present. Branching of the airway tree continues, and toward the end of this stage all the airway divisions down to the level of alveolar ducts are present (Kitaoka 1996). The proximal airways are lined by tall columnar epithelium, the first ciliated cells appear in the central airways, and in the peripheral airways the epithelium is lined with undifferentiated cells - until the alveolar stage. The arterial tree branches mostly in parallel with the airways, whereas the veins run in between the the airway branches in connective tissue septa. At the end of this stage, the hierarchical pattern of preacinar airways and blood vessels corresponds to that of the adult lung.

During the process of development of the lung bud into a fully developed airway tree, the lung bud undergoes a series of patterning events termed branching morphogenesis. The primary lung bud consists of undifferentiated epithelial cells surrounded by mesenchymal cells. Interactions between mesenchyme and epithelium are required for airway branching. Of the mediators, fibroblast growth factor family members - the most well-known being fibroblast growth factor 10 - and their receptors play a critical role in mediating these epithelial-mesenchymal interactions during airway branching (Bellusci et al 1997).

Canalicular stage (16-26 weeks). The transition between the pseudoglandular stage and the canalicular stage is marked by formation of the prospective gas-exchanging tissue, acinus. The early acinus consists of an airway stem and a spray of short tubules. The lung parenchyma becomes canalized by the multiplication of capillaries. These capillaries form a loose three-dimensional network in the mesenchyme, and come to lie closer to the epithelial layer, thus forming a peritubular network. The cuboidal epithelium in the tubules begins to flatten, type-II epithelial cells appear as well as type-I epithelial cells - the principal cells lining the alveoli - and

Figure 1.



Stages of human lung development and time-scale (Reviewed in Burri 1997).

areas of a thin air-blood barrier are formed. Type-I cells are considered to be derived from type-II cells during fetal lung development. The type-II cells start to accumulate lamellar bodies which serve in intracellular storage of surfactant.

Saccular stage (24 weeks to term). At this stage, the peripheral airways form terminal clusters of widened air spaces called saccules. All air spaces distal to the terminal bronchioles lengthen and widen. The final two or three generations of air spaces are formed by division of saccules, resulting in transitory ducts and the transitory sac. A massive increase results in the size of the prospective lung parenchyma. The volume of intervening interstitial tissue decreases, altering the the three-dimensional structure of the pulmonary capillary bed.

Alveolar stage (from week 28 to 6-24 months postnatally). At birth, the human lung is in its early phase of alveolarization. In humans, normal alveolarization may begin as early as 28 gestational weeks and proceeds rapidly, to achieve 20 to 50% of the adult number of alveoli at term (Hislop et al 1986). It is also suggested that alveolar formation is mainly a postnatal event. The number of alveoli at birth is not obvious; estimates vary from 0 to 50 million. Moreover, it is unclear when alveolarization ends; estimations vary between 6 to 24 months. Parallel to the alveolar formation the bilayered capillary network in the parenchymal septa transforms into a single-layered network forming in the end the mature gas-exchanging unit.

1.1. Alveolar formation

Formation of an alveolus - the architectural maturation of the lung's gas-exchange unit - takes place in the area of transitory ducts and saccules and consists of two intervening phases. First comes septation: the outgrowth of septa from the walls of the sac-

cules that compose the gas-exchange unit of the architecturally immature lung. Secondary crest development starts with the appearance of low ridges along both sides of the saccular walls, which extend to make new alveolar septa. These ridges incompletely subdivide the transitory ducts and saccules into smaller units, the alveoli. The secondary septa contain a central sheet of connective tissue, flanked on both sides by a capillary layer. The secondary septa are suggested to be formed by the upfolding of one of the two capillary layers on both sides of the primary septa. Development of the secondary cresta involves coordinated outgrowth of epithelial cells, the capillary network, and alveolar myofibroblasts at alveolar septal tips, and the volume fraction of alveolar type-I cells increases (Massaro et al 1996). Studies in rats have revealed that a critical period in development occurs in which septation results. (Massaro et al 1985). The serum concentration of glucocorticosteroid is low during the time septation occurs, and increases when septation ends and remodelling of the alveolar microvasculature begins (Massaro et al 1996).

The second process is alveolarization: thinning of the walls of distal air spaces through flattening of epithelial cells, reduction in epithelial cell number by apoptosis, and remodelling of the alveolar wall to form a single capillary network (Massaro et al 1996). Endothelial cell apoptosis occurs before capillary formation but not after vessels have formed; inhibition of apoptosis results in an impaired vascular tissue arrangement (Segura et al 2002). During alveolarization, all the inner air-space walls (i.e., primary and secondary septa) contain a capillary bilayer. In the adult, in contrast, the interalveolar septum contains only a single capillary layer occupying the entire width of the septum. In the primary septa, the two capillary layers possess interconnections, whereas in the secondary septa the capillary network is connected only at the tip of the septa. Alveolarization can proceed only where a capillary layer can be folded up, which is the case in all septa containing a capillary bilayer.

In developing rats, treatment with antiangiogenic agents results in decreased arterial density and alveolarization, suggesting that angiogenesis is necessary for alveolarization and that injury to the developing pulmonary circulation may result in lung hypoplasia (Jakkula et al 2000). Failure of alveolar formation results in emphysematous lungs. This is apparent in mice deficient in factors that participate in alveolar formation, e.g., transforming growth factor-ß and platelet-derived growth factor A (Kaartinen et al 1995, Bostrom et al 1996, Lindahl et al 1997).

1.2. Lung vascular development

The primary phase of vessel formation occurs during the canalicular stage. Two different processes have been identified in embryonic pulmonary blood vessel formation: angiogenesis, the budding and branching of vessels from pre-existing vessels, and vasculogenesis, the differentiation of endothelial cells from the mesoderm and organization into a vascular plexus which then expands and is remodelled into a vascular tree (Carmeliet et al 1999a, Conway et al 2001). In adults, neovascularization occurs mainly via angiogenesis, but postnatal neovascularization has been described as also occurring by vasculogenesis (Shi et al 1998). During development, vessels formed by central sprouting angiogenesis subsequently communicate with peripheral vessels that develop by vasculogenesis (Pardanaud et al 1987, Pardanaud et al 1989, deMello et al 1997). A third additional process is the process in which a luminal connection is established between these two separate processes (deMello et al 2000). The pre-acinar branches of the pulmonary artery develop as the airways divide, whereas the intra-acinar or respiratory surface vessels appear as

the alveoli multiply. Definite bronchial arteries develop between the 9th and 12th weeks; pre-acinar and resistance arteries are present by the 28th week. By the 28th week, a blood-gas barrier has developed of a thickness similar to that in the adult. The development and maturation of vascular smooth muscle tissue lags behind endothelial development (Woodcock-Mitchell et al 1993). Several growth factors - including members of vascular-endothelial, platelet-derived, basic- and transforming-growth factor families - play a role in pulmonary vascular development (Risau W 1997, Petrova et al 1999, Conway et al 2001).

Microvascular maturation. Following alveolar formation, the capillary network of the pulmonary parenchyma has to undergo maturation to assume the adult morphology. This structural remodelling represents the last step in lung development. This process involves the transformation of the bilayered capillary network in the parenchymal septa into a single layered network. The interstitial volume of the parenchymal septa undergoes a continuous reduction in mass during all developmental stages despite an increase in total lung volume. The connective tissue separating the capillary networks thins out, and the capillaries of both sides of the septum draw closer to each other. During this process, the capillary lumina are separated by the cytoplasmic extension of a single endothelial cell, suggesting that in the end these two capillaries merge into one. The thinning of the interstitial layer also results in direct cell-to-cell contact and merging of alveolar epithelial cells.

In humans, lung volume and the gas-exchange area increase about 20- to 25-fold between birth and adulthood (Zeltner et al 1987a). In the same period, capillary volume increases by over 35-fold. The capillary network is not just stretched to fit the growing gas-exchange surface, but new capillary segments are added within the capillary bed. (Caduff et al 1986). Microvascular maturation is thought to end at the age of 2 to 3 years (Zeltner et al 1987b).

2. Lung injury in the newborn

Before the 19th century, high infant mortality was considered inevitable. In the late 19th century, a closed incubator for premature infants was introduced, and in 1896 the first special hospital unit was founded for premature infants. The standard of care required minimal handling and treatment of sick premature infants. In the lungs of newborn infants dying of respiratory distress, hyaline membranes were first described in 1903. Before the use of mechanical ventilation, the natural course of respiratory distress syndrome (RDS) - acute respiratory failure - was either death or recovery by 7 days of age. Routine use of oxygen therapy became the common practise in the care of premature infants in the 1940's. In 1953, modern mechanical ventilation of newborn infants with respiratory failure was introduced, with the use of a negative-pressure ventilator, and in the 1960's, treatment with mechanical ventilation and supplemental oxygen became the standard. In the 1960's, RDS was the leading cause of death in newborn infants; it was defined as respiratory distress occurring in a newborn infant - mostly in those premature - after the start of breathing, within the first hours of life, and as being primarily due to a deficiency in the pulmonary surfactant system (reviewed in Northway 2000). Respiratory distress within the first minutes of life may quickly become life-threatening and require immediate respiratory and general supportive therapy (Verma 1995). Pulmonary edema and overperfusion resulting from a patent ductus arteriosus may further worsen the respiratory failure and aggravate surfactant deficiency.

2.1. Bronchopulmonary dysplasia (BPD)

2.1.1. Old BPD

2.1.1.1. Definition

BPD was first described in 1967 by Northway et al. They documented the clinical, radiological, and pathological changes in prematurely born infants with severe RDS who had been treated with prolonged mechanical ventilation and high concentrations of inspiratory oxygen. BPD was described as an injury and repair process occurring in the immature lung secondary to high concentrations of supplemental oxygen, pulmonary oxygen toxicity, and pressure-induced trauma (Bonikos et al 1976). The definition of BPD was revised in 1985; it was emphasised that such a combination of oxidant injury and mechanical ventilation resulted in inflammation, fibrosis, and smooth muscle hypertrophy in the airways (O'Brodovich et al 1985). The diagnostic criteria were revised in 1989 as a result of changes in BPD epidemiology from the requirement of supplemental oxygen at 36 gestational weeks to oxygen at the postnatal age of 28 days and a chest radiograph with findings characteristic of BPD (Shennan et al 1988). In addition to RDS, treatment of respiratory failure resulting from other causes, such as meconium aspiration pneumonia, congestive heart failure, the Wilson-Mikity syndrome, and congenital diaphragmatic hernia was recognized to lead to BPD (reviewed in Northway 2000).

The clinical course of BPD was originally divided into four stages: Stage I (2 to 3 days) was a period of acute RDS with respiratory failure, deposition of hyaline membranes, atelectasis, and metaplasia and necrosis of the bronchiolar mucosa. During Stage II (4 to 10 days) the infants were usually weaned from the respirator, but still needed high concentrations of oxygen. Histology showed emphysematous coalescence of alveoli, and increased bronchiolar necrosis. During Stage III (10 to 20 days), during the transition to the chronic stage of BPD, widespread bronchiolar metaplasia and hyperplasia, emphysematous alveoli, and atelectasis occurred. In Stage IV (beyond 1 month) histology showed hypertrophy of peribronchiolar smooth muscle, emphysema, and separation of capillaries from alveolar epithelium by thickening of the basement membranes (Northway et al 1967, Northway 2000).

2.1.1.2. Affecting factors

In several centres the association has been established between BPD and low birth weight and gestational age (Avery et al 1987, Kraybill et al 1987, Horbar et al 1988). Moreover, extremely low birthweight infants are at increased risk for BPD, regardless of the severity of RDS (Palta et al 1991). Mechanical ventilation produces pulmonary epithelial and endothelial injury, induces edema formation, and increases pulmonary inflammation (Dreyfuss et al 1985, Thome et al 1998). Barotrauma to the immature lung is a causative factor for development of BPD (Van Marter et al 2000). Free oxygen radicals generated during hyperoxic exposure in the lung play a role in the development of BPD. Immaturity is associated with development of pulmonary oxygen radicals, and protein oxidation in the neonatal lung is related to development of chronic lung disease (Pitkänen et al 1990, Varsila et al 1995). An inflammatory pulmonary reaction following acute lung injury is an early event in the development of BPD (Groneck et al 1995). Several proinflammatory mediators in the lung have been identified as associated with subsequent development of BPD (Merritt et al 1983, Groneck et al 1994). Another postnatal risk factor for BPD is infection. Maternal chorionamnionitis accelerates fetal lung maturation but also causes inflamma-

tion and subsequent lung injury (Watterberg et al 1996). Neonatal sepsis, and pulmonary Ureaplasma urealyticum colonialization are associated with subsequent development of BPD (Rojas et al 1995, Wang et al 1995). Patent ductus arterious (PDA) is a risk factor for development of BPD; moreover, PDA combined with infection provokes lung injury and promotes development of BPD (Rojas et al 1995, Gonzalez et al 1996). Obstetric and maternal risk factors for infant BPD include fetal asphyxia and poor intrauterine growth (Hakulinen et al 1988). Other neonatal risk factors for BPD include low Apgar score and male sex; RDS is more prevalent and more severe in male preterm infants (Avery et al 1987, Horbar et al 1988, Kraybill et al 1989, Palta et al 1991, Parker et al 1992).

2.1.1.3. Development of treatment

The introduction of surfactant therapy has reduced the severity of RDS and thereby reduced the effect of oxygen-induced lung injury. Surfactant treatment also reduces the severity of BPD (Parker et al 1992, Egberts et al 1997). Improvements in the management of premature infants, including advances in mechanical ventilation, in use of supplemental oxygen, and in antenatal glucocorticoid treatment have resulted in a dramatic reduction in mortality from RDS, so that it is no longer the leading cause of death in live-born premature infants. This effect is seen particularly in extremely low birth-weight infants (Avery et al 1991, Stevenson et al 1998, Northway 2000).

Infants surviving with BPD may suffer persistent pulmonary dysfunction, increased airway obstruction, airway hyperreactivity, and hyperinflation, low dynamic compliance, increased functional recidual capacity, and a permanent reduction in alveolar surface area (Northway et al 1990, Mitchell et al 1998, Jacob et al 1998). However, it seems that the milder course of BPD recently has improved long-term outcome as well in premature infants (Fitzgerald et al 2000).

2.1.2. New BPD

2.1.2.1. Epidemiology

A significant decrease has occurred in the incidence of BPD in infants weighing at birth over 1500 g, although overall incidence of RDS and of BPD has risen. This can be explained by the fact that survival of extremely low birth-weight infants (<1000 g) with BPD has increased (Parker et al 1992). Infants as small as birth weight 280 g have survived (Muraskas et al 1991). Preterm infants born at 24 weeks of gestation presently survive about half of the time; of these survivors, half develop BPD. It is now the most immature infants that develop BPD; these are infants with birth weights between 500 and 1000 g and those who are born at 24 to 28 weeks of gestation (Stevenson et al 1998).

At present, many of the small preterm infants who develop BPD have no preceding RDS or just a mild initial respiratory course. They require mechanical ventilation with low pressure and oxygen concentration. The mild RDS in these infants usually responds favourably to treatment with surfactant (Charafeddine et al 1999). However, many of these infants show progressive deterioration in lung function calling for increasing ventilatory and oxygen requirements. Bacterial or viral infections or patent ductus arteriosus (PDA) may act as the triggering agent for deterioration in pulmonary function (Rojas et al 1995).

2.1.2.2. Pathophysiology

Premature birth interrupts normal alveolar development. At the age of 24 gestational weeks, the preterm lung has completely branched airways, the potential gas exchange region is composed of saccular structures, and septation is just beginning, so

no alveoli are yet present. The epithelial cells are just starting to produce surfactant, which is still not mature. During the canalicular stage, capillaries form from mesenchymal progenitors and fuse in the interstitium, and the interstitium starts to thin (Langston et al 1984, Coalson et al 1989). The capillary bed is poorly developed and not closely opposed to epithelium.

Alveolar formation is disturbed by alterations in O2 partial pressure. In rats and in mice, hyperoxia diminishes septation, resulting in irregularly enlarged alveoli and a reduced developmental increase in gas-exchange surface area. (Shaffer et al 1987, Massaro et al 1990, Blanco et al 1991, Blanco et al 1993, Massaro et al 1996, Warner et al 1998). In addition, lung capillary development is disturbed by hyperoxia, resulting in decreased arterial concentration, in medial hyperotrophy in muscular arteries, and in a diminished number of alveolar capillaries (Wilson et al 1985, Randell et al 1990). In rats, hypoxia even for a short period, impairs septation and reduces gas exchange surface area, but accelerates thinning of the alveolar wall (Massaro et al 1989). Both preterm lambs and preterm baboons undergoing prolonged mechanical ventilation or ventilation with 100% oxygen show fewer alveoli, enlarged airspaces, and an arrest of alveolar development (Coalson et al 1992, Coalson et al 1995, Albertine et al 1999).

In the 1980's, Hislop et al had already noticed that infants who died after mechanical ventilation had fewer alveoli than did unventilated preterm infants dying of nonrespiratory causes (Hislop et al 1987). In contrast to findings in the 70's and 80's in larger surviving infants, recent findings include less airway epithelial disease, less severe vascular disease, varying degrees of interstitial fibrosis, and an abundance of large, simplified airspaces (Chambers et al 1989, Hislop et al 1990, Van Lierde et al 1991, Margraf et al 1991, Husain et al 1998). Findings in autopsies of very premature, extremely low birth-weight infants who develop fatal BPD include persistence of dilated terminal airspaces, a simplified distal lung acinus lined with cuboidal epithelium and separated by widened septa, and a consistent lack of significant alveolarization resulting in alveolar hypoplasia. Abnormal capillary configuration is also evident; capillaries are positioned subepithelially, vascular organization shows a dysmorphic pattern, and capillaries are extremely sparse in the saccular walls. Moreover, in preterm baboons treated prenatally with glucocorticoids and postnatally with surfactant, and subjected to low ventilatory settings and low inspiratory oxygen, a similar pattern of lung injury as in human autopsy specimens appears: minimal airway disease, diminished number of capillaries, and alveolar hypoplasia (Coalson et al 1999). Use of less oxygen and low peak airway pressures has been shown to result in decreased severity of interstitial fibrosis. Use of postnatal antenatal steroid or surfactant therapy does not, however, alter this arrest of alveolar development (Coalson et al 1997, Husain et al 1998, Coalson et al 1998).

Arrest of lung development. Pathological findings in the lungs of low birthweight infants with BPD include vascular arrest, alveolar hypoplasia, and adaptive dysmorphic changes in response to their premature adaptation to the extrauterine environment. The use of exogenous surfactant, with less barotrauma and oxygen injury, has resulted in a pattern of injury reflecting an extremely immature lung with impaired alveolar growth and development owing to developmental arrest, and subsequent abnormal reparative processes. Although multiple pathophysiological mechanisms, including inflammation and oxidant injury, take part in the development of BPD, the pathogenesis of new BPD encountered in very immature preterm infants may be caused primarily by arrest of normal lung development (Jobe 1999). Capillary development plays a role in septation and alveo-

lar maturation; inhibition of angiogenesis results in impaired alveolar development, indicating that angiogenesis is needed for alveolarization, and injury to the developing pulmonary circulation may result in lung hypoplasia (Jakkula et al 2000, Abman 2001).

2.2. Glucocorticoids and the preterm infant

2.2.1. Effects on lung development

In rat pseudoglandular-stage lung explants, dexamethasone treatment accelerates acquisition of several features of advanced maturation which normally accompany late stages of fetal development (Oshika et al 1998a). In rats, postnatal dexamethasone treatment in early life - during the period of normal septation - accelerates alveolar wall thinning (Massaro et al 1986). This treatment, however, inhibits outgrowth of new interalveolar septa in saccules and diminishes the extent of the increase in alveolar surface area, resulting in emphysematous-appearing lungs with fewer and larger airspaces (Massaro et al 1985, Blanco et al 1989). In addition, in rats, antenatal dexamethasone treatment suppresses alveolarization (Okajima et al 2001). The effect of glucocorticoids on fetal lung maturation is time-dependent as well as dose-dependent (Bunton et al 1984). Glucocorticoid treatment - like prenatal inflammation - results in an improvement in postnatal lung function but at the same time results in a decrease in alveolar volume and number of alveoli (Wíllet et al 2000). In rats, postnatal glucocorticoid treatment reduces interstitial tissue mass and accelerates capillary maturation, resulting in a capillary monolayer instead of a bilayer. In these same rats, a week after withdrawal of the treatment, the trend toward precocious maturation is partially reversed, interalveolar walls are thickened, and double capillary networks are again visible; however, this reversal is only partial, and the lungs still display their emphysematous condition (Tschanz et al 1995). These data suggest that dexamethasone treatment may interrupt normal alveolar development.

In preterm infants in the early postnatal period, in addition to effects on alveoli, glucocorticoids reduce in lung parenchymal cells both cell proliferation and apoptosis (Luyet et al 2002) and reduce the pulmonary inflammatory response (Groneck et al 1993).

2.2.2. Effects on preterm infant

A clear reduction in neonatal morbidity and mortality and a decrease in the incidence of RDS appear when antenatal glucocorticoids are administered in preterm labour (Crowley PA 1995). Maternally administered glucocorticoids cause fetal growth retardation but enhance lung compliance, lung volume, and surfactant production after preterm delivery (Jobe et al 1998). This beneficial effect is achieved by a single dose; repeated glucocorticoid courses seem to add nothing to the effect but rather to cause adverse effects (French et al 1999). Prenatal steroid therapy reduces risk for BPD, except in the smallest infants weighing less than 1000 g (Papageorgiou et al 1989, Van Marter et al 1990).

Early postnatal dexamethasone treatment in preterm infants has been shown to reduce lung morbidity, mortality, and severity of RDS, and to shorten the requirement for mechanical ventilation. Early postnatal dexamethasone seems to be of beneficial effect in reducing BPD, although this association is not yet clear. Postnatal dexamethasone, however, has adverse effects on infant growth and elevates the risk for hypertension, hyperglycaemia, and intestinal perforation. Moreover, early postnatal dexamethasone has adverse effects on long-term neurodevelopmental outcome (Shinwell et al 1996, Yeh et al 1997, Bhuta et al 1998, Garland et al 1999, Sinkin et al 2000, Stark et al 2001). In extremely low birth-weight

infants of less than 1000 g, postnatal dexamethasone has been found to show no beneficial effect on development of BPD and to have adverse effects on growth and on the intestinal perforation rate (Stark et al 2001).

2.3. Persistent pulmonary hypertension of the newborn

Persistent pulmonary hypertension of the newborn (PPHN) is a syndrome of acute respiratory failure characterized by systemic hypoxemia and elevated pulmonary artery pressure. PPHN is most common in infants with underlying diseases such as perinatal asphyxia, meconium aspiration, RDS, or lung hypoplasia; it may even be idiopathic (Morin et al 1995). PPHN is characterized by vascular intimal thickening related to increased migration and proliferation of vascular smooth muscle cells and by elevated pulmonary artery pressure associated with vascular intimal thickening of arteries of a diameter less than 200 μm, arteries which play an important role in pulmonary blood pressure and vascular resistance regulation (Rabinovitch et al 1986, Wagenwoort et al 1989, Morin et al 1995). Lung hypoplasia, seen as reduced alveolar count and pulmonary artery density, which is induced by perinatal hypoxia or dexamethasone, may augment the severity of pulmonary hypertension (le Cras et al 2000, Tang et al 2000).

3. Growth factors and the preterm lung

3.1. Vascular endothelial growth factor

3.1.1. Background

VEGF family. Members of the vascular endothelial growth factor (VEGF) family play a crucial role in the growth, differentiation, and regulation of vascular and lymphatic endothelial cells (reviewed in Carmeliet et al 1999a). The first member of the family, VEGF-A (referred to as VEGF, here) was discovered in 1983 (Senger et al 1983). It was found to be a potent endothelial cell-specific mitogen capable of regulating physiological and pathological angiogenesis and in 1989 termed as VEGF (Ferrara et al 1989, Plouet et al 1989, Leung et al 1989). A novel protein sharing marked similarity with VEGF was isolated in 1991 and termed placental growth factor PlGF (Maglione et al 1991). More members of the family, including VEGF-B (Olofsson et al 1996) and VEGF-C (Joukov et al 1996) were discovered in 1996. VEGF-C was identified as acting, in addition to its role in angiogenesis, as a regulator of lymphangiogenesis during development and in pathological conditions (Kukk et al 1996, Jeltsch et al 1997, Cao et al 1998, Eichmann et al 1998). VEGF-D was identified in 1998 (Achen et al 1998). Additional Orf-virus encoded members of the family were discovered in parapox virus in 1998 and termed VEGF-Es (Ogawa et al 1998, Meyer et al 1999).

VEGF-A isoforms. The human VEGF gene is located on chromosome 6p21.3 (Vincenti et al 1996). Alternative exon splicing of a single VEGF gene results in at least six different isoforms: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206 (Houck et al 1991, Tisher et al 1991, Shima et al 1996, Poltorak et al 1997, Jingjing et al 1999). VEGF165 is the predominant molecular species produced by the cells (Houck et al 1991). Whereas VEGF165 is basic, and is a heparin-binding protein, VEGF121 is weakly acidic and does not bind to heparin. VEGF189 and VEGF206 are more basic and bind to heparin with even greater affinity than does VEGF165 (Houck et al 1992). VEGF121 is a freely diffusible protein; VEGF165 is also secreted, although a significant fraction remains bound to the cell surface and the extracellular matrix. In contrast, although VEGF189 and VEGF206 are almost completely seques-

tered in the extracellular matrix, these isoforms may be released in a soluble form (Park et al 1993, Keyt et al 1996a). VEGF proteins may become available to endothelial cells by at least two different mechanisms: as freely diffusible proteins (VEGF121, VEGF165) or following protease activation and cleavage of the isoforms (VEGF189 and VEGF206). Loss of the heparin binding of VEGF results in reduction of mitogenic activity of vascular endothelial cells (Keyt et al 1996a).

VEGF receptors. VEGF-A has two receptors that bind to it with high affinity: Flt-1 (fms-like tyrosine kinase, VEGFR1) and Flk-1 (fetal liver kinase-1, VEGFR2). Both receptors have an extracellular domain, a single transmembrane region, and a tyrosine kinase domain. Flt-1 has higher affinity for recombinant human VEGF165 than does Flk-1 (de Vries et al 1992, Terman et al 1992, Veikkola et al 2000). An alternatively spliced soluble form of Flt-1 (sFlt-1) has also been identified that binds VEGF (Kendall et al 1993). VEGF mutants that bind selectively to Flk-1 are able to induce, in vivo, mitogenesis and chemotaxis in endothelial cells, and induce angiogenesis and permeability, whereas Flt-1 selective mutants cannot perform such abilities (Keyt et al 1996b, Gille et al 2001). Flk-1 activation has been shown to be required for the antiapoptotic effects of VEGF in endothelial cells, as well as for formation of capillary-like structures (Gerber et al 1998a, Koolwijk et al 2001). A third tyrosine kinase receptor that binds VEGF-C and VEGF-D was identified as Flt-4 (VEGFR3); in contrast to Flt-1 and Flk-1, expression of Flt-4 is largely restricted to lymphatic and venous endothelium during fetal development and to lymphatic endothelium in adults (Aprelikova et al 1992, Pajusola et als 1992, Shibuya et al 1995, Kaipainen et al 1995). There exists an additional isoform-specific receptor that binds VEGF165 but not VEGF121. This receptor is identical to human neuropilin-1, which is involved in regulation of neuronal cell guidance by semaphorins (Soker et al 1998)

Regulation of VEGF. Oxygen tension is a key regulator of VEGF gene expression (Shweiki et al 1992); VEGF is rapidly and reversibly induced by exposure to low pO2, and hypoxia induces both Flt-1 and Flk-1 expression in vitro (Minchenko et al 1994, Shima et al 1995, Brogi et al 1996, Waltenberger et al 1996). In acute hypoxia, VEGF mRNA in rat lungs increases within hours, whereas chronic hypoxia causes increased expression of Flt-1 and Flk-1 (Tuder et al 1995). Similarities exist between the mechanisms leading to hypoxic regulation of VEGF and of erythropoietin (Goldberg et al 1994). A hypoxia-specific enhancer is required for the hypoxia-inducibility of VEGF, and hypoxia-inducible factors (HIF)-1 and -2, essential mediators of O2 homeostasis, have been identified as these factors (Liu et al 1995). VEGF upregulation in response to hypoxia is also augmented postranscriptionally by increased mRNA stability (Ikeda et al 1995). A number of cytokines, hormones, and growth factors are able to upregulate VEGF mRNA expression in various cell types. A role in inflammation is suggested by upregulation of VEGF expression by inflammatory mediators (Ben-Av et al 1995, Nauck et al 1997, Horiuchi et al 1997). Epidermal growth factor, transforming growth factor-ß, keratinocyte growth factor, and platelet-derived growth factor have been shown to induce VEGF mRNA expression (Pertovaara et al 1994, Brogi et al 1994, Frank et al 1995). VEGF can upregulate its receptors Flt-1, Flk-1, and sFlt-1 (Barleon et al 1997, Kremer et al 1997, Shen et al 1998).

Biological activities of VEGF. VEGF is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, but it lacks significant mitogenic activity for other cell types (Ferrara 2001). VEGF is suggested to act as a paracrine fac-

tor, secreted by nonendothelial cells and modulating activities in adjacent vascular endothelium (Shifren et al 1994, Brogi et al 1996). VEGF is a major regulator of normal and pathological angiogenesis, endothelial cell differentiation, and maintenance of existing vessels, and is a survival factor for endothelial cells (Leung et al 1989, Pepper et al 1991, Shifren et al 1994, Alon et al 1995, Gerber et al 1998a). In addition to prosurvival activity, VEGF induces expression of antiapoptotic proteins in endothelial cells (Gerber et al 1998b). It induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators, as well as metalloproteinase interstitial collagenase in endothelial cells (Pepper et al 1991, Unemori et al 1992). VEGF is known also as a vascular permeability factor based on its ability to induce fenestration in endothelial cells and vascular leakage (Dvorak et al 1995, Roberts et al 1995).

In addition to effects on vascular endothelium, VEGF action may be involved in the survival or proliferation of alveolar epithelial cells. VEGF induces human fetal airway epithelial-cell proliferation in vitro, and treatment of rats with the VEGF-receptor blocker SU5416 enhances apoptosis in alveolar septal cells and leads to enlargement of air spaces (Kasahara et al 2000, Brown et al 2001).

Effect of corticosteroids on VEGF. Corticosteroids reduce VEGF expression and attenuate VEGF expression induced by inflammatory mediators or by hypoxia in vitro (Nauck et al 1997, Horiuchi et al 1997, Klekamp et al 1997, Nauck et al 1998). In contrast, in vivo, dexamethasone may raise pulmonary VEGF concentrations in preterm infants receiving postnatal dexamethasone (D'Angio et al 1999, Bhatt et al 2000).

3.1.2. VEGF and lung development

Prenatal expression of VEGF. VEGF is expressed at high levels in the lungs of normal human adults undergoing physiological endothelial turnover (Berse et al 1992). In human fetuses, mRNA for VEGF can be detected in all tissues, most abundantly in lung, kidney, and spleen. VEGF is, in fetuses and similarly in adults, localized in epithelial cells and myocytes, including the smooth muscle cells lining blood vessels (Shifren et al 1994, Acarregui et al 1999). Inactivation even of a single VEGF allele in mice results in early embryonic lethality. VEGF-/+ embryos are growth retarded and exhibit a number of developmental anomalies; formation of blood vessels is abnormal in heterozygous VEGF-deficient embryos and is even more impaired in homozygous VEGF-deficient embryos (Carmeliet et al 1996, Ferrara et al 1996). Part of this developmental block can be reversed by addition of exogenous VEGF (Bautch et al 2000). In an isoform-specific knockout of the VEGF gene, all of the VEGF120/120 mice die within 2 weeks after delivery (Carmeliet et al 1999b). In addition, the isoform-specific knockouts VEGF164-/- and VEGF188-/- display vascular defects in several tissues including in the pulmonary vasculature (Ng et al 2001), indicating that the functions of heparin-binding isoforms of VEGF cannot be replaced by VEGF120.

Prenatal expression of VEGF receptors. In mice, Flt-1 is expressed in both embryos and adults, in endothelium during vascular development (Peters et al 1993). In contrast, Flk-1 is expressed in vascular endothelium in mouse fetuses, but its expression is reduced in the adults (Terman et al 1992, Millauer et al 1993). Gene-targeting studies have demonstrated that in mice, both Flt-1 and Flk-1 are essential for the development of the embryonic vasculature. Mouse embryos homozygous for a targeted mutation in the Flt-1 or Flk-1 locus die in utero. In Flt-1-/- mice, endothelial cells develop but fail to organize in the normal vascular channels, whereas Flk-1-/- mice lack vasculogenesis,

fail to develop blood islands, present disrupted hematopoietic precursors, and fail to develop organized blood vessels (Fong et al 1995, Shalaby et al 1995). However, that mice lacking the tyrosine kinase domain of Flt-1 but still having the ligand-binding region develop normal vessels indicates that Flt-1 has, during development, primarly a non-signaling function (Hiratsuka et al 1998). PlGF potentiates the angiogenetic response to VEGF via binding to Flt-1; in mice deficient in PlGF, a factor binding to Flt-1 but not to Flk-1, embryonic angiogenesis is unaffected (Carmeliet et al 2001). Angiogenesis, plasma extravasation, and collateral growth in these mice is, however, impaired during ischemia, inflammation, wound-healing, and cancer, indicating a role for Flt-1 in the angiogenetic response to pathological conditions (Carmeliet et al 2001). Inhibition of Flk-1 with receptor blocker in rats induces alveolar septal cell apoptosis and leads to emphysematous enlargement of airspaces, indicating that Flk-1 signalling is required for maintenance of alveolar structures (Kasahara et al 2000).

Flt-4 expression is restricted to lymphatic endothelium during development (Kaipainen et al 1995). Flt-4 plays a crucial role in lymphatic development, as shown by inhibition of lymphangiogenesis in transgenic mice expressing soluble Flt-4 (Mäkinen et al 2001). That mice deficient in Flt-4 show cardiovascular failure suggests that Flt-4 plays an essential role in embryonic cardiovascular development before the emergence of lymphatic vessels (Dumont et al 1998).

VEGF and postnatal lung development. In preterm infants, VEGF levels in serum increase rapidly postnatally (Malamitsi-Puchner et al 1999). Early postnatal inactivation of VEGF in mice either by administration of sFlt-1 or by inducible gene targeting results in increased mortality, stunted body growth, and impaired organ development - and when the inhibition is more severe - in nearly complete growth arrest and lethality. Interestingly, after the fourth postnatal week, dependence on VEGF is eventually lost (Gerber et al 1999). Treatment of mice postnatally by an inhibitor of Flk-1 results in decreased alveolarization and arterial density, suggesting that the VEGF-Flk-1 system is required for normal postnatal alveolar development (Jakkula et al 2000).

3.1.3. VEGF and injury in the preterm lung

Lung injury. In animal studies, after prolonged exposure to hyperoxia, levels of VEGF mRNA and protein decrease, as does Flt-1 and Flk-1 expression, a phenomenon that has been suggested to contribute to the pathophysiology of oxygen-induced lung injury and to impaired vascular repair in such injury (Johnston et al 1996, Klekamp et al 1999). In newborn rabbits during hyperoxic lung injury, pulmonary expression of VEGF mRNA and protein are decreased, whereas during the recovery period in relative hypoxia, the expression of VEGF increases specifically in alveolar type-II cells (Maniscalco et al 1995, Maniscalco et al 1997). In mice overexpressing IL-13, survival after exposure to 100% oxygen is prolonged. In these mice, VEGF levels are increased in bronchoalveolar lavage, and this increase is even higher in those exposed to hyperoxia. That antibody neutralization of VEGF reduces survival of these mice indicates that production of VEGF protects against hyperoxic lung injury (Corne et al 2000).

BPD. In a premature-baboon model of BPD, capillary density does not increase, and capillaries are dysmorphic and not subepithelial; moreover VEGF and Flt-1 expression are significantly decreased (Maniscalco et al 2002). The lung specimens obtained by autopsy in infants dying of BPD show an abnormal distribution of alveolar capillaries and thickened alveolar septa in addition to decreased expression of VEGF

and Flt-1 (Bhatt et al 2001). These data suggest that development of BPD is associated with impaired lung microvasculature and that a possible mechanism is disruption of VEGF and Flt-1 expression. Loss of the hypoxia-inducible transcription factor-2-a (HIF-2-a), a promoter for VEGF, causes fatal RDS in neonatal mice due to insufficient surfactant production by alveolar type-II cells. VEGF levels in alveolar cells are decreased in these HIF-2-a-deficient fetuses; mice with a deficiency in the longer isoforms of VEGF or in the HIF-binding site in the VEGF promotor die of RDS. Capillary development in septa is impaired prior to birth: capillaries fail to remodel properly and are separated from the lumen. Intrauterine inhibition of Flk-1 results as well in defects in alveolarization and also in lung prematurity. Intrauterine or postnatal intratracheal instillation of VEGF stimulates conversion of glycogen to surfactant, improves lung function, and protects mice against RDS (Compernolle et al 2002). These data suggest a protective or reparative role for VEGF in neonatal lung injury in addition to its role in lung development.

Pulmonary hypertension. Flk-1 inhibition combined with prolonged hypoxia in rats results in severe pulmonary hypertension (Taraseviciene-Stewart et al 2001). Interestingly, VEGF expression is higher in lungs of newborn infants suffering from pulmonary hypertension. Shehata et al suggest this to be a reflection of an unsuccessful attempt to increase the pulmonary vascular bed in hypoplastic lungs to alleviate the associated pulmonary hypertension (Shehata et al 1999).

3.2. Hepatocyte growth factor

3.2.1. Background

Hepatocyte growth factor (HGF), also called scatter factor or lung fibroblast-derived mitogen, is a heterodimeric (69-kD a-chain and 34-kB ß-chain) heparin-binding growth factor with structural homology to plasminogen (Nakamura et al 1984, Nakamura et al 1989, Weidner et al 1991). HGF is synthesized in a variety of cell types, including fibroblasts, macrophages, smooth muscle cells, and epithelial cells (Mason 2002). It acts as a mesenchymal- or stroma-derived growth factor which stimulates cell growth, cell motility, and morphogenesis in epithelial cells in a wide range of organs (Stoker et al 1989, Gherardi et al 1989, Montesano et al 1991). In the lungs, HGF acts as a mitogen and motogen for alveolar type-II and bronchiolar cells and is suggested to be responsible for most of the stimulatory activity of type II cells in the lavage fluid (Panos et al 1993, Shiratori et al 1995, Mason et al 1996). HGF induces epithelial cell proliferation and formation of structures resembling alveolar and bronchial tissues in fetal rat and mice lung cultures (Itakura et al 1997, Sato et al 1997). In the developing lung, HGF and c-MET/HGF mRNA are expressed in mesenchyme and in epithelium, respectively. In fetal lung cultures, added HGF stimulates branching morphogenesis; moreover, HGF neutralization assays or translation arrest result in inhibition of epithelial branching (Ohmichi et al 1998). These data suggest a role for HGF in the morphogenesis both of alveolar and of bronchial epithelia during development. In addition, HGF stimulates growth and migration of endothelial cells (Bussolino et al 1992). Several growth factors induce HGF mRNA expression or HGF secretion, including epidermal growth factor, platelet-derived growth factor, basic fibroblast growth factor, acidic fibroblast growth factor, and transforming growth factor-a (Gohda et al 1994).

HGF receptor. A c-met protooncogene product possessing an intracellular tyrosine kinase domain has been identified as a cellular receptor for HGF (Bottaro et al 1991): c-met is expressed in epithelial cells in various organs, including type II pneumocytes

in alveoli; since HGF is detected mostly in mesenchymal or stromal cells, HGF is suggested to act primarily as a mesenchymal factor that affects the adjacent epithelia in a paracrine manner during development and affects other mesenchymal-epithelial interactions (Sonnenberg et al 1993, Panos et al 1993, Mason et al 1994, Shiratori et al 1995, Sato et al 1997). Expression of c-met has been detected also in endothelial cells (Bussolino et al 1992).

Effect of corticosteroids on HGF. In vitro, corticosteroids suppress HGF expression in human lung fibroblasts and HGF production in embryonic lung fibroblasts, they suppress HGF mRNA expression and HGF production in human skin fibroblasts and production of HGF in bone marrow stromal cells (Matsumoto et al 1992, Matsunaga et al 1994, Takai et al 1997). Growth factor-induced HGF mRNA expression and HGF secretion are inhibited by dexamethasone (Gohda et al 1994). In contrast, in the rat lung, c-met mRNA is elevated by dexamethasone (Oshika et al 1998a, 1998b).

3.2.2. HGF and lung development

Expression of HGF and c-met. HGF and c-met expression appear in virtually all of HGF and c-met is already detectable in human embryos at a gestational age of 6 weeks. HGF expression is not confined to mesenchymal tissues but is expressed during development in epithelial tissues, as well (Wang et al 1994, Kolatsi-Joannou et al 1997). In rats, HGF and c-met mRNA levels are low during late gestation and the neonatal period and increase markedly 3 ot 4 weeks postnatally; HGF is predominantly expressed in stromal cells, and c-met in epithelial cells in rats during late gestation and the early neonatal period (Kagoshima et al 1992). HGF is suggested to play a role in organ formation and maturation, and in the maintenance of tissue homeostasis during the postnatal period, presumably through its potential to act as a paracrine mitogen, motogen, and morphogen for epithelial cells (Montesano et al 1991, Kagoshima et al 1992).

Mice lacking the HGF gene fail to complete development. This mutation affects the embryonic liver, which is reduced in size and shows extensive loss of parenchymal cells. The mice die in utero with signs of apoptosis in their liver parenchymal cells. HGF is also essential for the development of several epithelial organs (Uehara et al 1995, Schmidt et al 1995). Mice lacking c-met fail to complete organogenesis and die with hypoplasia of the liver, leg muscles, and diaphragm (Bladt et al 1995).

3.2.3. HGF and injury in the preterm lung

HGF in lung injury. After pneumectomy in mice, alveolar and airway epithelial cells undergo compensatory DNA synthesis, and HGF mRNA and protein levels parallel this increase. The c-met expression in these mice is localized predominantly in alveolar type II and airway epithelial cells. Neutralization of HGF by an antibody suppresses the compensatory DNA synthesis in epithelial cells, whereas administration of recombinant HGF stimulates it, suggesting a pulmotrophic role for HGF in lung regeneration (Sakamaki et al 2002). In induced lung injury in rats, HGF increases in 3 to 14 days and peaks at 7 to 14 days, dropping sharply after 2 weeks: The high levels of HGF associate with bronchial and alveolar epithelial cell proliferation (Adamson et al 1999). Moreover, HGF protein level, expression of HGF mRNA, and HGF activity increase parallel to the DNA synthesis of alveolar epithelial cells. There also occurs a transient expression of c-met, after which it is down-regulated. Inhibition of HGF reduces the DNA synthesis of alveolar epithelial cells and aggravates lung injury (Yanagita et al 1993, Yamada et al 2000). In humans, in acute lung injury, the

HGF level is higher in the pulmonary edema fluid than in the plasma, indicating that the primary source of circulating HGF is the lung (Vergheseet al 1998). These data suggest that after acute lung injury, HGF is newly produced in the lung and that HGF is active in the alveolar space and may mediate early events in lung repair and play a role in lung regeneration (Panos et al 1996, Yanagita et al 1993, Vergheseet al 1998). In contrast, in adult ARDS (adult respiratory distress syndrome) patients, high levels of HGF are associated with poor prognosis; one possible explanation is that in ARDS, HGF may inhibit surfactant metabolism (Stern et al 2000, Vivekananda et al 2000).

Alveolar type-II cell proliferation occurs in response to lung injury, and is thought to play a critical role in alveolar epithelial repair (Piedboeuf et al 1996, Daly et al 1998). For airway epithelial cells and alveolar epithelial cells in vivo and in vitro, HGF is a potent mitogen. Intravenous injection of human recombinant HGF into mice with acute lung injury stimulates DNA synthesis of airway and alveolar epithelial cells (Ohmichi et al 1996). Exogenous HGF acts as a pulmotrophic factor in vivo in mice with severe lung injury induced by bleomycin and prevents the progression of bleomycin-induced lung injury when administered in either a simultaneous or delayed fashion (Yaekashiwa et al 1997). Intratracheal instillation of recombinant HGF induces a time- and dose-dependent increase in type II cell proliferation, although, in animals exposed to hyperoxia, this increase is less (Panos et al 1996). HGF may act as a pulmotrophic factor responsible for airway and alveolar regeneration during lung regeneration after acute lung injury (Ohmichi et al 1996). Intratracheal instillation of HGF may, after lung injury, provide a potential strategy to promote type II cell proliferation and augment alveolar epithelial repair (Panos et al 1996).

Aims of the study

- to evaluate the lung-lining fluid VEGF and the protein expression of VEGF and its receptor Flt-1 during the perinatal period.

- to evaluate in preterm infants the role of VEGF in acute and chronic lung injury.

- to evaluate in preterm infants the lung-lining fluid HGF during the perinatal period and in acute and chronic lung injury.

- to evaluate in preterm infants the effect of early prophylactic dexamethasone treatment on concentrations of the lung-lining fluids VEGF and HGF.

Material and methods

1.Material

1.1. Ethics

All studies were done with the approval of the Ethics Committee of the Hospital for Children and Adolescents, University Central Hospital, Helsinki. The aim of Study IV was to evaluate the effect of early postnatal dexamethasone on severity of RDS and development of BPD in preterm infants. However, in multiple trials at the time, several adverse effects from early dexamethasone treatment became evident, and its effect in reducing risk for BPD became controversial (Bhuta et al 1998). The clinical trial was therefore discontinued at the stage at which only 30 preterm infants had been enrolled.

1.2. Patients in tracheal aspirate studies

All the infants included were treated in the neonatal intensive care unit of the Hospital for Children and Adolescents, Helsinki University Central Hospital. Those infants enrolled were intubated at birth because of failure to establish spontaneous ventilation and all underwent mechanical ventilation during the study period. Those with major anomalies were excluded. All infants were treated according to the standard protocols of the neonatal intensive care unit. BPD was defined, in all studies, as the need for supplemental oxygen at the age of 36 gestational weeks, in association with chest radiographic findings typical for BPD (Shennan et al 1988).

1.2.1. Preterm infants

For Studies I, II, and III, a total of 70 infants were enrolled between August 1993 and October 1997. Of these infants, six were included in both Studies I and II. Patients in Studies II and III were mostly the same; patients were selected for these two studies so that there were equal numbers of infants developing BPD and surviving without BPD. These groups were matched according to gestational age and birth weight (Table 1).

1.2.2. Term infants without primary lung injury

Healthy term infants. Study II comprised 35 healthy term infants (18 males, 17 females, gestational age 39.5±2.3 wk, birth weight 3478±482 g) from normal pregnancies. Of these, 22 were intubated for tracheal suctioning because of meconium-stained amniotic fluid. In none of them was any significant amount of meconium found in the trachea. Of the other 13, blood samples were taken together with samples for clinical analyses during the first postnatal week.

Intubated term infants. Five other term infants (3 males, 2 females, gestational age 38.9±2.2 wk, birth weight 3324±651 g) who had cardiac anomalies without pulmonary pathology and had cardiac surgery during the first 10 postnatal days were enrolled between June 1998 and February 1999. A TAF sample was collected before surgery. These infants had no prenatal complications, and none had infections.

1.2.3. Infants in the dexamethasone study

This open-label study (IV), carried out between August 1997 and July 1999, comprised 30 preterm infants (19 males, 11 fe-

Table 1. Patient data in preterm infants (Studies I to III)

Parameter	Study		
	I (N=44)	II (N=31)	III (N=32)
Prenatal			
Antenatal betamethasone	36 (82%)	25 (81%)	28 (88%)
Pre-eclampsia [1]	8 (18%)	7 (23%)	9 (28%)
PROM [2] or chorionamnionitis [3]	16 (36%)	14 (45%)	14 (44%)
At birth			
Mode of delivery (section/vaginal)	25 / 19	19 / 12	18 / 14
Gestational age (wks)	27.3 ± 2.0	26.7±1.8	27.0 ± 1.7
Birth weight (g)	962 ± 319	819 ± 167	906 ± 248
Gender (M / F)	23 / 21	16 / 15	18 / 14
Apgar score 1 min	5 ± 2	5 ± 2	5 ± 2
Umbilical cord pH	7.26 ± 0.11	7.26 ± 0.12	7.28 ± 0.12
Umbilical cord base excess	-3.9 ± 3.9	-3.5 ± 3.7	-3.3 ± 4.0
Postnatal			
Initial arterial / alveolar ratio	0.21 ± 0.14	0.23 ± 0.20	0.21 ± 0.12
Surfactant therapy	35 (80%)	24 (77%)	25 (78%)
Doses of surfactant	3 ± 1	3 ± 1	2 ± 1
Indomethacin therapy	34 (77%)	27 (87%)	28 (88%)
Postnatal dexamethasone during study period [4]	3 (7%)	7 (23%)	6 (19%)
Mean inhaled oxygen during during study period (%) [4]	34 ± 15	35 ± 15	35 ± 13
Duration of mechanical ventilation (d)	16 ± 14	24 ± 18	22 ± 18
BPD [5]	13 (30%)	16 (52%)	17 (53%)
Death	3 (7%)	2 (7%)	2 (6%)

1 Pre-eclampsia: elevated maternal blood pressure and proteinuria.

2 PROM: Premature rupture of the membranes > 24 hrs ante partum.

3 Chorionamnionitis: clinical signs, leukocytosis (B-leuk>14x109/L), and C-reactive protein concentration in plasma >50mg/L.

4 1 week in Study I, 10 days in Study II, 2 weeks in Study III.

5 Bronchopulmonary dysplasia, defined as need for supplemental oxygen at 36 gestational weeks, in association with typical chest radiographic findings.

In Studies II and III, patients selected to include equal numbers of infants subsequently developing BPD and those surviving without it.

males, gestational age 29.2±1.1 wk, birth weight 1241±154 g) randomized to receive dexamethasone (n=15) or to serve as controls (n=15). Inclusion criteria were birth weight 1000 to 1500 g and respiratory distress syndrome that required mechanical ventilation and surfactant treatment. Dexamethasone, given as a 4-day course, was started at the age of 12 to 24 hours at a dose of 0.5 mg/kg/day for 2 days and 0.25 mg/kg/day for the subsequent 2 days (Study IV, Table 1).

1.2.4. Infants with PPHN

For Study II, 23 infants with PPHN (12 males, 11 females, gestational age 37.2±3.8 wk, birth weight 3111±964 g) were enrolled between 1993 and 1997. PPHN was diagnosed as Doppler ultrasound-confirmed

iso- or suprasystemic pulmonary artery pressure and persistent hypoxia despite optimal conventional therapy.

1.3. Patients in immunohistochemistry studies

Subjects for immunohistochemistry studies were collected between 1985 and 1999. Of the 14 infants, 7 were born prematurely: 3 of these died of acute respiratory distress syndrome during the first postnatal week and 4 of bronchopulmonary dysplasia. Of the 7 term infants, 4 died of cardiac anomalies and 2 of PPHN, and a lung biopsy was obtained from an infant suffering alveolar-capillary dysplasia. In addition, the 4 fetuses enrolled were aborted because of major extrapulmonary anomalies; all had microscopically and macroscopically normal lungs. None of the infants had pneumonia at the time of death. Those term infants who died of congenital cardiac anomalies had macroscopically and microscopically normal lungs. (Study II, Table 1).

2. Methods

2.1. Sample collection

TAF. Samples of tracheal aspiration fluid (TAF) were collected once daily by standardised routine tracheal lavage. One mL of sterile isotonic saline was instilled into the endotracheal tube, the patient was manually ventilated for 3 breaths, and the trachea was suctioned twice for 5 seconds. For analysis of tracheal aspirates, secretions were collected into a trap and transferred into tubes containing 500 I.U. of aprotinin (Trasylol®, Bayer, Leverkusen, Germany) and 5 mg of deferoxiamine (Desferal®, Ciba, Basel, Switzerland). The tubes were stored at -20°C until analysis.

In Studies I and II, a total of 341 TAF samples were collected from 69 preterm infants during their first 10 postnatal days. In Study II, 27 samples were collected during postnatal weeks 3 to 5 from 8 infants who developed BPD; from 22 healthy term infants came 22 samples taken at birth; from 5 intubated term infants with cardiac anomalies and without primary lung injury, 9 samples during the first 10 postnatal days; and from 12 PPHN infants, 54 samples during the first 10 postnatal days. In Study III, 172 TAF samples were collected from 32 preterm infants during the first 2 postnatal weeks. In Study IV, 41 samples were collected from 15 infants in the dexamethasone group, and 49 samples from 15 infants in the control group during the first postnatal week.

Plasma. In Study I, 24 blood samples were taken from 9 preterm infants through radial artery catheters into EDTA tubes. In Study II, 13 blood samples were taken from 13 healthy term infants from peripheral veins and 29 blood samples from 11 PPHN infants through peripheral arterial catheters into EDTA tubes. All tubes were centrifuged (3000 rpm for 10 min), and plasma was stored at -20°C until analysis.

2.2. Assays from tracheal aspirate samples

All assays from tracheal aspirate samples were performed in the Scientific Laboratory in the Hospital for Children and Adolescents, University of Helsinki.

VEGF and HGF. VEGF was analyzed by the Quantikine Human VEGF Immunoassay (R&D Systems, Oxon, UK). HGF in TAF was analyzed by the Quantikine Human HGF Immunoassay (R&D Systems, Oxon, UK).

Analyses for dilution of the samples. In Study I, a correction for dilution of samples was calculated by using the ratio of urea-N in TAF and in each corresponding serum sample.

For Studies II to IV, in order to eliminate the effect of dilution of TAF samples, the concentration of the secretory component of immunoglobulin-A (IgA-SC) was determined by direct ELISA. Concentration

of IgA-SC in lung secretions is independent of capillary leakage, and the concentration of IgA-SC in tracheal aspirates is independent of respiratory distress, gestational age, or postnatal age (Watts et al 1992). Secretory IgA isolated from human colostrum served as the standard, and the results were standardised with the help of Dr. B. Götze-Speer and Prof. Ch. Speer (University Children's Hospital, Wyrzburg, Germany). Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at +4°C with 100-µL aliquots of 1:2000 diluted anti-human secretory component (Dako, Glostrup, Denmark) in 50 mM Na bicarbonate, pH 9.5. After washing with 200 µL of 20-mM tris-500 mM NaCl, pH 7.5 (TBS), the plates were blocked for unspecific protein binding by incubation with 200 µL of 2% bovine serum albumin (BSA) in TBS and washed with 0.05% Tween 20 in TBS (TTBS). TAF samples were diluted to between 1:10 to 1:500 in diluting buffer (1% BSA in TTBS), and 100-µL aliquots were added to the wells. After incubation overnight at room temperature, the plates were washed 3 times with TTBS; 100 µL of peroxidase-conjugated rabbit anti-human SC (Dako), diluted 1:400 in diluting buffer, was added, and the plates were incubated for 4 hours at room temperature. After washing with TTBS, the plates were developed with 100 µL of substrate solution containing 8 mg of orthophenylenediamine (Dako) and 5 µL of 30% H2O2 in 12 mL water. After 30 minutes at room temperature, the optical densities of the plates were read at 450 nm.

Measurements for surfactant maturity. In Study I, a tracheal aspirate sample was taken within 3 hours postnatally, before the infants' treatment with surfactant for determination of the lecithin/sphingomyelin ratio (LS-ratio), and presence of ceramide lactoside (Rauvala et al 1984, Hallman et al 1989).

2.3. Immunohistochemistry

All immunostainings were performed in the Haartman Institute Laboratory, University of Helsinki. Autopsies were performed within 2 days after death. The samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and kept in dry storage at room temperature. After sectioning, the slides were used for immunoperoxidase stainings within 2 weeks. For stainings, after drying overnight at 37°C, 4- or 5-µm sections were cut on coated slides, deparaffinized, and rehydrated. The sections were either treated with trypsin or microwaved. Endogenous peroxidase was quenched by incubating the sections in methanol and hydrogen peroxidase. After applying blocking serum, the sections were incubated overnight at +4°C with the primary antibody. Immunohistochemical stainings were performed by use of commercial rabbit, goat, or mouse Elite ABC kits (Vectastain, Vector Laboratories, Burlingame, CA, USA). The following day, the sections were incubated with the biotinylated secondary antibody and the peroxidase-labeled ABC for 30 minutes each. Bound peroxidase was visualized by incubation in a 3-amino-9-ethylcarbazole (AEC) solution (Sigma A-5754). Finally, the sections were stained with hematoxylin. Antibodies used were: VEGF: A-20, Santa Cruz, 1:100 dilution, and Flt-1: C17, Santa Cruz sc 316, 1:100 dilution. A negative control was included in each staining round. In these slides, staining was performed by the identical protocol without primary antibody.

2.4. Statistical analyses

In all studies, patient data are given as mean±SD, and results as mean±SEM. When data were not normally distributed, logarithmic transformations of the data were performed. P-values less than 0.05 were con-

sidered statistically significant. All calculations were done with StatView 4.1 (I) or StatView 5.0 (II-IV).

Study I. Kruskal-Wallis one-way ANOVA and the Mann-Whitney U-test served for analyses between concentrations of VEGF in TAF and categorical variables. Simple regression analysis was used for analyses between concentrations of VEGF in TAF and continuous variables. The chi-square served for analyses between categorical variables. The Bonferroni correction served for post-hoc comparisons.

Study II. One-way ANOVA and Student's t-test served for analyses between concentrations of VEGF in TAF and categorical variables. Simple regression analysis was used for analyses between concentrations of VEGF in TAF and continuous variables. The Bonferroni correction served for post-hoc comparisons.

Study III. One-way ANOVA and Student's t-test served for analyses between concentrations of HGF in TAF and categorical variables. Simple regression analysis was used for analyses between concentrations of HGF in TAF and continuous variables. A multiple regression analysis was performed using concentration of HGF as the dependent variable and all significant univariate repeated-measurements-adjusted correlations as independent variables. The chi-square was used for analyses between infants developing BPD and those surviving without BPD in categorical variables. The Bonferroni correction served for post-hoc comparisons.

Study IV. One-way ANOVA and Student's t-test served for analyses between concentrations of VEGF or HGF in TAF and categorical variables. Simple regression analysis was used for analyses between concentrations of VEGF or HGF in TAF and continuous variables. Student's t-test was used in analyses of differences between the dexamethasone group and the control group in continuous variables. The chi-square test served for analyses between the dexamethasone group and the control group for categorical variables. The Bonferroni correction served for post-hoc comparisons.

Results

1. VEGF during the perinatal period (Studies I and II)

VEGF in TAF in preterm infants. The mean concentration of VEGF in TAF increased from 25±12 pg/mL on the first day to 526±120 pg/mL on day 7. In Study I, a correction for dilution of the tracheal aspirates was performed in 69 of the 189 samples. In these samples, the actual mean concentrations of VEGF were estimated to be 4.1±0.9 ng/mL during days 1 to 3, and 16.3±2.3 ng/mL during days 4 to 7. In Study II, the secretory component of immunoglobulin A (IgA-SC) was measured, and data were corrected for dilution by adjustment with IgA-SC before all statistical analyses. The dilution-adjusted mean concentration of VEGF at birth for preterm infants was 4±2 pg/mL/IgA-SC unit on day 1, and 65±17 pg/mL/IgA-SC unit on day 10, and the mean concentration of VEGF during the first 10 postnatal days was 54±6 pg/mL/IgA-SC (Study II, Figure 2).

In preterm infants, no correlations were seen between VEGF in TAF and gestational age or birth weight. Mean VEGF correlated inversely with Apgar score, and with pH and base-excess in blood-gas analysis from the umbilical cord artery. In preterm infants born to mothers with premature rupture of the membranes or with chorionamnionitis, or with both, VEGF was higher and in preterm infants born to mothers with preeclampsia lower than in preterm infants without these antenatal complications. In 32 preterm infants, a tracheal aspirate sample was obtained within 3 hrs after birth for determination of surfactant maturity. In these samples, the LS-ratio showed a correlation with VEGF. In 7 of these 32 infants, ceramide lactoside was present, and they had higher VEGF. The presence of ceramide lactoside was associated with premature rupture of the membranes and with chorionamnionitis (P=0.0026) (Table 2).

VEGF in TAF in term infants without primary lung injury. For healthy term infants, the mean concentration of VEGF at birth was 4±1 pg/mL (dilution-adjusted mean concentration 0.5±0.2 pg/mL/IgA-SC unit). For intubated term infants, the mean concentration of VEGF during the first 10 postnatal days was 434±246 pg/mL (dilution-adjusted mean concentration 13.7±8.6 pg/mL/IgA-SC unit) (Study II, Figure 2).

VEGF in plasma. The mean concentration of VEGF in plasma during the first postnatal week was for preterm infants 48±6 pg/mL, and for healthy term infants 138.5±39.4 pg/mL (Study II, Figure 2).

VEGF immunohistochemistry. For VEGF in fetuses and in premature infants, positive immunostaining was apparent in bronchial epithelial cells, in cuboidal cells in the alveolar epithelium, and in vascular endothelial cells; in addition, some alveolar macrophages were positive. In term infants, VEGF staining was visible in bronchial epithelial cells and in alveolar macrophages (Study II, Figure 1).

Flt-1 immunohistochemistry. In fetuses, positive staining was apparent in capillaries, veins, and small arteries in the endothelial cells, and in the intima of the small arteries, and a staining reaction was visible in alveolar epithelium in bronchial epithelial cells and cuboidal cells. In premature infants, a similar staining reaction was visible in vessels and bronchi. In term infants, positive staining for Flt-1 was seen in the endothelial lining of veins and capillaries and arteries, as well as in the intima of the small arteries, and in bronchial epithelial

Table 2. VEGF in TAF and perinatal factors in preterm infants (Studies I and II)

Parameter	Study I (pg/mL) D 1-3	Study II (pg/mL/IgA-sc unit) D 4-7	D 1-10
Prenatal			
Antenatal betamethasone	NS	NS	NS
Pre-eclampsia [1]	NS	88±18 vs 308±50; P=0.0001 [4]	NS
PROM [2] or chorionamnionitis [3]	NS	504±68 vs 308±50; P=0.0058 [4]	NS
At birth			
Gestational age (wks)	NS	NS	NS
Birth weight (g)	NS	NS	NS
Apgar score 1 min	R=-0.30, P=0.012	P=-0.28, R=0.0028	NS
Umbilical cord pH	NS	NS	R=-0.36, P=0.050
Umbilical cord base excess	R=-0.46, P=0.0084	R=-0.30, P=0.024	R=-0.54, P=0.0001
LS-ratio [5]	R=0.29, P=0.031	NS	-
Presence of ceramide lactoside	NS	623±111 vs 243±25; P=0.0009	-

1 Pre-eclampsia: maternal elevated blood pressure and proteinuria (vs infants without maternal complications).

2 PROM: Premature rupture of the membranes > 24 hrs ante partum.

3 Chorionamnionitis: clinical signs, leukocytosis (B-leuk>14x109/L), or C-reactive protein concentration in plasma >50mg/L.

4 vs. infants without maternal complications.

5 Lecithin/sphingomyelin ratio, measured from tracheal aspirate sample obtained within 3 hrs after birth.

- Data unavailable.

cells (Study II, Figure 1).

2. VEGF in lung injury in preterm infants (Studies I and II)

VEGF in TAF and parameters of respiratory distress. In Studies I and II, although no correlation existed between initial arteriolar-alveolar ratio and VEGF, patients who required surfactant therapy had lower VEGF than those who survived without surfactant. Moreover, an inverse correlation also appeared between mean VEGF and number of surfactant doses required. Inverse correlations existed between VEGF and both mean inspiratory oxygen and duration of mechanical ventilation. In neither study was any difference found in VEGF concentrations between patients receiving glucocorticoid treatment (antenatal or postnatal) or not (Table 3).

VEGF in TAF in infants developing BPD. In Study I, during days 4 to 7, those 13 patients who developed BPD had a lower mean VEGF than those surviving without BPD (Figure 2, Table 3). In Study II, preterm infants who developed BPD had lower VEGF in TAF during the first 10 postnatal days than did those who survived without it, but this difference did not reach statistical significance (Table 3). In Study II, for those 8 BPD infants there was a tendency towards lower VEGF concentrations during weeks 3 to 5 than during first 10 postnatal days (33.4±3.6 pg/mL / IgA-SC unit vs. 49.6±9.1 pg/mL / IgA-SC unit, P=0.061).

VEGF and Flt-1 immunohistochem-

Table 3. VEGF in TAF and parameters of respiratory distress (Studies I and II)

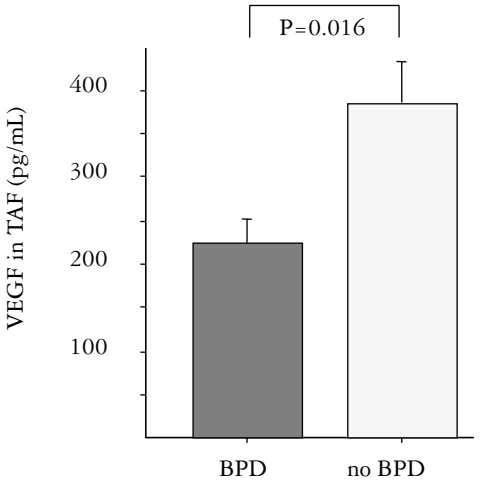
Parameter	Study I days 1 to 3 (pg/mL)	Study I days 4 to 7 (pg/mL)	Study II days 1 to 10 (pg/mL/IgA-sc unit)
Surfactant therapy (+ vs -)	NS	290±34vs653±135, P=0.0025	36±4vs97±27, P=0.0011
Doses of surfactant	R=-0.26,P=0.034	R=-0.35, P=0.0022	R=-0.26, P=0.0077
Dexamethasone therapy (+ vs -)	NS	NS	NS
FiO2 (%)[1]	NS	R=-0.29, P=0.0092	NS
Duration of intubation (d)	NS	NS	R=-0.25, P=0.01
BPD (+ vs -)[2]	NS	235±31vs383±50, P=0.016	46±7vs64±17, P=0.28

1 FiO2: Mean supplemental oxygen during study period.

2 Defined as need for supplemental oxygen at 36 gestational weeks, in association with typical chest radiographic findings.

istry in infants developing BPD. Positive immunostaining for VEGF appeared in BPD infants in bronchial epithelial cells and alveolar macrophages, in vascular endothelium, and in cells apparently representing type-II pneumocytes. For Flt-1, in BPD infants, positive staining was visible throughout the walls of small arteries, and in the endothelial lining of veins and capillaries. In addition, bronchial epithelium was positive, as were cells apparently representing type-II pneumocytes in the alveoli, and alveolar macrophages (Study II, Figure 1).

Figure 2.



Mean VEGF concentrations in TAF during postnatal days 4-7 in preterm infants developing BPD and in those surviving without BPD. (Study I)

VEGF in PPHN-infants. In PPHN infants, the mean concentration of VEGF in TAF during the first 10 postnatal days was 205±30 pg/mL (dilution-adjusted mean concentration 19.6±3.5 pg/mL/IgA-SC unit vs. 13.7±8.6 pg/mL/IgA-SC unit in intubated term infants without primary lung injury; P=0.10). In plasma, the mean concentration of VEGF for PPHN infants was 43.5±6.7 pg/mL vs. 138.5±39.4 pg/mL in healthy term infants, P=0.039) (Study II, Figure 2).

In immunohistochemistry in PPHN infants, staining for VEGF was apparent in bronchial epithelium and vascular endothelial cells. Additional staining was visible in cells apparently representing type-II pneumocytes in alveolar epithelium. In the infant with alveolar-capillary dysplasia, strong staining appeared in bronchial epithelium and vascular endothelial cells; also alveolar macrophages were positive. In PPHN infants and in the infant with alveolar-capillary dysplasia, staining for Flt-1 was apparent throughout the vascular walls in capillaries, arteries, and veins. Bron-

chial epithelium and cells apparently representing type-II pneumocytes in alveolar epithelium were positive, as well as alveolar macrophages (Study II, Figure 1).

3. HGF during the perinatal period and in lung injury in preterm infants (Study III)

HGF in TAF and perinatal parameters. In preterm infants, the mean concentration of HGF was 1113±597 pg/mL on the first postnatal day, 981±382 pg/mL on day 14, and 619±64 pg/mL during the first 2 postnatal weeks. IgA-SC, the secretory component of immunoglobulin A, was measured, and data were corrected for dilution by adjustment with IgA-SC before analysis. The dilution-corrected values were: day 1, 80.5±27.3 pg/mL/IgA-SC unit; day 14, 50.2±16.37 pg/mL/IgA-SC unit; and during the first 2 postnatal weeks, 71.4±15.7 pg/mL/IgA-SC unit.

No differences existed between mean concentrations of HGF during the first 2 postnatal weeks in patients born to mothers with premature rupture of the membranes or with chorionamnionitis, in patients born to mothers with pre-eclampsia, or in infants without these maternal complications. Of the mothers of these infants, 28 received treatment with an antenatal glucocorticoid, and the number of treatments correlated negatively with mean HGF concentration (R=-0.22, P=0.021). During the first 2 postnatal weeks, mean HGF correlated negatively with gestational age (R=-0.32, P=0.0001), but not with birth weight. No correlations existed between mean HGF during the first 2 postnatal weeks and 1' and 5'-minute Apgar scores, or base-excess and pH in blood-gas analysis from the umbilical cord artery (Study III, Table 2).

HGF in TAF and parameters of respiratory distress. Those 7 infants surviving without surfactant therapy had higher mean HGF concentrations than did those needing surfactant (145.9±49.6 pg/mL/IgA-SC unit vs. 49.6±13.7 pg/mL/IgA-SC unit; P=0.0001). A tendency toward a negative correlation existed between mean HGF concentration and number of surfactant doses required (R=-0.16, P=0.06), and between HGF and duration of mechanical ventilation. No association appeared between mean HGF concentration and need for postnatal dexamethasone, or between mean HGF concentration and indomethacin treatment (Study III, Table 2).

HGF in TAF and development of BPD. The 15 infants who survived without BPD showed higher mean HGF concentrations during the first 2 postnatal weeks than did those who subsequently developed BPD (101.8±31.9 pg/mL/IgA-SC unit vs. 44.5±8.9 pg/mL/IgA-SC unit, P=0.028 (Study III, Figure 1 and Table 2).

4. Effects of dexamethasone on VEGF and HGF (Study IV)

Dexamethasone and clinical parameters. In the dexamethasone group, less BPD was observed (1/15 vs. 7/15 in control group, P=0.01), but no differences existed between dexamethasone and control groups in regard to other perinatal parameters

Because TAF samples were not available every day from every patient, the mean values on postnatal days 1 to 2, 3 to 4, and 5 to 7 served for analysis. The secretory component of immunoglobulin A (IgA-SC) was measured, and data were corrected for dilution by adjustment with IgA-SC before analysis.

Dexamethasone and VEGF. No differences existed between the dexamethasone and control groups in mean VEGF levels on days 1 to 2 (18.4±7.5 vs. 14.8±5.0 pg/mL/IgA-SC unit, respectively, P=0.70), on days 3 to 4 (35.4±5.6 vs. 38.7±10.7 pg/mL/IgA-SC unit, P=0.77), or on days 5 to 7 (48.8±9.0 vs. 37.9±9.0 pg/mL/IgA-SC unit, P=0.45) (Study IV, Figure 1).

Dexamethasone and HGF. No differ-

ences existed in mean HGF concentrations between the dexamethasone and control groups on days 1 to 2 (62±8 vs. 110±32, pg/mL/IgA-SC unit, respectively, P=0.16). The dexamethasone group had a lower mean HGF concentration than the control group on days 3 to 4 (41±5 vs. 96±20 pg/mL/IgA-SC unit, respectively, P=0.0022) and on days 5 to 7 (47±11 vs. 123±49 pg/mL/IgA-SC unit, P=0.030) (Study IV, Figure 1).

Infants who had received antenatal betamethasone had a tendency towards a lower mean HGF on days 1 to 2 than did those given no betamethasone (69±11 vs. 144±65 pg/mL/IgA-SC unit, P=0.064).

Discussion

1. VEGF and lung development

VEGF plays a pivotal role during fetal development: inactivation of even a single VEGF allele in mice results in early embryonic lethality: development of vascular endothelial cells is blocked, and the formation of blood vessels is abnormal, including the pulmonary vasculature (Carmeliet et al 1996, Ferrara et al 1996, Bautch et al 2000, Ng et al 2001). VEGF plays a role, in addition to development, also postnatally, in physiological angiogenesis and in vascular maintenance (Leung et al 1989, Pepper et al 1991, Shifren et al 1994). The concentrations of VEGF in TAF in our studies in preterm infants fell into the same range as that shown in vitro to induce proliferation and differentiation of human fetal airway epithelial cells (Acarregui et al 1998). The level of VEGF in almost all infants studied was low after birth but increased steadily during the early postnatal period. Its concentrations in TAF correlated neither with gestational age nor with birth weight, but VEGF levels were higher at birth and during the first 10 postnatal days in preterm than in term infants. Moreover, we discovered a correlation between the functional maturity of alveolar type II cells, defined as the LS-ratio, in a TAF sample (Rauvala et al 1984), and VEGF in TAF, suggesting that concentration of VEGF may reflect the functional maturity of the preterm lung.

We found constant expression for VEGF in all fetuses and infants in bronchial epithelium and alveolar macrophages, and in addition, in fetuses and preterm infants also in alveolar epithelium. Pulmonary VEGF expression during development has been found by immunohistochemistry in nonendothelial cells including alveolar and bronchial epithelial cells and alveolar macrophages, and in smooth muscle cells including those lining blood vessels (Shifren et al 1994, Brogi et al 1996, Acarregui et al 1999, Bhatt et al. 2001). In situ hybridization has shown pulmonary VEGF expression during development most strongly in alveolar epithelial cells but also in bronchial epithelial cells and smooth muscle cells (Hirose et al 2000, Bhatt et al 2001). Pulmonary Flt-1 staining during development has been found in immunohistochemistry mainly in vascular endothelium (Peters et al 1993), and for Flt-1, we found positive staining in endothelial cells lining capillaries, veins, and small arteries. Moreover, we discovered that VEGF level was significantly higher in TAF than in plasma in preterm infants. These data, in line with previous findings, suggest a paracrine role for VEGF, secreted by nonendothelial cells and modulating activities in adjacent vascular endothelium.

In contrast to previous findings, we found additional staining for VEGF in the vascular endothelium in fetuses and in preterm infants and for Flt-1 in bronchial epithelial cells in fetuses and in infants. One explanation for these surprising results may be due to nonspecific staining during immunohistochemistry. A negative control, in which staining was performed by an identical protocol without a primary antibody, was included in each staining round, and in these slides no staining was apparent. It is possible, however, that the polyclonal antibodies we used (for VEGF, Santa-Cruz A-20, and for Flt-1, Santa-Cruz C-17) exhibit some nonspecific binding properties. Other antibodies directed against these proteins could be used for comparative stainings along with antigen-blocked antiserum as a

control. On the other hand, in situ hybridization could be used to confirm the cells that produce the respective RNAs. One likely explanation for VEGF staining in the endothelium may be that in these cells the ligand is bound to its receptor.

The persistent appearance of VEGF and Flt-1 during the perinatal period from the 16th gestational week supports a pivotal role for VEGF in the development of the human lung. We suggest that the pulmonary VEGF level in preterm infants may be gestational-age dependent and that the increase in early postnatal VEGF in preterm and term infants may represent a physiological phenomenon belonging to the early neonatal period.

2. VEGF in lung injury in preterm infants

Antenatal complications. We detected lower VEGF in TAF postnatally in preterm infants from mothers with pre-eclampsia. Similarly, in pregnancies complicated by pre-eclampsia, VEGF mRNA levels in the placentas are lower (Cooper et al 1996). In premature infants and in fetal growth restriction, pre-eclampsia correlates with low concentrations of surfactant protein A and with a higher incidence of RDS (Schiff et al 1993, Kari et al 1995, Odegård et al 2000). Our finding of lower VEGF may be related to the postnatal respiratory difficulties seen in these infants, because VEGF induces surfactant protein expression in fetal alveolar epithelial cells in vitro and in vivo (Acarregui et al 1998, Compernolle et al 2002).

We found higher VEGF in TAF in preterm infants from pregnancies complicated by chorionamnionitis, and in addition, in tracheal aspirates, found ceramide lactoside, a marker for the presence of neutrophils indicative of antenatal infection (Hallman et al 1989), to be associated with high pulmonary VEGF. Chorionamnionitis has been associated with less severe RDS, and this effect has been ascribed to the effect of prenatal inflammation in accelerating lung maturation and in possible induction of the surfactant system (Watterberg et al 1996, Kitajima et al 1992). In experimental animals, pulmonary VEGF expression is induced by proinflammatory mediators (Pertovaara et al 1994, Brogi et al 1994), many of which have been shown to be higher in the lungs of preterm infants born to mothers with premature rupture of the membranes or with chorionamnionitis. (Groneck et al 1994, Watterberg et al 1996). Because pulmonary VEGF expression is induced by proinflammatory mediators (Pertovaara et al 1994, Brogi et al. 1994) it therefore seems that inflammation and infection also in preterm infants may raise pulmonary VEGF levels.

Hypoxia is a major stimulator of VEGF expression (Shweiki et al 1992). We found that in preterm infants, asphyxia at birth was associated with higher VEGF in TAF. Hyperoxia reduces pulmonary expression of VEGF in rats with hyperoxia-induced lung injury, and its expression increases during recovery to normoxia (Maniscalco et al 1995, Maniscalco et al 1997). We found an inverse correlation between mean inspiratory oxygen and VEGF in TAF in preterm infants postnatally. On the basis of our present data, it appears possible that in the lung of the preterm infant, hypoxia and hyperoxia both affect VEGF levels.

Lung injury. We discovered in preterm infants high pulmonary concentrations of VEGF during the end of the first postnatal week. In RDS, acute lung injury begins to resolve within the first postnatal days, after which a recovery phase usually begins (Verma 1995). In premature infants with RDS, pulmonary events associated with development of chronic lung injury, including enhanced pulmonary inflammation, are suggested to occur during the recovery phase at the end of the first week (Merritt et al 1983, Watts et al 1992, Groneck et al 1994). We found that in preterm infants

with more severe RDS (defined as lower initial arterial/alveolar ratio, greater need for surfactant, higher mean inspiratory oxygen, and longer duration of mechanical ventilation), VEGF in TAF was lower. In animal studies, VEGF has been suggested to participate in repair of lung injury and in protection of the lung against injury. Loss of HIF-2-a, a promoter for VEGF, causes fatal RDS in neonatal mice; in these mice, intrauterine or postnatal intratracheal instillation of VEGF stimulates surfactant production, improves lung function, and protects against RDS (Compernolle et al 2002). Prolonged exposure to hyperoxia in animals results in a decrease in VEGF (Johnston et al 1996, Klekamp et al 1999). Hyperoxia itself may explain our finding, or another explanation may be that infants with severe pulmonary injury may be incapable of responding to the inflammatory stimuli with an increase in VEGF. Because our data, however, suggest that the lower pulmonary VEGF in preterm infants associates with more severe RDS, VEGF may play a protective or a reparative role in neonatal lung injury in addition to its role in human lung development .

We found that preterm infants subsequently developing BPD had lower VEGF during the early postnatal period. Immunohistochemistry for VEGF and Flt-1 showed staining in type-II pneumocytes in alveolar epithelium only in infants with BPD. Rabbits recovering from hyperoxic lung injury have type-II pneumocytes exclusively expressing VEGF mRNA; this suggests a role for VEGF in the regulation of microvascular endothelial cell proliferation after oxidant injury (Maniscalco et al 1997). Thus, the presence of VEGF in the alveolar epithelium of our infants with BPD may be associated with the healing process.

Arrest of lung development. A significant change has occurred in the epidemiology and pathophysiology of BPD. The classical severe form of BPD has become less common, replaced by less severe forms of lung disease that affect the smallest preterm infants (Charafeddine et al 1999, Parker et al 1992, Rojas et al 1995). A newborn of 24 gestational weeks suffers from pulmonary prematurity: there are no alveoli yet present, surfactant production is just starting, and the capillary bed is poorly developed. The pathological pulmonary findings in infants with fatal BPD include a consistent lack of significant alveolarization, and a thickened alveolar septa resulting in emphysematous-looking lungs (Chambers et al 1989, Hislop et al 1990, Van Lierde et al 1991, Margraf et al 1991, Husain et al 1998). In addition, capillaries do not develop normally (Bhatt et al 2001). Inhibition of capillary growth, a central event during septation and alveolar maturation, may affect alveolarization in very premature infants and result in the pathological findings in infants with fatal BPD (Abman 2001). The pathogenesis of new BPD in very immature preterm infants has therefore been suggested to be caused primarily by arrest of normal lung development (Jobe 1999) and our finding of lower pulmonary VEGF during the early neonatal period in preterm infants developing BPD supports this hypothesis.

3. HGF during the perinatal period and in lung injury in preterm infants

HGF and lung development. We discovered in TAF in preterm infants significant amounts of HGF, the level of HGF postnatally in our preterm infants did not increase. However, we found higher HGF in TAF from more immature infants. HGF, a mesenchymal-derived growth factor for epithelial cells, is suggested to participate in organ formation and maturation during fetal development (Montesano et al 1991, Kagoshima et al 1992). In the lung, HGF elicits mitogenic and morphogenic actions in fetal alveolar type II cells and bronchiolar epithelial cells (Itakura et al 1997, Sato et al 1997). Addition of HGF stimulates

branching morphogenesis in alveolar and bronchial epithelia of the fetal rat lung, and HGF inhibition assays result in decreased epithelial branching (Ohmichi et al 1998a, 1998b). The rapid postnatal increase in HGF and c-met mRNA reported in neonatal rats suggests a role for HGF in lung development also postnatally (Kagoshima et al 1992). Our finding of a significant amount of lung-lining fluid HGF in human preterm infants supports a role for HGF in lung development.

Lung injury. In our study, infants with more severe RDS had lower HGF concentrations in TAF. This may represent an impaired response to the stimuli induced by lung injury, or it may be explained by damage to the cells producing HGF, or it may represent an insufficient capability of the immature lung to respond to lung damage. Acute respiratory distress is characterized by diffuse lung damage and alveolar epithelial injury (Piedboeuf et al 1996, Daly et al 1998). In experimental animals suffering acute lung injury, expression of HGF mRNA and HGF activity increase, and the HGF concentration in lung lavage fluid increases, both of which actions are associated with both bronchial and alveolar epithelial cell proliferation. In these animals, inhibition of HGF reduces the DNA synthesis of alveolar epithelial cells and aggravates the lung injury (Yanagita et al 1993, Adamson et al 1999, Yamada et al 2000). After pneumectomy in mice, neutralization of HGF suppresses the compensatory DNA synthesis in epithelial cells, whereas administration of recombinant HGF stimulates it (Sakamaki et al 2002). These data suggest that after acute lung injury, HGF production is augmented and that HGF as a pulmotrophic factor may mediate airway and alveolar regeneration in lung repair.

BPD. In our study, those infants who developed BPD had less HGF in TAF than did those who survived without BPD. Intravenous injection of human recombinant HGF stimulates DNA synthesis of airway and alveolar epithelial cells and prevents injury progression (Ohmichi et al 1996, Yaekashiwa et al 1997), and intratracheal instillation of recombinant HGF induces a time- and dose-dependent increase in type II cell proliferation (Panos et al 1996). We therefore suggest that since HGF is a pulmotrophic factor responsible for alveolar regeneration during lung repair, it plays a role in repair in lung injury also in human preterm infants; we further suggest that a relative lack of HGF may impair alveolar repair and thus contribute to the development of BPD.

4. Dexamethasone and VEGF and HGF

Dexamethasone and VEGF. Corticosteroids downregulate VEGF expression in vitro (Nauck et al 1997, Horiuchi et al 1997, Klekamp et al 1997), but in preterm infants, postnatal dexamethasone may raise VEGF levels in TAF (D'Angio et al 1999, Bhatt et al 2000). We found no differences, however, in concentrations of VEGF in TAF in preterm infants who were randomized to receive dexamethasone or not. More studies are therefore needed to evaluate in preterm infants the effect of corticosteroids on pulmonary VEGF in vivo.

Dexamethasone and HGF. We found lower HGF in TAF from preterm infants randomized to receive dexamethasone, in line with in vitro studies, showing that corticosteroids such as dexamethasone suppress HGF mRNA expression, and HGF production and secretion (Matsumoto et al 1992, Matsunaga et al 1994, Gohda et al 1994, Takai et al. 1997). Moreover, a tendency toward lower HGF was detectable in infants receiving antenatal betamethasone. In extremely low birth-weight infants, postnatal dexamethasone does not reduce the risk for development of BPD (Stark et al 2001). Glucocorticoid treatment improves postnatal lung function and reduces mortality from RDS, but at the same time may lead to per-

manent lung damage due to alveolar hypoplasia (Wíllet et al 2000). Glucocorticoids have been shown to inhibit septation and diminish the extent of the increase in alveolar surface area, resulting in emphysematous-looking lungs (Massaro et al 1985, Blanco et al 1989, Okajima et al 2001). It may be that the beneficial effect of dexamethasone is related to its effect on acute respiratory insufficiency; extremely low birthweight infants may however, have mild RDS or no RDS at all, yet still develop BPD. Postnatal dexamethasone may therefore show no beneficial pulmonary effects in extremely immature infants; conversely, it may worsen arrest of alveolar development. Since HGF has been shown to participate in epithelial repair after lung injury, we suggest that inhibition of HGF may be related to the adverse influences of dexamethasone on pulmonary development and on repair of acute injury in the preterm lung.

5. VEGF in PPHN

In contrast to findings in term infants without lung injury, we observed in PPHN infants positive staining for VEGF and for Flt-1 in type-II pneumocytes in alveolar epithelium. Moreover, we found a tendency toward higher concentrations of pulmonary VEGF protein in PPHN infants than in term infants without lung disease. In rats recovering from lung injury, pulmonary VEGF protein level increases and alveolar type-II cells exclusively express VEGF (Maniscalco et al 1995). The higher VEGF in TAF in PPHN infants and the additional expression of VEGF in alveolar epithelium may represent an enhanced production of VEGF due to impaired endothelial function in PPHN. However, circulatory VEGF was lower in our PPHN infants than in our term infants without lung disease. Since VEGF is a paracrine factor, it has been suggested that VEGF leaks from different tissues into the circulation (Shifren et al 1994). Lower circulatory VEGF in PPHN infants may therefore reflect an overall disturbance in vascular development. Another explanation is that the pulmonary bed may be an important source of circulating VEGF. In severe PPHN, there is a right-to left-shunt through the foramen ovale and also via the ductus arteriorus if it remains open. Consequently, pulmonary blood flow is diminished, which may contribute to low plasma concentrations in these infants.

Conclusions

Our findings of persistent pulmonary expression of VEGF and Flt-1 during the perinatal period, higher VEGF in TAF in preterm than in term infants, and a postnatal increase in VEGF in TAF in preterm infants all suggest a physiological role for VEGF in the developing lung. Those preterm infants who suffered more severe RDS and those who subsequently developed BPD had lower VEGF in TAF. These data indicate a role for VEGF in the preterm lung in protection or in recovery from acute lung injury. Lower VEGF in these infants may be related to the arrest of development seen in infants developing BPD.

The significant amount of HGF in TAF supports the notion of a role for HGF in human lung development. Lower HGF in human preterm infants with more severe RDS and in those subsequently developing BPD suggests a protective or regenerative role for HGF in their lung injury. Reduced levels of HGF but not of VEGF in their tracheal aspirates during the early postnatal period were evident in infants receiving early postnatal dexamethasone therapy. The suppressive effects of glucocorticoids on lung development may in part be mediated by a reduction in pulmonary HGF.

Future prospects

VEGF. Development of new BPD has been suggested to be caused by arrest of normal lung development: inhibition of capillary growth and impairment of septation and alveolarization (Jobe 1999, Abman 2001). VEGF plays a pivotal role in vascular development. A targeted deletion of VEGF, of its hypoxia-inducible promoter HIF-2-a, or of VEGF receptors; or inhibition of VEGF all result in severe developmental abnormalities and embryonic lethality (Fong et al 1995, Shalaby et al 1995, Carmeliet et al 1996, Ferrara et al 1996, Bautch et al 2000, Compernolle et al 2002). Inhibition of the VEGF receptor Flk-1 results in impaired alveolarization, alveolar septal cell apoptosis, and emphysematous lungs (Kasahara et al 2000, Compernolle et al 2002). The insufficient surfactant production and fatal RDS in HIF-2-a knockout mice can be reversed by intrauterine or postnatal intratracheal instillation of VEGF (Compernolle et al 2002). Because VEGF is also a factor able to induce vascular permeability (Dvorak et al 1995), administration of VEGF can induce permeability-caused pulmonary edema. In mice, however, intratracheally administered VEGF does not stimulate vascular leakage or bronchial edema. Moreover, such VEGF remains restricted to the alveolar compartment and does not leak in significant amounts into the circulation (Compernolle et al 2002). These data indicate that VEGF administration has protective and reparative roles in lung injury in neonatal mice. It may therefore be the case that in extremely preterm human infants at risk for development of BPD, intratracheal treatment with VEGF will improve lung function and prevent development of BPD.

HGF. Alveolar type-II cell proliferation occurs in response to lung injury and is thought to play a critical role in alveolar epithelial repair (Daly et al 1998, Piedboeuf et al 1996). HGF treatment in experimental animal lung injury stimulates DNA synthesis of airway and alveolar epithelial cells, induces time- and dose-dependent alveolar type-II cell proliferation, and acts as a pulmotrophic factor preventing the progression of injury (Panos et al 1996, Ohmichi et al 1996, Yaekashiwa et al 1997). On the other hand, in the adult ARDS patient, a high level of HGF is associated with poor prognosis (Stern et al 2000), Moreover, in adult rats with oxygen-induced lung injury, HGF administration inhibits surfactant metabolism in type-II cells in vitro (Vivekananda et al 2000). Before one can speculate as to the potential therapeutic use of HGF in human preterm infants, in vivo animal studies are needed to reveal whether HGF treatment does inhibit surfactant metabolism.

Acknowledgements

This work was carried out at the Hospital for Children and Adolescents, University of Helsinki, Finland, during the years 1996 to 2002.

Professor Emeritus Jaakko Perheentupa and Veli Ylitalo, the former and present Heads of the Hospital for Children and Adolescents; Professor Mikael Knip, Head of that hospital; Professor Erkki Savilahti, Head of its Research Laboratory; and Professor Markku Heikinheimo are thanked for providing an excellent research atmosphere and research facilities.

I wish to express my gratitude to all of you who made this work possible and I especially wish to acknowledge:

Professor Sture Andersson, my supervisor, for introducing me the world of science and for showing that it does not have to be dull. For all the cigars and three-star Jaloviina shots during the good times, and for all the laughs and jokes during the bad times... You are an inspiring example of a scientist.

Professors Steven Abman and Kari Alitalo, the official reviewers of this thesis, thank you for the thorough review of the manuscript and for your comments and criticism that improved this book.

My co-authors, for rewarding collaboration: Maila Turanlahti and Irmeli Nupponen, for enjoyable time spent together doing our theses during these years; Päivi Heikkilä, for help with immunohistochemistry and pathology; Professor Leif Andersson, for all those admirable ideas and for teaching me the scientific way of thinking; Ari Ristimäki, for aid with this work as well as for ideas for other studies; Professors Olavi Ylikorkala and Lasse Viinikka, for guidance during the beginning of my scientific career; Annikki Sarnesto and Kristina von Boguslawski, for skilled methodological assistance; Anneli Kari and Maija Pohjavuori, for assistance with the clinical aspects of this work.

Marjatta Vallas, Elina Laitinen, and Merja Lahtinen, for excellent laboratory work.

Carol Norris, for intensive and skilful revision of the language.

Professor Sirpa Asko-Seljavaara and others at the Department of Plastic Surgery for letting me have time off from work for finishing my thesis.

My parents, Eila and Allan, and brother Jan, for supporting me during these years. My parents-in-law, Ulla and Matti, and my sister- and brother-in-law Tuuli and Olli, for taking me into their family.

All my friends in different fields: Kalle for sharing with me interests in interior design and carpentery as well as in plastic surgery; Arno for reminding me of all the other things in life besides work; Harri and the boys from the Band; Tuomas and Hessu for being my friends during these years.

Heini, I thank you for sharing with me this academic life, for being in Lönnrothinkatu 20 in September 1994.

The study was financially supported by grants from the Academy of Finland, the Duodecim Research Fund, the Ella and Georg Ehrnrooth Foundation, Finska Läkaresällskapet, the Foundation for Pediatric Research, the Helsinki University Central Hospital Research Fund, and the Orion Research Fund.

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