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Balance of growth factors in the human perinatal lung

Implications for physiological lung development and link to bronchopulmonary dysplasia

Joakim Janér, M.D.

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki in Lecture Hall 2, Biomedicum Helsinki, on February 27th 2009, at 12 noon.

Helsinki 2009

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Kopiotaito Oy
Helsinki 2009

To my family; Nina-Maria, Alexander and Iris

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Original publications

This Thesis is based on the following publications:

I Janér J, Andersson S, Haglund C, Karikoski R, Lassus P. Placental growth factor and vascular endothelial growth factor receptor-2 in human lung development. Pediatrics 2008 Aug;122(2):340-346.

II Janér J, Andersson S, Haglund C, Lassus P. Pulmonary endostatin perinatally and in lung injury of the newborn infant. Pediatrics. 2007 Jan;119(1):e241-6.

III Janér J, Andersson S, Kajantie E, Lassus P. Endostatin concentration in cord plasma predicts the development of bronchopulmonary dysplasia in very low birth weight infants. Pediatrics 2009; in press.

IV Janér J, Lassus P, Haglund C, Paavonen K, Alitalo K, Andersson S. Pulmonary vascular endothelial growth factor-C in development and lung injury in preterm infants. Am J Respir Crit Care Med. 2006 Aug 1;174(3):326-30.

The publications are referred to in the text by their Roman numerals.

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Abbreviations

ΔNΔC-VEGF-C	mature proteolytically processed human VEGF-C lacking the N-terminal and C-terminal propeptides
ABC	avidin-biotin complex
BE	base excess
BPD	bronchopulmonary dysplasia
BSA	bovine serum albumin
BW	birth weight
CPAP	continuous positive airway pressure
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
EMAP-II	endothelial monocyte-activating polypeptide-II
FiO_2	mean supplemental fraction of inspired oxygen during the first 2 postnatal weeks
GA	gestational age
IgA-SC	secretory component of IgA
IL	interleukin
LC	lactosyl ceramide
LYVE-1	lymphatic vessel endothelial HA receptor-1
nCPAP	nasal CPAP
OR	odds ratio
PlGF	placental growth factor
Prox1	prospero-related homeobox1
RDS	respiratory distress syndrome
SD	standard deviation
SEM	standard error of the mean
TAF	tracheal aspirate fluid
TBS	20-mM tris-500 mM NaCl, pH 7.5
TGF-ß	transforming growth factor-ß
Tie	tyrosine kinase with Ig and EGF homology domains
TTBS	0.05% Tween 20 in TBS
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLBW	very low birth weight

Abstract

The aims of this Thesis was to evaluate the role of proangiogenic placental growth factor (PlGF), antiangiogenic endostatin and lymphangiogenic vascular endothelial growth factor (VEGF) -C as well as the receptors vascular endothelial growth factor receptor (VEGFR) -2 and VEGFR-3 during lung development and in development of lung injury in preterm infants. The studied growth factors were selected due to a close relationship with VEGF-A; a proangiogenic growth factor important in normal lung angiogenesis and lung injury in preterm infants.

The thesis study consists of three analyses. I: Lung samples from fetuses, preterm and term infants without lung injury, as well as preterm infants with acute and chronic lung injury were stained by immunohistochemistry for PlGF, endostatin, VEGF-C, VEGFR-2 and VEGFR-3. II: Tracheal aspirate fluid (TAF) was collected in the early postnatal period from a patient population consisting of 59 preterm infants, half developing bronchopulmonary dysplasia (BPD) and half without BPD. PlGF, endostatin and VEGF-C concentrations were measured by commercial enzyme-linked immunosorbent assay (ELISA). III: Cord plasma was collected from very low birth weight (VLBW) (n=92) and term (n=48) infants in conjuncture with birth and endostatin concentrations were measured by ELISA.

I: All growth factors and receptors studied were consistently stained in immunohistochemistry throughout development. For endostatin in early respiratory distress syndrome (RDS), no alveolar epithelial or macrophage staining was seen, whereas in late RDS and BPD groups, both alveolar epithelium and macrophages stained positively in approximately half of the samples. VEGFR-2 staining was fairly consistent, except for the fact that capillary endothelial staining in the BPD group was significantly decreased.

II: During the first postnatal week in TAF mean PlGF concentrations were stable whereas mean endostatin and VEGF-C concentrations decreased. Higher concentrations of endostatin and VEGF-C correlated with lower birth weight (BW) and associated with administration of antenatal betamethasone. Parameters reflecting prenatal lung inflammation associated with lower PlGF, endostatin and VEGF-C concentrations. A higher mean supplemental fraction of inspired oxygen during the first 2 postnatal weeks (FiO_2) correlated with higher endostatin concentrations.

III: Endostatin concentrations in term infants were significantly higher than in VLBW infants. In VLBW infants higher endostatin concentrations associated with the development of BPD, this association remained significant after logistic regression analysis.

We conclude that PlGF, endostatin and VEGF-C all have a physiological role in the developing lung. Also, the VEGFR-2 expression profile seems to reflect the ongoing differentiation of endothelia during development. Both endostatin and VEGFR-2 seem to be important in the development of BPD. During the latter part of the first postnatal week, preterm infants developing BPD have lower concentrations of VEGF-A in TAF. Our findings of disrupted VEGFR-2 staining in capillary and septal endothelium seen in the BPD group, as well as the increase in endostatin concentrations both in TAF and cord plasma associated with BPD, seem to strengthen the notion that there is a shift in the angiogenic balance towards a more antiangiogenic environment in BPD. These findings support the vascular hypothesis of BPD.

Introduction

Advances in neonatology during the latter part of the 20th century and the first decade of the 21st century have led to a decreasing mortality rate among ever-smaller preterm infants. Currently mortality rates for infants born at a gestational age (GA) of 22 weeks are reported to be about 95%, at 24 weeks 60% and at 26 weeks 25% (Moser et al, 2008). Due to an increase in the overall preterm delivery rate (Goldenberg et al, 2008) and the decreased mortality rate, there are more preterm infants surviving than ever before. Thus, despite advances in treatment, morbidity remains high and remains a significant challenge when treating preterm infants.

Since the 1940s, preterm infants have been able to survive due to the use of supplemental oxygen and mechanical ventilation. In the early days ventilation strategies had to be aggressive to achieve appropriate oxygenation and ensure survival of the infant. The aggressive treatment led to the development chronic lung disease, BPD first described in 1967 (Northway et al, 1967). The disorder was characterized by inflammation, fibrosis and smooth muscle hypertrophy in the airways.

Ever since BPD was first described in 1967, knowledge about the disorder and appropriate treatment strategies have been strenuously studied. Advances in mechanical ventilation, better methods for administration of supplemental oxygen, the introduction of surfactant and antenatal glucocorticoid therapy have changed the whole survival profile of preterm infants from that seen in the 1970s. It has also led to a temporal shift in BPD from infants with GA of around 34-37 weeks, to infants with GA below 32 weeks. Subsequently the whole pathogenesis of BPD has changed. Currently less inflammation and fibrosis of the airways is seen. Instead autopsy findings in infants with fatal BPD reveal a persistence of simple terminal air spaces, consistent lack of significant alveolarization and dysmorphic pattern of vascular organization which all in all leads to emphysematous appearing lungs (Husain et al, 1998; Bhatt et al, 2001). As highlighted by these autopsy findings, birth at the early part of the third trimester interrupts the normal development of the lung and the development of BPD is believed to be due to a disruption of vascular development in preterm infants (Jobe, 1999; Abman, 2001; Zoban & Cerny, 2003; Abman, 2008).

Previously, VEGF-A has been shown to play an important part in normal angiogenesis in addition as having a role in the development of BPD (Lassus et al, 1999). We chose to study growth factors and receptors associated with VEGF-A in order to increase our understanding of normal lung angiogenesis as well as the development of BPD.

Review of the literature

1. Normal development of the human lung

1.1 Development of the lung

Traditionally, lung development is divided into five distinct but overlapping stages based on the histologic appearance of the developing epithelium (Figure 1). The basis of lung formation through various biologic processes and genetic pathways is laid down during gastrulation at around a GA of 5 weeks when the embryo forms three specific germ layers. One of these germ layers, the endoderm, organizes and forms the gut tube out of which several organ domains then bud (reviewed in Haddad et al, 2002). Genetic transcription during lung development has been studied intensely during the last decade. The complexity surrounding the regulation of transcription is evident from the various cell types that exist in the lung, ectodermal, mesenchymal and endodermal, all in appropriate numbers and locations to ultimately enable respiration.

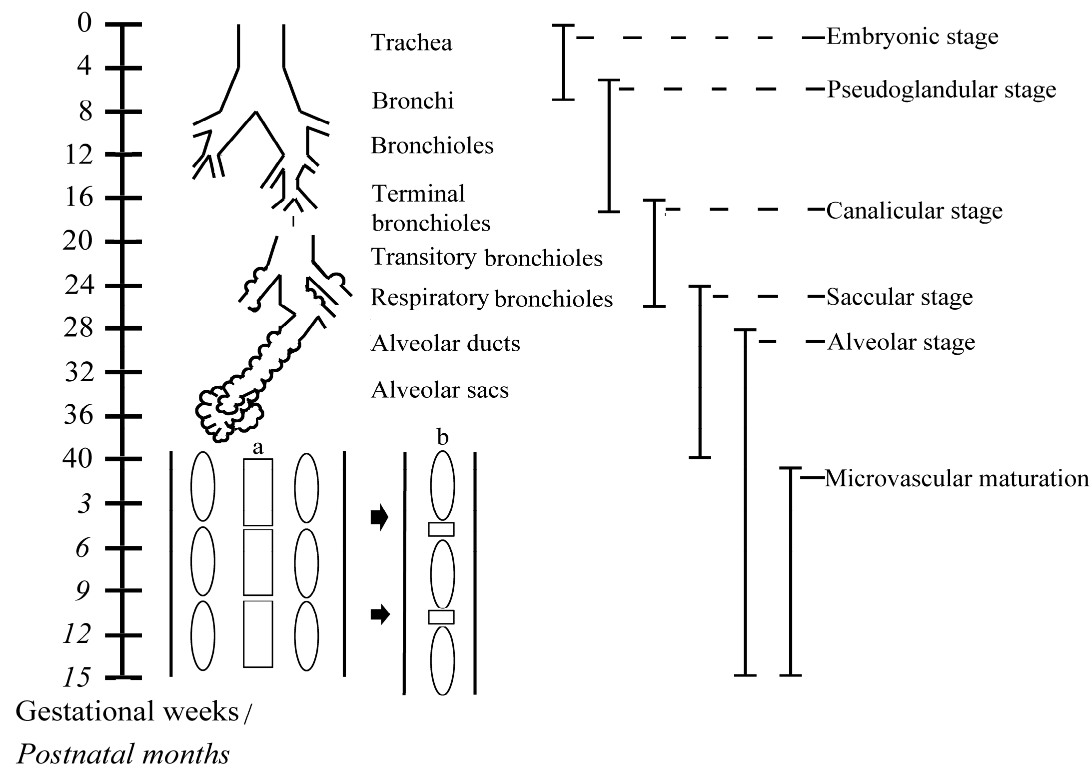

Figure 1. *The various stages of lung development with respect to gestational age/postnatal months. In the schematic drawing of microvascular maturation the oval circles depict endothelial cells and the rectangular forms connective tissue. Microvascular maturation proceeds from a double-capillary layer sandwiching connective tissue (a) to a single capillary layer with connective tissue intertwined (b).*

1.1.1 Embryonic stage (GA 3 to 7 weeks)

The human lung buds out of the gut tube during the embryonic stage of lung development. On gestational day 26, lung and trachea can be recognized as a ventral outpouching of the foregut. This structure consists of the future trachea and two primordial lung buds. The lung buds then initiate branching morphogenesis. By the fifth week, outgrowth, elongation and branching of the two primordial bronchial lung buds create five bronchial stems, three on the right and two on the left. These stems are the foundation for the future lung lobes of the mature lung.

1.1.2 Pseudoglandular stage (GA 5 to 17 weeks)

The pseudoglandular stage is characterized by continuation of branching morphogenesis of the pulmonary tree until the pre-acinary level. At the end of this developmental stage at around a GA of 17 weeks, the lung has completed its branching morphogenesis and bronchopulmonary segments have been formed. At this stage, all the airway divisions down to the level of alveolar ducts are present (Kitaoka et al, 1996). Branching morphogenesis can be considered to create the backbone of the lung with an extensive three-dimensional network of bronchi and bronchioles. It transforms the primordial buds consisting of undifferentiated epithelial cells surrounded by mesenchyme into a highly organized tree-like organ. This is the basis for the extensive gas-exchanging unit that is the mature human lung. The process requires dynamic and reciprocal interactions between the epithelium and the surrounding mesenchyme. On a cellular level, fibroblast growth factor-10, bone morphogenic protein 4, sonic hedgehog and transforming growth factor-β (TGF- β) all play important roles in this process. Simplified, branching morphogenesis consists of bud outgrowth followed by an elongation of the bud and finally subdivision of the terminal portion of the bud to form new buds and continue the branching process (Weaver et al, 2000).

1.1.3 Canalicular stage (GA 16 to 26 weeks)

The canalicular stage of development initiates the formation of structures that are capable of gas-exchange in the lung. First, terminal bronchioles divide to form two respiratory bronchioles. The respiratory bronchioles then branch into 3 to 6 primitive alveolar ducts, which end in terminal sacs. This completes the formation of the prospective gas-exchanging tissue, the acinus. At this stage, the acinary structure is immature but contains the cells needed for respiration. Within the acinus bronchiolar cells start to differentiate, initially to become type II pneumocytes. The type I pneumocyte may then develop from a type II pneumocyte, if the type II pneumocyte is situated in close proximity to a capillary artery. Importantly, capillary arteries begin to proliferate and come into closer contact with epithelial cells; canalizing the lung parenchyma.

1.1.4 Saccular stage (GA 24 weeks to term)

During the saccular stage, the terminal sacs dilate and branch to form further generations of terminal sacs, finally forming transitory ducts and transitory saccules. The walls of the saccules are still immature and contain a central layer of connective tissue surrounded by a double capillary network. This immature wall of the saccule is known as the primary septum. This is considered the basis from which mature lung-exchanging structures bud during alveolarization. Type-I cells continue to flatten and spread, increasing the surface area available for gas exchange.

1.1.5 Alveolar stage (GA 28 weeks to 1-2 years postnatally)

The alveolar stage, alveolarization, can be divided into two separate, yet overlapping processes; septation and microvascular maturation. Alveolarization represents a very important developmental step in evolution, increasing the lung gas exchanging surface area of humans about 20-fold between birth and adulthood.

The process of alveolarization begins relatively late in gestation and continues for 1-2 years postnatally. The phenomenon of alveolarization is currently under scrutiny as late secondary alveolar growth after the process of microvascular maturation, so called late alveolarization, is being suggested as a significant contributor to overall alveolarization (Burri, 2006; Mund et al, 2008; Schittny et al, 2008).

1.1.6 Alveolarization

Septation initiates alveolarization. A secondary crest, containing a connective tissue layer surrounded by a double capillary layer, starts to grow into the airspace from the primary septum, dividing the saccule into what are now termed alveoli. Septation involves coordinated outgrowth of epithelial cells, a capillary network and alveolar myofibroblasts at the alveolar septal tips. The sheet of connective tissue in the middle of the septum is in part made of elastic fibers. Elastic fiber formation in the lung peaks as septation of the distal air spaces occurs (Mariani et al, 2002), suggesting that the elastic matrix is critical in this process.

There is a critical timeframe for septation and any interruption of septation could lead to hypoplasticity and a decreased gas-exchanging area. In rats, treatment with dexamethasone at the time of septation prevented septation, withdrawal of dexamethasone treatment did not lead to spontaneous re-establishment of septation (Massaro & Massaro, 1992; Blanco & Frank, 1993).

After septation, the second part of alveolarization, microvascular maturation, commences. Ultmately it transforms alveolar septa from having a double-capillary layer separated by connective tissue, into septa with a single capillary layer that contains intertwining connective tissue. This encompasses thinning of alveolar septa of distal airspaces by epithelial cell flattening and apoptosis (Massaro & Massaro, 1996). It is

difficult to assess exactly when microvascular maturation begins and when it is complete. Ultrastructural and morphometric studies in the rat show that the merging of the two capillary layers is initiated by a decrease of the dividing septal connective tissue. The two capillary layers merge their lumina after coming into a close contact with each other. The process of preferential growth then expands capillary structures, further increasing the surface area for gas exchange (Burri, 2006).

As mentioned earlier, the issue of late alveolarization has been raised. Subpleural areas of the lung represent sites suited for the addition of new alveoli even during adulthood. On careful examination of the adult lung, disseminated septa with an immature aspect, i.e. a double capillary layer surrounding connective tissue, can be found (Burri, 2006). Whether the capacity for late alveolarization can significantly increse gas-exchange capacity in preterm infants who have developed BPD is unknown. This issue is perhaps of greater interest in recovery from adult lung insult than it is in the pathogenesis of BPD and the aspect of this Thesis.

1.2 Vascular development in the human lung

The pulmonary artery and pulmonary veins are established at the end of the embryonic stage of lung development. However, it is during the next stage of development, the pseudoglandular stage, that vascular development of the lung truly accelerates. At the same time that branching morphognesis forms the basis of epithelial structures, vascular development lays down the foundation for endothelial structures critical in later development of the gas-exchanging parenchyma of the lung.

The lung vasculature develops through both angiogenesis, in which vessels are formed from pre-existing vessels, as well as by vasculogenesis, in which blood lakes appear *de novo.* Initially these two systems are separated; on day 50.5 (GA of 7 weeks) five to six generations of airway branches have formed and blood-lakes are in abundance in the subpleural mesenchyme. However, by this stage the pulmonary artery has only reached the third or fourth generation of airway branches. On day 54, many weeks before a connection between the developing pulmonary artery and the capillary network is formed, a hilar vein is seen connecting to the peripheral lakes, establishing venous drainage. Between a GA of 12-16 weeks, the peripheral blood-lakes have established an extensive network of capillaries surrounding the most peripheral lung buds (deMello & Reid, 2000).

By the GA of 22-23 weeks, the capillary network approaches and bulges into the airspace. Additionally, at this stage the pulmonary artery accompanies even the most distal airway branch under the pleura. It seems that fusion between angiogenesis and vasculogenesis occurs during this, the canalicular, stage of development (deMello & Reid, 2000). The capillary network then continues to expand and becomes more complex. The development of lung vasculature ends with microvascular maturation described in the section on alveolarization above.

1.3 Lymphatic development in the human lung

The lymphatic vasculature is important for the collection of protein-rich exudate leaking from blood vessels, as well as for playing a part in the body's immune response. The lymphatic system is an open-ended linear system through which tissue fluid (lymph) is drained from the interstitial space of most organs. The lymph is then transported from thin initial capillaries to the larger collecting lymphatics that are eventually connected by means of the thoracic duct to the inferior vena cava for recirculation (reviewed in Hong et al, 2004).

The development of the lymphatic system is relatively poorly understood. In 1902, Floorence Sabin proposed that lymphatic sacs bud out of veins during embryogenesis and that the lymphatic vasculature proceeds to grow from this early basis (Sabin, 1902). This theory has recently been proved to be correct by studies on mice. In these studies, the prospero-related homeobox 1 (Prox1) gene was required for a subset of venous endothelial cells (ECs) in the embryonic cardinal veins to migrate out, to form the initial lymphatic vessels during early embryogenesis (Wigle & Oliver, 1999; Wigle et al, 2002). These budding lymphatic ECs eventually gave rise to the primary lymph sacs, from which lymphatic vessels then spread to peripheral tissues of the embryo (Wigle & Oliver, 1999). It is believed that all venular ECs may originally be bipotent, being able to differentiate towards becoming either venous or lymphatic ECs. But when these cells simultaneously express lymphatic vessel endothelial HA receptor-1 (LYVE-1), Prox1, VEGFR-3 and secondary lymphoid chemokine, they lose their bipotency and become irreversibly commited to the lymphatic EC lineage (Wigle et al, 2002).

2. Proangiogenic growth factors in the preterm lung

2.1 Overview of proangiogenic growth factors

Angiogenesis in the human lung involves intricate and complex regulation by a wide variety of factors. The role of transcriptional factors and genes and other proangiogenic molecules are immensely important for vascular development but are not within the scope of this Thesis. Instead, we shall focus our attention on growth factor-receptor pairs. While the VEGF-A-VEGFR system is regarded as the most important during vascular development, the angiopoietin and ephrin systems in addition to platelet-derived growth factor are all recognized as important in lung development.

Ephrin receptors are divided into Ephrin A and Ephrin B kinases according to sequence homology and binding specificity to membrane bound ephrin ligands (Miao & Wang, 2008). In total, the ephrin family consists of 14 known receptors and at least 8 ligands and it functions in the growth and development of the neuronal and vascular systems (O'Leary & Wilkinson, 1999; Adams & Klein, 2000; Pasquale, 2005). Of the ligands, ephrinA1 has been shown to have angiogenic properties (Pandey et al, 1995; Daniel et al, 1996) and recently to increase EC permeability (Larson et al, 2008) and ephrinB2 acts as an arterial cell marker during early embryonic development (Wang et al, 1998).

Platelet derived growth factor is an important regulator of angiogenesis by acting to increase proliferation and survival of smooth muscle cells and pericytes (Claesson-Welsh, 1994). In addition, platelet derived growth factor has been shown to induce direct EC growth (Shibuya, 2008).

2.2 Vascular endothelial growth factor-A (VEGF-A)

2.2.1 Background

VEGF-A was the first member of the VEGF family to be identified (Figure 2). The family also includes VEGF-C, VEGF-D and PlGF discussed later, as well as less studied growth factors VEGF-B (Nash et al, 2006), VEGF-E (Meyer et al, 1999) and VEGF-F (Suto et al, 2005). VEGF-A was discovered in 1983 by Senger and coworkers as a vascular permeability factor secreted by tumor cells (Senger et al, 1983). It was found to be a potent EC mitogen capable of regulating physiological and pathological angiogenesis, and in 1989, it was termed VEGF (Ferrara & Henzel, 1989; Leung et al, 1989; Plouet et al, 1989).

The human VEGF-A gene is located on chromosome 6p21.3 (Vincenti et al, 1996). Alternative exon splicing of a single VEGF gene results in six isoforms of VEGF-A. Four mature isoforms; VEGF121, VEGF165, VEGF189 and VEGF206, as well as two less commonly expressed isoforms; VEGF145 and VEGF183 (Houck et al, 1991; Tischer 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). VEGF165, VEGF189 and VEGF206 all bind to heparin. Loss of this heparin biding ability results in a reduction of mitogenic activity of vascular ECs (Keyt et al, 1996).

2.2.2 Biological activity of VEGF-A

VEGF-A exerts its biologic effect through interaction with cell-surface receptors. These receptors are transmembrane tyrosine kinase receptors VEGFR-1 (fms-like tyrosine kinase-1) as well as VEGFR-2 (fetal liver kinase-1/kinase insert domain receptor), selectively expressed on vascular ECs to which VEGF-A binds with high affinity (Veikkola et al, 2000). VEGFR-1 and VEGFR-2 are discussed in more detail below.

VEGF-A is the most potent proangiogenic protein described to date. It induces proliferation, sprouting and tube formation of ECs (Ferrara et al, 2003). It is also a potent survival factor for ECs, and has been shown to induce the expression of antiapoptotic proteins in these cells (Benjamin & Keshet, 1997; Gerber et al, 1998).

VEGF-A is regulated by several separate factors and pathways. Oxygen tension is a key regulator of VEGF-A gene expression (Shweiki et al, 1992). Low pO_2 rapidly and reversibly induces VEGF-A expression through hypoxia inducible factor-α (Liu et al, 1995). A number of cytokines, hormones and growth factors are able to up-regulate VEGF-A messenger ribonucleic acid (mRNA) expression in various cell-types. In addition,

VEGF-A seems to have a role in inflammation, as suggested by the up-regulation of VEGF-A expression by inflammatory mediators (Ben-Av et al, 1995; Horiuchi & Weller, 1997; Nauck et al, 1997).

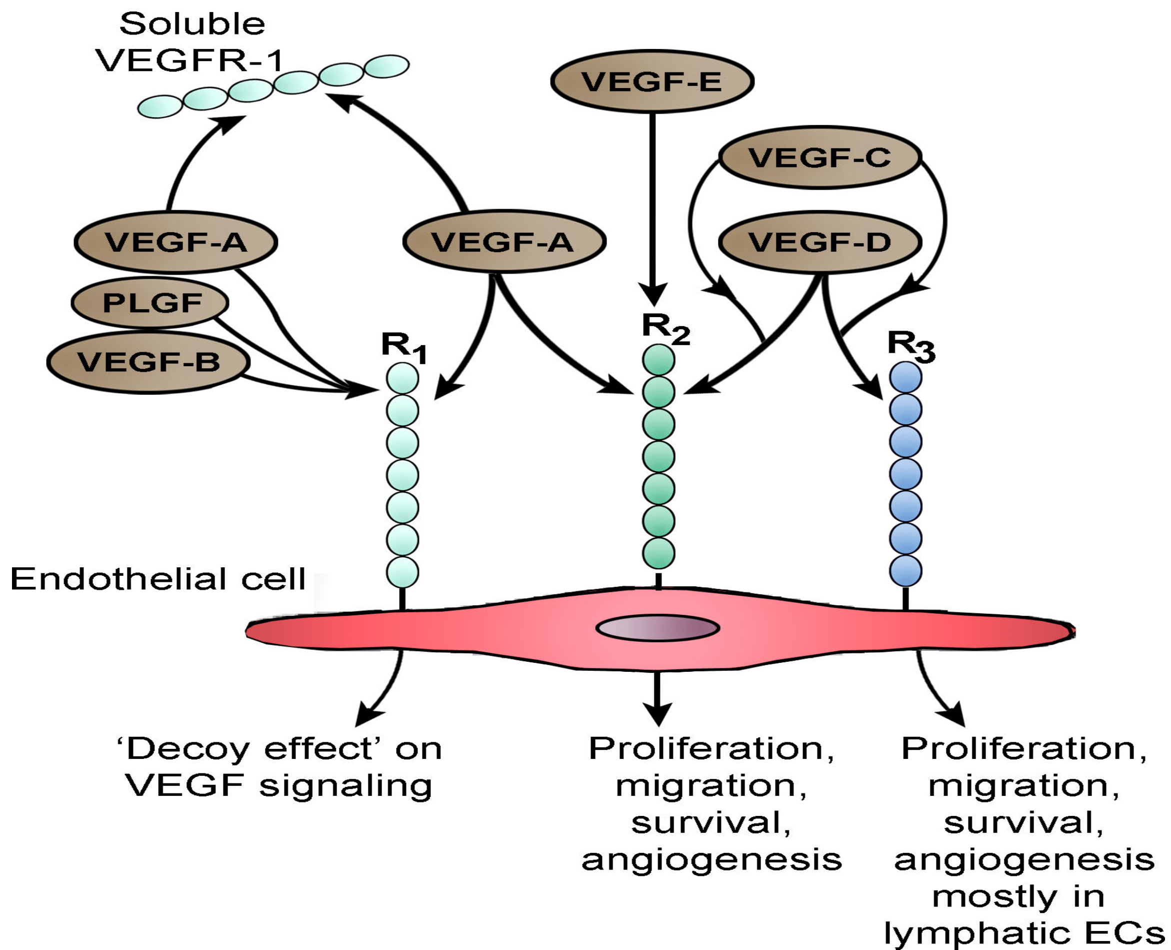

Figure 2. *VEGF family of growth factors and receptors. R_1 denotes VEGFR-1, R_2 VEGFR-2 and R_3 VEGFR-3. Modified and reproduced here with permission from the copyright holder (Ferrara et al, Nat Med 9: 669-676; 2003).*

2.2.3 VEGF-A in physiological angiogenesis

In human fetuses VEGF-A mRNA can be detected in all tissues, most abundantly in lung, kidney and spleen. VEGF-A is localized in epithelial cells and myocytes, including smooth muscle cells lining blood vessel walls (Shifren et al, 1994; Acarregui et al, 1999). High levels of VEGF-A mRNA (Kaipainen et al, 1993) and protein (Shifren et al, 1994) have also been localized in airway epithelial cells in human fetal lung during the second trimester. Increased VEGF-A gene expression in distal airway epithelial cells has been shown to associate with the spontaneous differentiation of human fetal lung *in vitro*, and VEGF-A seems to direct the development of the alveolar capillary bed (Acarregui et al, 1999). Inactivation even of a single VEGF-A allele in mice results in early embryonic

lethality. VEGF-A$^{-/+}$ embryos are growth retarded and exhibit a number of developmental anomalies; formation of blood vessels is abnormal in heterozygous VEGF-A deficient embryos, and is even more impaired in homozygous VEGF-A deficient embryos (Carmeliet et al, 1996; Ferrara et al, 1996).

2.3 Placental growth factor (PlGF)

2.3.1 Background

Human PlGF was initially located in the human placenta (Maglione et al, 1991), but has since been located also in the heart and lung. PlGF binds mainly to VEGFR-1 (Yla-Herttuala & Alitalo, 2003). The proangiogenic action of PlGF is mediated indirectly through VEGFR-2. VEGFR-2 is a receptor tyrosine kinase (Terman et al, 1991), which binds VEGF-A, VEGF-C, and VEGF-D, and is recognized as the primary receptor transmitting signals in ECs (Wise et al, 1999; Zachary & Gliki, 2001). Since VEGF-A binds to both VEGFR-1 and VEGFR-2, it is suggested that PlGF boosts angiogenesis by binding to VEGFR-1, thus decreasing the amount of free VEGFR-1. A larger percentage of VEGF-A instead binds to VEGFR-2 (Park et al, 1994). In addition, PlGF seems to have a distinct unique angiogenic signalling pathway through VEGFR-1 (Cao et al, 1996; Neufeld et al, 1999; Shibuya et al, 1999) (Figure 3).

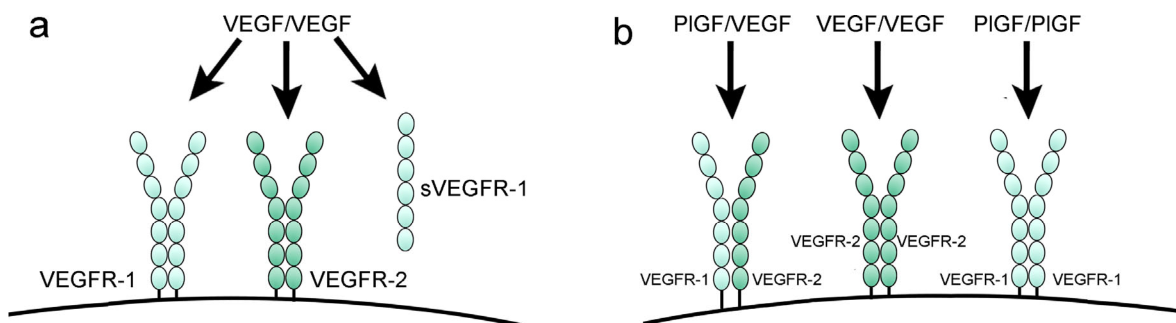

Figure 3. *The binding pattern of VEGF-A and PlGF during angiogenesis. a) During physiological angiogenesis VEGF-A binds equally to VEGFR-1, VEGFR-2 and sVEGFR-1. b) In pathological angiogenesis PlGF binding to VEGFR-1 allows an increased number of VEGF-A molecules to bind to VEGFR-2. In addition, PlGF may stimulate angiogenesis directly by binding to VEGFR-1, as well as by forming a heterodimer pair with VEGF-A, inducing the formation of VEGFR-1/VEGFR-2 receptor heterodimers. Modified and reproduced here with permissionfrom the copyright holder (Tjwa et al, Cell Tissue Res 2003;314:5-14).*

2.3.2 PlGF in physiological development

PlGF knockout mice do not exhibit any abnormalities in developmental angiogenesis or vasculogenesis, however, these mice suffer from impaired angiogenesis in states of ischemia such as myocardial infarction, inflammation, wound healing and tumor growth (Carmeliet et al, 2001).

2.4 Vascular endothelial growth factor receptors 1&2 (VEGFR-1 and VEGFR-2)

Both VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a tyrosine kinase domain (Terman et al, 1991; de Vries et al, 1992). VEGFR-1 was the first receptor tyrosine kinase to be recognized as a VEGF family receptor in 1992 (de Vries et al, 1992). VEGFR-1 expression is upregulated by a hypoxia inducible factor-α dependent mechanism (Gerber et al, 1997). In addition to VEGF-A, VEGFR-1 also binds to PlGF (Park et al, 1994) and VEGF-B (Olofsson et al, 1998), both of which do not bind to VEGFR-2. VEGFR-1 undergoes only weak tyrosine autophosphorylation in response to VEGF-A binding (de Vries et al, 1992; Waltenberger et al, 1994). This finding seems to indicate that VEGFR-1 acts as a "decoy" receptor by binding VEGF-A, thus preventing VEGF-A from binding to its major signal transducing receptor, VEGFR-2 (Park et al, 1994). This decoy effect can also be performed by the soluble form of the receptor, s-VEGFR-1 (Carmeliet et al, 2001). The importance of VEGFR-1 in early fetal development is illustrated by lethality of VEGFR-1-null mice, which die *in utero* between embryonic day 8.5 and 9.5 (Fong et al, 1995; Fong et al, 1999). These mice exhibit an excessive proliferation of angioblasts and failure of ECs to organize into channels (Fong et al, 1999).

VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. The importance of VEGFR-2 in vascular development is highlighted by the fact that VEGFR-2-null mice fail to develop blood-islands and vessels altogether, dying *in utero* between embryonic day 8.5 and 9.5 (Shalaby et al, 1995). However, at least *in vitro*, endothelial/hematopoietic precursor cells can be derived from VEGFR-2-deficient embryonic stem cells, demonstrating that VEGFR-2 is not required for the formation of the common hematopoietic/endothelial progenitor cell, the so-called hemangioblast (Hidaka et al, 1999; Schuh et al, 1999). VEGFR-2 thus seems to be critical in EC commitment as well as VEGF-A directed hemangioblast migration to suited environments in the developing embryo (Shalaby et al, 1997; Hidaka et al, 1999; Schuh et al, 1999). In addition, VEGFR-2 has previously been detected on lymphatic ECs (Makinen et al, 2001a).

In summary, VEGF-A binds VEGFR-2 and forms the developing vasculature which, at least in early development, is shaped and directed by the activity of VEGFR-1.

2.5 Angiopoietin 1

Angiopoietin 1 is expressed by many different cell types, and angiopoietin 1 mRNA expression has been detected early in fetal development in myocardium, followed by expression in mesenchyme surrounding blood vessels (Davis et al, 1996). In transgenic mice, angiopoietin 1 induces more abundant, more highly branched and larger blood vessels than in wild type mice (Suri et al, 1998). The phenotypes of angiopoietin 1 and tyrosine kinase with Ig and EGF homology domains (Tie) -2, the receptor of all angiopoietins including angiopoietin 1, deficient mice suggest a role for this ligand-receptor system in maintaining the communication between ECs and the surrounding mesenchyme. This communication is critical in order to establish stabilization of the formed blood vessel wall, and is established by cellular and biochemical interactions

between ECs and mesenchyme (Dumont et al, 1994; Puri et al, 1995; Suri et al, 1998; Thurston et al, 1999). The importance of this stabilization is highlighted by the fact that VEGF-A gene treatment alone leads to improved vessel generation, but the vessels are immature and leaky (Kunig et al, 2005b; Thebaud et al, 2005; Kunig et al, 2006b). Combined VEGF-A and angiopoietin 1 gene transfer preserves alveolarization, and enhances angiogenesis with more mature blood vessels that are less permeable, reducing the vascular leakage seen in VEGF-A-induced vessels (Thebaud et al, 2005).

2.6 Tyrosine kinase with Ig and EGF homology domains (Tie) receptors

There are two members of the Tie class of receptor tyrosine kinases, Tie-1 and Tie-2. Both receptors are predominantly expressed by vascular ECs (Loughna & Sato, 2001). Whereas all the angiopoietins bind Tie-2, Tie-1 has no known ligands to date (Davis et al, 1996; Maisonpierre et al, 1997; Valenzuela et al, 1999). Tie-2 activation triggers several signalling pathways with downstream targets leading to an antiapoptotic, anti-inflammatory, antipermeable, prosprouting effect on ECs (Eklund & Olsen, 2006). Tie-$2^{-/-}$ mice die between embryonic day 9.5 and 10.5 due to lack of remodelling of the primary capillary plexus. The development of the heart also shows severe defects with poor associations between ECs and the underlying extracellular matrix (Dumont et al, 1994; Sato et al, 1995). There is an absolute requirement for Tie-2 in endocardium on embryonic day 10.5, otherwise both Tie-1 and Tie-2 are initially, at least partly, dispensable for the development of the rest of the vasculature (Puri et al, 1999). However, both play a role during late organogenesis in the development of the microvasculature as well as in virtually all adult blood vessels and postnatal bone marrow hematopoiesis (Puri & Bernstein, 2003).

The function of Tie-1 has been elusive, mostly because a binding ligand has been difficult to identify. Tie-1 is unphosphorylated, and it does not induce tyrosine phosphorylation of cellular proteins in ECs, indicating that Tie-1 does not act via ligand-induced kinase activity (McCarthy et al, 1999; Marron et al, 2000b). However, a soluble angiopoietin 1 chimeric protein, COMP-angiopoietin 1, has recently been shown to induce phosphorylation of Tie-1. This effect was strengthened by the ability of Tie-1 to forms heterodimeric complexes with Tie-2 (Saharinen et al, 2005). Heterodimerization could be a way in which Tie-1 might modulate both its own, and Tie-2, signalling in a ligand independent fashion (Marron et al, 2000a; Marron et al, 2000b; Tsiamis et al, 2002; Saharinen et al, 2005). Before these mechanisms of Tie-1 action were discovered, *in vivo* studies illustrated a role for Tie-1 in vascular development; EC survival and extension of the vascular network during late embryogenesis, particularly regions of capillary angiogenic growth, required Tie-1 activation. Mice lacking Tie-1 die between embryonic day 13.5 and the immediate postnatal period due to severe hemorrhage and edema (Puri et al, 1995; Sato et al, 1995).

3. Antiangiogenic growth factors in the preterm lung

3.1 Overview of antiangiogenic growth factors

In contrast to proangiogenic growth factors, much less is known about the function of antiangiogenic growth factors in the development of lung vasculature and about their potential role in lung injury of preterm infants. Most known antiangiogenic growth factors have been discovered in conjuncture with cancer research as potential targets for tumor therapy.

Angiostatin (Holmgren et al, 1995; Ruegg et al, 2006), pigment epithelium-derived factor (Dawson et al, 1999; Zhang et al, 2005) and maspin (Zou et al, 1994; Zhang et al, 2000; Solomon et al, 2006) are all recognized as regulators of pathologic angiogenesis, particularly tumor angiogenesis. In addition, pigment epithelium-derived factor has been shown to be downregulated during pathologic neovascularization of the retina (Gao et al, 2001) and in pulmonary fibrosis (Cosgrove et al, 2004). In developing mice, lack of pigment epithelium-derived factor results in increased stromal vasculature and epithelial cell hyperplasia both in the prostate and kidney (Doll et al, 2003). The role of these growth factors in the developing human lung is unknown.

Endothelial monocyte-activating polypeptide-II (EMAP-II) also acts in an antiangiogenic fashion. EMAP-II is known to be expressed in the mouse lung *in utero*, and decreasing levels of EMAP-II coincide with rapid vascularization. Postnatally, concentrations in the lung remain low except for the period of microvascular maturation (Schwarz et al, 1999b). Increased levels of EMAP-II have also been associated with decreased vasscularization and development of BPD in preterm baboons (Quintos-Alagheband et al, 2004).

3.2 Endostatin

3.2.1 Background

Endostatin is a 20 kDa proteolytic fragment of the C-terminal nontriple-helical domain of collagen XVIII (O'Reilly et al, 1997). It was discovered in cancer research and is the first endogenous inhibitor of angiogenesis to be identified in a matrix protein (Folkman, 2006). Endostatin has been identified as a component in nearly all endothelial and epithelial basement membranes (Muragaki et al, 1995; Fukai et al, 2002). Early experimentation utilized endostatin from tumor bearing mice. Endostatin was then produced in *Escheria coli* (O'Reilly et al, 1997). Experimentation in mice showed a dramatic inhibitory effect on tumors when soluble endostatin was administered subcutaneously (Boehm et al, 1997). These results were difficult to duplicate and many remained sceptical towards the antitumor effects of endostatin. These problems were overcome with the production of soluble endostatin in yeast (*Pichia pastoria*) (Folkman, 2006). Problematically, the effect of bolus administration of the soluble form of endostatin on tumors was not as great as that

of the insoluble form. This problem could be overcome by administering soluble endostatin continuously. This proved to be just as efficient as administration of the insoluble form (Capillo et al, 2003). This suggests that endostatin has a short half-life and circulating concentrations need to be elevated in order for endostatin to achieve maximum efficiency in tumor inhibition.

The inhibitory effect of endostatin on ECs includes inhibition of proliferation (O'Reilly et al, 1997), migration (Dhanabal et al, 1999; Yamaguchi et al, 1999) and induction of cell apoptosis (Dhanabal et al, 1999; Dixelius et al, 2000).

3.2.2 Antiangiogenic action of endostatin

Endostatin's mechanism of action has been described as broad-spectrum antiangiogenic. Abdollahi and coworkers showed, using custom microarrays covering over 90% of the human genome that 12% of all genes were significantly regulated in human microvascular ECs exposed to endostatin. Angiogenesis inhibitors were upregulated, while angiogenesis stimulators were downregulated (Abdollahi et al, 2004).

Since the discovery in 1997, numerous studies have shown that the physiological actions of endostatin are diverse and broad. Endostatin levels are elevated in certain types of cancer, in intratumoral fluid and malignant ascites (van Hensbergen et al, 2002), as well as in chronic inflammatory diseases such as rheumatoid arthritis (Hebbar et al, 2000) and diabetic retinopathy (Funatsu et al, 2001). As mentioned above, endostatin has also been shown to inhibit growth and proliferation of certain tumours (Ramchandran et al, 2002).

3.2.3 Endostatin in physiological development

Several studies have implicated, that while endostatin may be a factor in physiological angiogenesis, it is not a critical one. The impact of endostatin on physiological angiogenesis has been studied in a mouse model (Li & Olsen, 2004). Apart from ocular abnormalities, endostatin knockout mice exhibited no major vascular abnormalities. However, aortic explants from these mice showed a twofold increase in the number and length of microvessels, suggesting a more proangiogenic environment. Endostatin modulates cell-matrix interactions locally and acts as an antiangiogenic regulator. This action destabilizes vessel walls and can lead to vessel regression (Li & Olsen, 2004). Endostatin is critical for human retinal development and normal blood vessel formation in the eye (Sertie et al, 2000; Fukai et al, 2002); it seems to affect EC migration and guidance in the developing retina. $Col18a1^{-/-}$ mice exhibited abnormal bending of major retinal blood vessels, but no perfusion deficits or vascular leakage were observed (Marneros & Olsen, 2003). Thus, the abnormal retinal vasculature in these mutant mice did not seem to affect retinal function or morphology (Marneros et al, 2004).

3.3 Angiopoietin 2

Angiopoietin 2 is a protein containing 496 amino acids (Maisonpierre et al, 1997), and expression of angiopoietin 2 has been detected at sites of active angiogenesis (Maisonpierre et al, 1997; Holash et al, 1999b). Although angiopoietin 2 and angiopoietin 1 share a similar protein structure, their biological activities differ greatly. Angiopoietin 2, in contrast to angiopoietin 1, does not stimulate EC proliferation (Witzenbichler et al, 1998). Mice overexpressing angiopoietin 2 die at embryonic day 9.5 due to a disruption in vessel formation (Maisonpierre et al, 1997). Similarly to angiopoietin 1, angiopoietin 2 also binds the Tie-2 receptor, but whereas angiopoietin 1 induces phosphorylation and activation, angiopoietin 2 does not activate the receptor and thereby acts as a competitive inhibitor of angiopoietin 1 (Davis et al, 1996; Maisonpierre et al, 1997). Angiopoietin 2 destabilizes vessel walls and promotes active remodeling; regression in the absence of growth factors or vessel sprouting in the presence of growth factors, most notably VEGF-A (Maisonpierre et al, 1997; Holash et al, 1999a; Holash et al, 1999b; Zagzag et al, 1999).

Although early studies characterized angiopoietin 2 as antiangiogenic, this is not always strictly the case. In certain *in vitro* assays, angiopoietin 2 has been shown to have functions similar to angiopoietin 1. In these assays, angiopoietin 2 is able to induce Tie-2 receptor phosphorylation (Maisonpierre et al, 1997; Kim et al, 2000). Thus, depending on the surroundings, angiopoietin 2 could act as a proangiogenic as well as an antiangiogenic growth factor.

In addition to effects on vascular development, angiopoietin 2 seems to play a role in lymphatic development (Gale et al, 2002). Mice lacking angiopoietin 2 show lymphatic defects. These defects can be compensated and the lymphatic phenotype can be rescued if angiopoietin 1 is placed in the angiopoietin 2 locus. These findings seem to indicate, that in addition to being factors in angiogenesis, both angiopoietin 2 and angiopoietin 1 play some part in lymphangiogenesis as agonists.

The function of the angiopoietins, especially angiopoietin 2, in vascular development remains slightly controversial and seems to vary depending on the surrounding environment and signalling by other growth factors. Certainly more information is needed on the subject.

4. Lymphangiogenic growth factors in the preterm lung

4.1 Overview of lymphangiogenic growth factors

Lymph sacs first appear at about week 6-7 of gestation. Prox1 (Hong et al, 2002) and VEGF-C are crucial for early lymphatic development.

Other factors that may contribute to lymphatic development include VEGF-D, podoplanin (Schacht et al, 2003), and receptors VEGFR-3 and LYVE-1 (Banerji et al, 1999). VEGF-D knockout mice have a decrease in VEGFR-3 positive vessels adjacent the muscular surface of bronchioles. However, this decrease is not large enough to change the

difference between wet and dry lung weight, suggesting that VEGF-D is not crucial for normal lymphatic development (Baldwin et al, 2005). Also in mice, VEGF-D is evident from embryonic day 13.5 in the pseudoglandular stage of lung development and remains active until birth, but is not seen postnatally (Greenberg et al, 2002).

Mediators contributing to the development of the lymphatic system have been difficult to investigate. The problem seems to center around the fact that there have been no specific markers for lymphatic ECs. Although VEGFR-3 is highly lymph specific later in development, during embryogenesis it is also expressed by blood vascular ECs. LYVE-1 seemed to be specific for lymphatic ECs throughout development (Banerji et al, 1999), although this has also been placed in doubt (Gordon et al, 2008).

4.2 Vascular endothelial growth factor C (VEGF-C)

4.2.1 Background

VEGF-C is a member of the VEGF family of vascular endothelial growth factors (Tammela et al, 2005). Together with VEGF-D, VEGF-C was the first characterized growth factor to induce growth of new lymphatic vessels *in vivo* (Joukov et al, 1996; Jeltsch et al, 1997; Oh et al, 1997). VEGF-C is produced as a 61 kDa pre-propeptide and is proteolytically processed to form a homodimer of 21 kDa, which has a high binding affinity for VEGFR-3 and VEGFR-2 (Joukov et al, 1996; Joukov et al, 1997). Only fully processed VEGF-C has the ability to bind VEGFR-2 and hence act on blood EC function, as well as lymphatic endothelium. Proteolytic cleavage of the immature VEGF-C molecule is hence an important step in the regulation of VEGF-C action (Joukov et al, 1997; Joukov et al, 1998). The binding affinity of the short 21 kDa form of VEGF-C to VEGFR-3 is 4-5 times stronger than to VEGFR-2 (Makinen et al, 2001b).

4.2.2 VEGF-C in physiological development

During development VEGF-C is localized particularly to regions where lymphatic vessels sprout from embryonic veins. In these regions, VEGF-C is produced by smooth muscle. High levels of VEGF-C have also been detected in the developing murine mesenterium, lung, heart and kidney (Kukk et al, 1996). VEGF-C is essential for the embryonic development of the lymphatic system, as gene-targeted mice lacking the VEGF-C gene are embryonic lethal due to fluid accumulation in tissues. VEGF-$C^{+/-}$ mice survive into adulthood, but display severe lymphatic hypoplasia (Karkkainen et al, 2004). The importance of VEGF-C for the developing lymphatic vasculature is demonstrated in VEGF-$C^{-/-}$, $Prox1^{+}$ mice, where lymphatic ECs arise normally in the cardinal vein, but do not sprout from their initial location (Baldwin et al, 2005).

4.3 Vascular endothelial growth factor receptor 3 (VEGFR-3)

Before the onset of lymphatic vascular differentiation, VEGFR-3 (fms-like tyrosine kinase-4) is highly expressed in blood vascular ECs, but its expression becomes gradually restricted to lymphatic ECs after midgestation (Kaipainen et al, 1995; Kukk et al, 1996; Dumont et al, 1998). Mice deficient in VEGFR-3 gene expression show abnormal remodelling of vascular plexuses and die on embryonic day 9.5 (Dumont et al, 1998). Soluble VEGFR-3 in mice effectively inhibits lymphangiogenesis and leads to regression of existing fetal lymphatic vessels *in vivo*, without affecting vascular development (Makinen et al, 2001a). This illustrates the high lymphatic specificity of VEGFR-3 and the need for continuous VEGFR-3 stimulation in lymphatic development. In the adult, VEGFR-3 is located primarily on lymphatic ECs (Kukk et al, 1996). Missense mutations deactivating VEGFR-3 have been shown to lead to primary lymphedema both in humans and in mice (Karkkainen et al, 2000).

Recent studies of postnatal lymphangiogenesis have shown that lymphatic capillaries require VEGFR-3 to be activated by soluble ligands for up to two weeks after birth, after which lymphatic capillaries become insensitive to VEGFR-3 inhibition. This indicates that after a certain period lymphatic capillaries become mature and do not depend on VEGFR-3 expression for continued survival (Karpanen et al, 2006).

5. BPD in the 21st century

5.1 Historical perspective on BPD

Hyaline membranes were first described in 1903, found in the lungs of infants dying from respiratory distress. Little or no therapy was available for treatment of RDS with infants either recovering or dying by 7 days of age.

From the 1940s onward, routine use of oxygen became common practice followed by positive pressure mechanical ventilation in the 1950s, and mechanical ventilation combined with supplemental oxygen in the 1960s. This development led to higher survival rates, but also to the development of a new chronic lung disorder, BPD, first described by Northway in 1967 (Northway et al, 1967). The condition discovered and described in the 1960s differs greatly from the one seen today. High percentage inspiratory oxygen resulted in inflammation, fibrosis and smooth muscle hypertrophy in the airways (O'Brodovich & Mellins, 1985).

In 2001, the diagnostic criteria were revised due to changes in BPD epidemiology, from the requirement of supplemental oxygen at postnatal age of 28 days to need for supplemental oxygen at 36 weeks postmenstrual age, in addition to a chest radiograph with findings characteristic of BPD (Jobe & Bancalari, 2001).

The clinical course of BPD was originally divided into four stages: Stage I (2 to 3 days), was essentially RDS; respiratory failure, deposition of hyaline membranes,

atelectasis, and metaplasia and necrosis of the bronchiolar mucosa. During stage II (4-10 days), the infants were usually weaned from the respirator, but still needed high concentrations of inspired oxygen. Histological appearance of the lungs showed emphysema at alveolar level as well as bronchiolar necrosis. Stage III (10-20 days), was characterized by widespread bronchiolar metaplasia and hyperplasia, emphysematous alveoli and increasing atelectasis. During this stage the changes occurring began transforming the lung injury towards a more chronic state, BPD.

In Stage IV (after 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, 2001).

Towards the end of the century, new forms of treatment again altered the clinical aspect of BPD. Surfactant replacement therapy was an important discovery which served to "soften" the clinical course of BPD. In addition, the use of antenatal steroids, continuous positive airway pressure (CPAP) to decrease time on mechanical ventilation, oxygen saturation monitors to minimize oxygen exposure, combined with less aggressive ventilation strategies, and improved nutrition all improved the clinical outcome of preterm infants with respect to lung morbidity. Whereas before infants typically suffered from severe respiratory distress during the first postnatal days now the course of the disease is milder with less severe symptoms (Avery & Merritt, 1991; Parker et al, 1992; Egberts et al, 1997; Stevenson et al, 1998; Northway, 2001). This change in the clinical course of the disease has not decreased its occurence, only shifted it towards more immmature infants with more immature lungs.

5.2 Pathogenesis of BPD; prenatal events

The pathogenesis of BPD is considered to consist of prenatal and postnatal components. The main components can be seen in Figure 3.

5.2.1 Prenatal infection and inflammation

There is strong evidence indicating that infection and inflammation play important roles in the pathogenesis of BPD. An imbalance between proinflammatory and anti-inflammatory mechanisms *in utero* seems to predispose the infant for later development of BPD. Subclinical and clinical intrauterine infection and the inflammatory response created in the preterm lung are important in the etiology of preterm labor and preterm premature rupture of membranes (Lahra & Jeffery, 2004).

Although prenatal infection correlates to a lower rate of postnatal RDS it also correlates to a higher incidence of BPD (Watterberg et al, 1996). The fetal lung does not normally mount inflammatory responses, and the components that contribute to a mature inflammatory response to injury are deficient in the fetus. The preterm fetal lung contains almost no macrophages or granulocytes, and host defense proteins such as surfactant protein A and surfactant protein D are also deficient (Stahlman et al, 2002).

Proinflammatory cytokines do not cross the placenta and all existing cytokines in the preterm lung *in utero* are thus synthesized there (Aaltonen et al, 2005). Cytokines are synthesized by alveolar macrophages, airway epithelial cells, fibroblasts, type II pneumocytes and ECs. Hypoxia, hyperoxia, micro-organisms, endotoxin, other bacterial cell wall constituents and biophysical factors such as barotrauma and volutrauma all activate cytokine synthesis in the preterm lung (Speer, 2006). Important proinflammatory cytokines include interleukin (IL) -8, tumor necrosis factor-α, IL-1 and IL-6 (Coalson et al, 1999). The increased expression of proinflammatory cytokines could be a reflection of an inability of the preterm lung to regulate inflammation through anti-inflammatory cytokines such as IL-4, IL-10, IL-12, IL-13 and IL-18 (Jones et al, 1996; Jonsson et al, 2000; Baier et al, 2003; Kakkera et al, 2005; Nakatani-Okuda et al, 2005). Inflammation in the preterm lung has a delayed clearance rate and causes injury followed by maturation (Jobe et al, 2000; Moss et al, 2002; Willet et al, 2002).

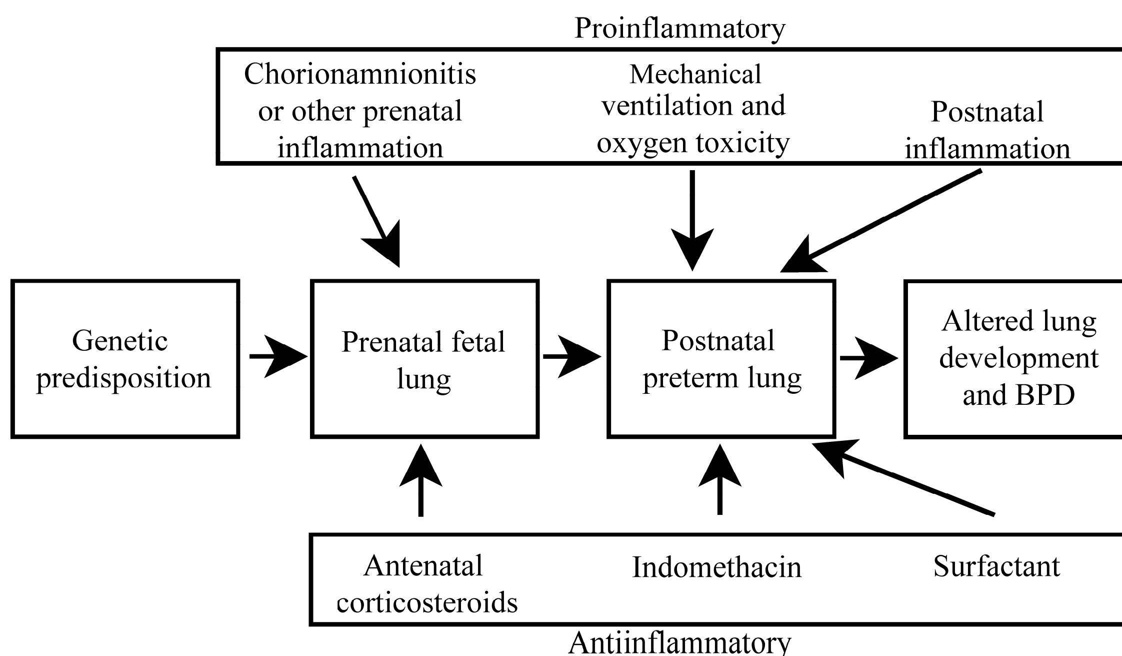

Figure 4. *The main components in the pathogenesis of BPD.*

5.2.2 Antenatal steroids

Antenatal glucocorticoid treatment decreases the incidence of RDS while it does not seem to affect the incidence of BPD. This lack of effect of antenatal glucocorticoid treatment on BPD has been explained by the increased survival of infants mostly at risk of BPD (Crowley, 1995; Jobe, 2000). Glucocorticoid exposure of the fetal lung upregulates numerous genes and downregulates others; for some genes, such as surfactant protein A, regulation is dose dependent, low doses upregulate and high doses suppress gene expression (Liley et al, 1988). Early lung maturational responses to glucocorticoids can occur in the fetal primate lung by midgestation. However, high or prolonged fetal exposures cause an inhibition of subsequent

fetal lung development. Newborn mice and rats are born with saccular lungs at term, and postnatal glucocorticoid treatments cause a delay in alveolar and vascular development that persists as the animals age (Massaro & Massaro, 2000). Repeated doses of antenatal glucocorticoid treatment have been shown to have a beneficial effect on lung maturation and growth, but at the same time an adverse effect on brain function and also contribute to overall growth retardation (Aghajafari et al, 2002).

It may be that excessive exposure of the preterm lung to glucocorticoids could prime the lung for later insults, such as inflammation and oxygen exposure. This might be the underlying reason why antenatal glucocorticoids therapy has failed to decrease the incidence of BPD, and why antenatal steroids should be administered with care.

5.3 Pathogenesis of BPD; postnatal events

5.3.1 Surfactant treatment

The introduction of widespread surfactant treatment for RDS beginning with the first clinical trials at the beginning of the 1980s has contributed greatly towards decreasing mortality and morbidity in preterm infants (Soll, 2000a; Soll, 2000b). Before the surfactant era, BPD was always preceded by aggressive RDS. This entailed destruction of existing pulmonary structures, inflammation and fibrosis, affecting infants of GA 32-36 weeks. Since surfactant decreased the incidence of RDS, it also led to decreased BPD among these more mature preterm infants. BPD was still occurring, but now in more immature preterm infants than before (GA 24-28 weeks). Hence, surfactant treatment is indirectly an important component in the new form of BPD.

5.3.2 Postnatal infection and inflammation

Postnatal inflammation in the lung is usually the result of continued intrauterine infection and inflammation combined with postnatal oxidative stress and mechanical ventilation. The inflammatory response injures the lung parenchyma and injured cells are removed by apoptosis, programmed cell death. This may result in arrested growth of the parenchyma, as cells removed certainly include progenitor cells or cells that are needed for cell-cell interactions. If such damage occurs at critical times in development, for example when the lung is building the framework for later alveolarization during the canalicular stage, lung development may be irreversibly halted (Le Cras et al, 2004; Massaro & Massaro, 2004).

Associations between early onset systemic bacterial infections (Groneck et al, 1996; Groneck et al, 2001), as well as systemic nosocomial infections (Rojas et al, 1995; Groneck et al, 1996; Cordero et al, 1997) and the development of BPD have also been established.

5.3.3 Oxidative stress and barotrauma

The toxicity of both oxygen and barotrauma have been shown in several studies. In term, ventilated neonatal piglets, treatment with hyperoxia alone causes less damage than hyperoxia combined with hyperventilation, but more damage than hyperventilation alone (Davis et al, 1991). Premature baboons equivalent to approximately GA of 30 weeks in humans, and ventilated with oxygen to maintain normal arterial oxygen concentrations have significantly less damage than those ventilated with 100% oxygen (Delemos et al, 1987).

Two randomized trials have shown a higher incidence of BPD in groups that were treated with a higher inspired oxygen ratio, although the results were not completely conclusive (STOP-ROP trial, 2000; Askie et al, 2003). Hyperoxia seems to lead to an arrest in alveolarization in part mediated by changes in TGF-β-bone morphogenic protein signaling in the lung (Alejandre-Alcazar et al, 2007). This in turn up-regulates the gene expression of p53. p53 down-regulates the expression of VEGF-A, which impairs angiogenesis. p53 also induces the transcription of the cyclin-dependent kinase inhibitor p21(WAF/CIP1) mRNA. p21 activation is known to lead to cell cycle arrest and could possibly inhibit proliferation of lung cells (Maniscalco et al, 2005). However, this interesting mechanism for arrest in alveolarization is still incompletely understood and further study is needed to strengthen and clarify the subject.

In an interesting study closely mimicking current state of the art treatment protocols, the lung pathology of premature baboons equivalent to about GA of 26 weeks in humans was studied. Despite treating the preterm baboons using minimal ventilatory stretch and limiting the amount of inspired oxygen, the premature baboons developed alveolar hypoplasia and hypoplastic capillaries (Coalson et al, 1999). This lung pathology is very reminiscent of the pathology seen in human preterm infants with a BW under 1000 g that succumb to BPD. This might indicate that very small amounts of oxygen and barotrauma could have a long lasting detrimental effect on the developing preterm lung. Recent studies and workshops have tried to identify the optimal ventilation strategy that allows for survival and improved clinical outcome whilst avoiding complications such as BPD. The main points of ventilatory strategies are to reduce the overall duration and need for mechanical ventilation by introducing milder ventilation techniques such as nasal CPAP (nCPAP) (Ambalavanan & Carlo, 2006). In fact, Miksch and coworkers showed that early introduction of nCPAP instead of conventional ventilation (Miksch et al, 2008) and Bhandari and coworkers that switching from convential ventilation to synchronized nasal intermittent positive pressure ventilation (Bhandari et al, 2007), greatly reduced the incidence of BPD in preterm infants while not affecting other outcomes. Also, the initial use of nasal intermittent mandatory ventilation instead of nCPAP was shown to decrease the need for intubation and hence decrease the incidence of BPD (Kugelman et al, 2007). However, it may be that ventilatory strategies are reaching a limit where they cannot be significantly improved with respect to decreasing the development of BPD.

5.4 Pathogenesis of BPD; genetics

There are several known genetic factors contributing to the pathogenesis of BPD. Already in 1996 Parker and coworkers showed in a study on preterm twins that the BPD status in one twin was a highly significant predictor of BPD in the other twin (adjusted odds ratio (OR) = 12.3, $p < 0.001$), irrespective of birth order, Apgar scores or other factors (Parker et al, 1996). In a study on monozygotic and dizygotic twins Bhandari and coworkers observed a significantly higher concordance of BPD in monozygotic twins than expected (Bhandari et al, 2006a). A deletion in the gene for surfactant protein B was found to associate with higher risk for BPD (Rova et al, 2004). This association was only significant in singletons or presenting multiples, and became stronger after adjustment for lower BW, highlighting the requirement of both genetic and environmental factors for BPD to develop. These studies show a significant genetic susceptibility for BPD in preterm infants. A wide variety of candidate genes have been investigated, but no single gene has proven to be significant on its own. This seems logical since BPD is considered to be polygenic (Bhandari & Gruen, 2006). Despite the increase in knowledge within genomics, relatively little is known about genetic susceptibility underlying BPD, and the studies conducted have contained a restricted patient material. In a recent study Lavoie and coworkers (Lavoie et al, 2008) underlined the role of genes in the development of BPD. In twin pairs, genes accounted for the susceptibility for BPD in approximately 80% of cases. Clearly this is an area of BPD that needs further investigation.

5.5 The new BPD; a halt in development

BPD is characterized by persistent respiratory signs, prolonged need for mechanical ventilation or oxygen therapy, recurrent hospitalizations for respiratory infections and distress, exercise intolerance, and other problems that reach beyond childhood (McLeod et al, 1996). The exact incidence of BPD is difficult to assess, but recent estimates evaluate the incidences according to BW as follows; 52% in infants with a BW between 501 and 750 g, 34% in infants with a BW between 751 and 1000 g, 15% in infants with a BW between 1001 and 1250 g, and 7% in infants with a BW between 1251 and 1500 g (Ehrenkranz et al, 2005). Overall, the average age of preterm infants developing BPD has markedly decreased since the introduction of modern treatment strategies (antenatal betamethasone, surfactant, ventilation and early treatment of infection). BPD is currently rare in infants with a BW > 1500 g and GA > 30 weeks (Ehrenkranz et al, 2005). Because of this temporal shift of BPD from more mature infants to infants with less developed lungs, the underlying mechanisms in the pathogenesis of the disease have changed. Maybe most importantly for the change in the pathogenesis of BPD is that histological changes now represent injury to the lung at an earlier (early saccular stage, GA 24-28 weeks) stage of development (Parker et al, 1992; Husain et al, 1998). Pathologic findings in the lungs of infants with "new" BPD are very different from those found in "classic" BPD. Instead of the destruction of already existing structures and inflammation seen in "classic" BPD, a

halt in development without widespread inflammation and destruction is seen in the new form of BPD.

Recent evidence suggests that blood vessels in the lung actively promote normal alveolar development (Jakkula et al, 2000; Abman, 2001) and contribute to the maintenance of alveolar structures throughout life (Kasahara et al, 2000). When an infant is born prematurely, the normal development of the lung is disrupted. Due to advances in treatment, premature infants of GA 24-28 weeks survive, but at the same time are at the greatest risk for the development of BPD. Infants born during this stage of development have lungs that are in the late canalicular to early saccular stage of development. The initiation of the gas exchanging unit has only recently begun, with vascular vessels and pulmonary structures coming into contact. Thus, birth at this stage of development disrupts the whole base of the gas exchanging function of the lung. This is reflected by decreased septation and alveolarization, which in turn decreases the overall gas exchanging surface of the lung. The alveoli are larger in size and fewer in number (Le Cras et al, 2002). Another typical feature is dysmorphic vascular growth (Tomashefski et al, 1984).

Previously, an association between abnormalities in pulmonary circulation, specifically pulmonary hypertension, and increased risk for development of BPD have been documented (Hislop & Haworth, 1990; Parker & Abman, 2003). Current results from both animal and clinical studies suggest that there is a link between impairment of angiogenesis during critical periods of development and the disruption of lung epithelial development, most importantly alveolarization, leading to the development of BPD. Support for the vascular hypothesis of BPD is extensive and has been summarized in a review by Thébaud and Abman (Thebaud & Abman, 2007).

The disruption of angiogenesis disrupts alveolarization in several animal models. Treatment with angiogenesis inhibitors in newborn and infant rats compared to vehicle-treated control rats clearly decrease not only vascular density, but also alveolarization and lung weight (Jakkula et al, 2000; Le Cras et al, 2002). Mice lacking in two out of three important isoforms of VEGF-A show marked decrease of not only vasculature, but also air-blood barriers and airspace-parenchyma ratio compared to wild type mice (Galambos et al, 2002). Inhibition of VEGF-A and VEGFR-2 in rats decrease angiogenesis and impairs alveolarization. Alveolar structures become oversimplified, resembling those seen in human lung pathology in BPD (Thebaud et al, 2005). Downregulation of VEGF-A and VEGFR-1, as well as VEGFR-2 and Tie-2 receptors, have been reported to lead to dysmorphic vascular development and to decrease septation and alveolarization in a way that is characteristic of BPD (Maniscalco et al, 1997; Bhatt et al, 2000; Bhatt et al, 2001; Lassus et al, 2001; Maniscalco et al, 2002).

Prolonged exposure to hyperoxia decreases VEGF-A mRNA levels, a phenomenon believed to contribute to oxygen induced lung injury and impaired vascular repair (Johnston et al, 1996; Klekamp et al, 1999). Similarly, in newborn rabbits, VEGF-A mRNA expression is decreased during hyperoxia, whereas during the recovery period in relative hypoxia, VEGF-A expression is increased (Maniscalco et al, 1995; Maniscalco et al, 1997). The lungs of ventilated preterm infants show a decrease in VEGF-A and angiopoietin 1 mRNA, but an increase in endoglin mRNA when compared to age-matched controls (De Paepe et al, 2008). Higher angiopoietin 2 concentrations in TAF of human

preterm infants have been shown to associate with a higher risk of developing BPD (Aghai et al, 2008). Angiopoietin 2, predominantly exhibiting antiangiogenic action, expression has been found to be induced by hyperoxia. Interestingly, angiopoietin $2^{-/-}$ knockout mice are protected against the injury caused by the hyperoxic conditions (Bhandari et al, 2006b). Neutralization of VEGF-A during hyperoxia clearly decreases survival in adult mice. In these mice overexpression of IL-13, an anti-inflammatory mediator, increases VEGF-A levels in bronchoalveolar lavage and this increase is even more marked in hyperoxia (Corne et al, 2000). However, in newborn transgenic mice neither IL-13 (Choo-Wing et al, 2007) nor VEGF-A (Bhandari et al, 2008) has similar protective effects against the development of BPD. This demonstrates the disruption of normal angiogenesis in a hyperoxic environment and highlights the dysmorphic character of angiogensis in BPD. Certainly, VEGF-A expression is downregulated by hyperoxia, and yet unknown mediators might be able to reverse this downregulation. Thus, the role of other regulators of angiogenesis, such as endoglin, together with VEGF-A could act to protect the lung against hyperoxic injury.

In fetal preterm lambs, intra-amniotic infusion of endotoxins markedly reduces VEGF-A expression as well as the expression of other important factors associated with proangiogenic activity in the lung (Kallapur et al, 2004). In preterm baboons prolonged mechanical ventilation also decreases VEGF-A levels (Maniscalco et al, 2002; Asikainen et al, 2006b). In summary, several factors known to contribute to the pathogenesis of BPD; inflammation, hyperoxia and mechanical ventilation, all alter the profile of angiogenesis in the lung. Indeed preterm infants with lower pulmonary concentration of VEGF-A during the latter part of the first postnatal week have a higher incidence of BPD (Lassus et al, 1999; Bhandari et al, 2008). Also, in lung tissue from human infants who died from BPD, the typical pattern of alveolar simplification along with dysmorphic microvascular features are associated with a lower VEGF-A and angiopoietin 1, but higher lung endoglin mRNA expression (Bhatt et al, 2000; De Paepe et al, 2008).

Upregulation of PlGF has been observed in several states of pathologic angiogenesis. Increased expression of PlGF in skin of transgenic mice results in a significant increase in number and size of skin blood vessels as well as increasing blood vessel permeability (Odorisio et al, 2002; Oura et al, 2003). PlGF deficiency results in a diminished and shortened inflammatory response which leads to reduced inflammatory angiogenesis and edema formation (Oura et al, 2003). PlGF also contributes to the pathogenesis of preeclampsia. Decreased levels of PlGF in plasma of pregnant women seem to increase the risk of preeclampsia, although this result remains controversial (Widmer et al, 2007). In a study measuring PlGF from cord plasma in preterm infants at birth Tsao and coworkers found higher PlGF concentrations to be associated with the development of BPD (Tsao et al, 2004). Thus, PlGF seems to be upregulated by inflammation and in turn PlGF upregulates the inflammatory response. Also, a higher concentration of PlGF in cord plasma at birth seems to predict the development of BPD.

Studies on the effect of endostation and VEGF-C on lung injury in preterm infants have been limited. For both, current knowledge is based on mouse models focusing either on pathologic angiogenesis (endostatin) (Fukai et al, 2002; Li & Olsen, 2004), or lymphangiogenesis (VEGF-C) (Karkkainen et al, 2004).

In VEGF-A transgenic mice mortality rates are increased both when VEGF-A expression increases prenatally (Akeson et al, 2005) as well as postnatally (Le Cras et al, 2004), in part due to increased vascular permeablity. Bhandari and coworkers demonstrated that the increased vascular permeability mediated by VEGF-A in transgenic mice is at least partly nitric oxide dependent, while effects on surfactant production are nitric oxide independent (Bhandari et al, 2008). Any possible intervention on angiogenesis needs to be carefully planned and executed in order to be both safe and effective. It seems that a combination of several regulatory systems could prove most effective. Certain studies have demonstrated improved vascular and alveolar growth in experimental BPD models by enhancing angiogenesis. Rats treated with exogenous recombinant human VEGF-A during hyperoxic conditions initially showed increased emphysema, which was followed by improved vascularization during the recovery period (Kunig et al, 2006a). Treated rats also exhibited enhanced alveolarization (Kunig et al, 2005a). Likewise, postnatal intratracheal adenovirus mediated VEGF-A gene therapy improves survival, promotes lung capillary formation, preserves alveolar development and regenerates new alveoli in a model for irreversible lung injury in newborn rats (Thebaud et al, 2005). In preterm baboons, activation of hypoxia inducible factors leads to an increase in expression of proangiogenic platelet-endothelial cell adhesion molecule 1 and VEGF-A but does not increase alveolar epithelial structures (Asikainen et al, 2006a).

A recent study by De Paepe and coworkers shows that an initial decrease in ECs after brief ventilation is followed by a marked increase during prolonged ventilation in BPD, despite a decrease in vessel number. The study was quantitative in nature and thus did not look at vessel organization (De Paepe et al, 2006). Typically, a failure of septation also leads to a failure in microvascular maturation, an intricate process. It seems likely that despite the marked EC proliferation observed by De Paepe, that lack of organization and regulation of EC migration, proliferation and vessel formation would lead to dysmorphic vascular development. Animal models as well as clinical studies have been focused to better investigate the new form of BPD.

Clearly there are unresolved issues regarding the pathogenesis of BPD and details surrounding the vascular hypothesis. However, on the basis of information currently available it seems clear that angiogenesis and alveolarization are closely intertwined during development of the functional gas-exchanging apparatus of the lung. A disturbance in angiogenesis will disrupt physiological alveolarization and *vice versa*.

Aims of the study

The aim of this study was to investigate the roles of proangiogenic, antiangiogenic and lymphangiogenic growth factors in normal lung development as well as in the development of BPD by using human samples. Each growth factor was selected because of its close relationship to VEGF-A, known to have an established role in both normal angiogenesis and in the pathogenesis of BPD.

By using three separate analyses: 1. immunohistochemistry on lung samples (I, II and IV), 2. TAF (I, II and IV), and 3. cord plasma (III) the specific aims of the study were to:

1. clarify the role of PlGF, endostatin, VEGF-C, VEGFR-2, and VEGFR-3 in physiological lung development.

2. investigate whether any of PlGF, endostatin, VEGF-C, VEGFR-2, or VEGFR-3 participates in the pathogenesis of lung injury, specifically BPD.

Material and methods

1. Material

1.1 Ethics

Studies I, II and IV were approved by the Ethics Committee of the Hospital for Children and Adolescents, University Central Hospital (Helsinki, Finland). Study III was approved by the Ethics Committee of the Department of Obstetrics and Gynaecology, University Central Hospital (Helsinki, Finland). All studies comply with the principles stated in the Declaration of Helsinki (2004).

1.2 Patients in immunohistochemistry studies (I, II and IV)

For immunohistochemistry analysis all lung tissue samples were collected between March 1991 and June 2000. Immunohistochemistry analysis was included in studies I, II and IV. Study I included 39, study II 45 and study IV 48 subject samples (Table 1).

The infants from which the samples were analyzed were divided into groups according to GA or time of abortion as well as lung pathology at the time of death. The diagnoses of these infants can be seen in Table E1 of study IV. Fetuses as well as preterm and term controls had macroscopically and microscopically normal lungs at the time of death. In other words, no hyaline membranes or macroscopical edema or inflammation, which are both indicators of acute lung injury; RDS, were present in the samples from these infants.

The early RDS group consisted of preterm infants who died within 2 days of birth. The late RDS group consisted of preterm infants who died later than 10 days after birth but did not fill the criterion for BPD. BPD was diagnosed defined clinically as the need for supplemental oxygen at the age of 36 postmenstrual weeks in association with chest radiographic findings typical of BPD (Jobe & Bancalari, 2001), and post mortem histologically. The patients in both RDS and BPD groups died because of their lung disease.

1.3 Patients in TAF studies (I, II and IV)

TAF analysis was included in study I, II and IV. A total of 59 infants were enrolled between December 1993 and March 2002. Study I included 20, study II 59, and study IV 54 infants. Patients were selected on the basis of development of BPD, defined clinically as the need for supplemental oxygen at the age of 36 postmenstrual weeks in association with chest radiographic findings typical of BPD (Jobe & Bancalari, 2001). Approximately half of the infants in the study group developed BPD while half survived without it. The BPD vs. no BPD group was matched with respect to GA and BW (Table 2).

Lactosyl ceramide (LC) has previously been demonstrated in large amounts in granulocytes and inflamed fetal membranes. It was measured by thin layer chromatography as previously described (Hallman et al, 1989).

All the infants included were treated in the neonatal intensive care unit of the Hospital for Children and Adolescents, Helsinki University Central Hospital. Infants included in the study had to be intubated at birth because of failure to establish spontaneous ventilation. Infants were mechanically ventilated during the whole study period. Infants exhibiting major anomalies were excluded from the study. The standard protocol for treatment of the neonatal intensive care unit was followed in the treatment of all infants.

1.4 Patients in cord plasma study (III)

Samples from a study population consisting of 140 newborn infants born at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland were collected. The study population was divided and analyzed according to BW and GA; a VLBW group whose BW was under 1500 g and GA under 32 wk (n=92), and a healthy term group with BW over 1500 g and GA over 37 wk (n=48) which served as a controls. Analysis was done both between the groups as well as within the group. Infants whose mothers had pre-eclampsia or diabetes mellitus were excluded from the control group to ensure as healthy a control group as possible (Table 2).

Table 1. *Patient data for immunohistochemistry (I, II and IV)*

	Fetus	*Preterm control*	*Term control*	*Early RDS*	*Late RDS*	*BPD*
N, I	9	4	8	5	7	6
N, II	9	5	9	7	7	8
N, IV	10	5	10	8	7	8
GA , I	18.1±2.6	24.6±2.9	38.9±1.7	26.0±2.1	26.8±1.7	28.2±2.3
GA, II	18.6±2.9	24.2±2.7	39.1±2.1	26.0±2.1	26.8±1.7	27.7±2.1
GA, IV	18.5±2.1	24.2±2.7	38.2±2.7	25.8±2.0	26.8±1.7	27.7±2.1
BW, I	194±122	788±341	3411±608	799±464	692±151	892±177
BW, II	226±162	698±357	3406±569	727±399	692±151	908±218
BW, IV	224±153	698±357	3276±662	713±372	692±151	908±218
TAD, I	-	0.1±0.1	1.0±1.0	1.3±0.7	11.3±2.8	207±82
TAD, II	-	0.1±0.1	1.0±0.9	1.5±0.6	11.3±2.8	180±86
TAD, IV	-	0.1±0.1	0.8±0.8	1.4±0.9	11.3±2.8	143±88

Abbreviations: N: number of subjects included in each group of each study, GA: gestational age expressed in weeks, BW: birth weight expressed in grams, TAD: time at death expressed in days. STUDY I: PlGF and VEGFR-2, II: endostatin, IV: VEGF-C and VEGFR-3.

Table 2. *Patient data for ELISA (I-IV)*

	I PlGF TAF	***II ES TAF***	***III ES cord***	***IV VEGF-C TAF***
	N=20	N=59	N=92	N=54
Antenatal parameters				
Betamethasone	14	46	85	43
Number of doses (1 vs. 2 doses)	7 vs. 5	26 vs. 13	54 vs. 16	32 vs. 11
time of administration before delivery (days)	6.6±6.7	5.2±5.2	5.9±8.5	5.1±5.3
Antenatal dg.				
Chorioamnionitis	7	11	10	12
Preeclampsia	4	15	20	17
C-section	15	37	52	39
Parameters reflecting maturity				
Gestational age (weeks)	27.7±2.3	27.3±2.0	27.9±2.3	27.2±1.8
Birth weight (g)	1088±409	948±323	1015±285	920±287
Birth weight (SDS)	0.0±1.6	-1.2±1.6	-1.2±1.4	-1.5±1.6
L/S ratio (0-10) †	7±3	6±3	NA	6±3
Perinatal parameters				
Apgar score at 1 min	5±2	5±2	7±2	5±2
Indomethacin	14	42	27	40
LC †	5	10	NA	9
Umbilical arterial cord pH	7.30±0.07	7.31±0.08	7.28±0.09	7.29±0.10
Umbilical arterial cord BE	-2.3±3.0	-2.3±2.9	-3.0±3.0	-2.7±3.5
Respiratory distress parameters				
aA ratio	0.16±0.14	0.22±0.21	NA	0.20±0.18
Surfactant	19	49	28	44
Extubation (days)	16±13	18±17	23±18	20.5±17
FiO_2 (%)	38±18	36±16	54±21	39±16
Development of BPD	9	27	19	27

Continuous data given as mean±SD. Definition of abbreviations: ES = endostatin, chorioamnionitis = clinical signs, leukocytosis (B-leuk>14x10^9/L), or c-reactive protein concentration in plasma > 50mg/L, NA = not applied, L/S = lecithin/sphingomyelin, †: fetal lung maturity was measured from a tracheal aspirate sample within 3 hours after birth, aA = arterial-alveolar. STUDY I: PlGF, II: endostatin, III: endostatin in cord plasma (VLBW group), IV: VEGF-C.

2. Methods

2.1 Immunohistochemistry of lung samples (I, II and IV)

Lung samples from all infants included in the study were collected at autopsy. The samples were then fixed by 10% neutral buffered formalin and immersed in formalin. Samples were stored in dry conditions at room temperature until use. When samples were sectioned for use, the slides were stained within 2 weeks after sectioning. 5 μm sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol and distilled water. The sections were then treated in a 700 W microwave oven for 4x5 min in Tris-EDTA solution, and the slides were cooled at room temperature for 20 min and washed in 1:10 PBS distilled water solution. Specific antibodies were then applied, discussed individually for the different studies below. Bound antibody was visualized by the avidin-biotin complex (ABC) immunoperoxidase technique (Vectastain, Elite ABC Kit, Vectastain; Vector, Burlingame, CA). Sections were incubated with the biotinylated second-layer antibody and peroxidase labeled ABC for 30 minutes each. All dilutions were made in PBS (pH 7.2) and all incubations in the ABC method were carried out in humid chambers at room temperature. Between each step in the staining process, slides were rinsed in 3 changes of PBS. Peroxidase staining was visualized with 3-amino-9-ethyl-carbazole (A-5754; Sigma, St Louis, MO), 0.2 mg/mL in 0.05 M acetat buffer containing 0.03% perhydrol (pH 5.09 at room temperature for 15 min. Sections were rinsed in tap water for 10 min. To complete the process sections were counterstained in Mayer's haematoxylin, cleared in tap water and mounted in aqueous mounting medium (Aquamount; BDH, Poole, UK).

Negative controls were performed by omission of the primary antibody, and a known positive section (human liver) for the antibody was included as a positive control.

PlGF antibody was used at a 1:100 dilution (ab9542, rabbit polyclonal to human PLGF, Novus Biologicals, Inc., Littleton, CO). VEGFR-2 antibody was used at a 1:60 dilution (AF357, R&D Systems Anti-human VEGF R2 (KDR) Antibody, R&D Systems Inc., MN). Endostatin antibody was used at 1:200 dilution (PK-6105, Vectastain Elite ABC Kit (Goat IgG), Vector Laboratories Inc., CA). Before staining with the specific antibody sections to be stained for VEGF-C were treated with Trizma®base-HCl (pH 8.5) in 4 cycles of 5-min and microwaved. VEGF-C antibody was used at 1:100 dilution (Z-CVC7, polyclonal VEGF-C antibody, Zymed Laboratories Inc., CA) and VEGFR-3 antibody at 1:300 dilution (SC-321, Santa Cruz Biotechnology, Santa Cruz, CA).

2.2 Sample collection for TAF (I, II and IV)

A standardized routine of tracheal lavage was used once daily to collect TAF samples. After placing 1 mL of sterile isotonic saline into the endotracheal tube, the patient was then manually ventilated for 3 breaths after which the trachea was suctioned twice for 5 seconds each. Secretions were then collected into a trap and transferred into tubes containing 500

I.U. of aprotinin (Tyrasol®, Bayer, Leverkusen, Germany) and 5 mg of deferoxiamine (Desferal®, Ciba, Basel, Switzerland). Until the time of analysis the tubes were stored at -20°C.

In study I, a total of 70 samples from 20 patients, in study II, a total of 223 samples from 59 patients and in study IV, a total of 191 samples from 54 patients all during the first postnatal week were collected and used for analysis. In addition, in study II 23 samples during week 2 and 22 samples during weeks 3 to 5 and in study IV, 13 samples during week 2 and 23 samples during weeks 3 to 5 were collected from 6 patients who would later develop BPD. These samples were also included in the analysis for development of BPD.

2.3 Assays for TAF (I, II and IV)

All ELISA from TAF samples were performed in the laboratory facility of the Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland.

PlGF was measured with the Human PlGF Immunoassay Kit (R&D Systems Inc.). Endostatin was measured with the Human Endostatin Immunoassay Kit (R&D Systems Inc.). VEGF-C was measured with the VEGF-C ELISA Kit (Zymed Laboratories Inc.).

2.4 Analysis for dilution of the samples (I, II and IV)

In TAF analysis, to negate the effect of dilution of the sample and thus estimate the true *in situ* pulmonary concentration of each growth factor, the concentration of secretory component of IgA (IgA-SC) in TAF was used. IgA-SC concentration in TAF is independent of capillary leak as well as respiratory distress or GA (Watts et al, 1992). The method was standardized by using IgA-SC standards, which were isolated from human colostrums, kindly provided by Dr. B. Götze-Speer and Prof. Ch. Speer (University Children's Hospital, Würzburg, Germany).

In TAF IgA-SC concentration was determined by direct ELISA as follows. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μL aliquots of 1:2000 diluted anti-human secretory component (Dako, Glostrup, Denmark) in 50mM Na bicarbonate, pH 9.5 overnight at +4°C. The plates were then washed with 200 μL of 20-mM tris-500 mM NaCl, pH 7.5 (TBS). To block unspesific protein binding the plates were incubated with 200 μL of 2% bovine serum albumin (BSA) in TBS. After incubation the plates were washed with 0.05% Tween 20 in TBS (TTBS). 100 μL aliquots of diluted TAF samples were then added to the wells. TAF samples were diluted to between 1:10 to 1:500 in diluting buffer (1% BSA in TTBS). The plates were incubated overnight at room temperature. After incubation the plates were washed 3 times with TTBS. After washing 100 μL of diluted (1:400) peroxidase-conjugated rabbit anti-human SC (Dako) was added and the plates were incubated for 4 hours at room temperature. After incubation the plates were washed with TTBS. After washing 100 μL of substrate solution containing 8 mg of orthophenylenediamine (Dako) and 5 μL of 30% H_2O_2 in 12 mL water was added in order

to develop the plates. The optical densities of the plates were read at 450 nm after 30 min incubation at room temperature.

2.5 VEGF-C ELISA assaying (IV)

We wanted to make sure that the commercial ELISA kit for VEGF-C recognized only the appropriate human forms of VEGF-C. In order to do this we tested the ability of the VEGF-C kit to recognize different spliced forms of VEGF-C. The procedure is described in full in the following segments.

2.5.1 ELISA assaying

We assayed for ability of the VEGF-C ELISA kit to recognize conditioned media from cells stably transfected with full length VEGF-C, mature proteolytically processed human VEGF-C lacking the N-terminal and C-terminal propeptides (ΔNΔC-VEGF-C) or mock vector, described in segment 2.5.2 and 2.5.4. We also assayed for the ability to recognize human full-length VEGF-C and human ΔNΔC-VEGF-C by using the conditioned media from the adenoviral infections with full-length VEGF-C, ΔNΔC-VEGF-C and β-galactosidase (control), described in segments 2.5.2, 2.5.3 and 2.5.4. We finally assayed for recognition of recombinant human full-length VEGF-C and ΔNΔC-VEGF-C, described in segment 2.5.5. In summary, the ELISA kit recognized all full-length human VEGF-C proteins tested but not ΔNΔC-VEGF-C, mock vectors or controls.

2.5.2 Cell culture

For analysis of stably transfected VEGF-C, ΔNΔC-VEGF-C or mock vector, T293 cells were used. The cells were grown in Dulbecco's Modified Eagle's Media in the presence of 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) under selection with 1 ug/mL puromycin.

For analysis of adenoviral transfected full-length VEGF-C, ΔNΔC-VEGF-C and β-galactosidase (control), HeLa cells were used. The cells were grown in Modified Eagle's Media in the presence of 10% fetal calf serum, 2 mM L-glutamine, 0.1mM non-essential amino acids (Sigma-Aldrich) and 1mM sodium pyruvate (Sigma-Aldrich), penicillin (100 U/mL) and streptomycin (100 µg/mL).

2.5.3 Generation of adenoviral vectors

By cloning full-length human VEGF-C156S cDNA as a BamHI/NotI fragment into the corresponding sites of the pAD BglII vector, adenoviral vectors encoding for the full-length human VEGF-C, ΔNΔC-VEGF-C or ß-galactosidase (control) could be generated.

For transfection, 293T cells were used to grow replication-deficient E1-E3 deleted adenoviruses. The adenoviruses were then concentrated by ultracentrifugation. Adenoviral preparations were then inspected and were found to contain no helper viruses, lipopolysaccharide, or any bacteriological contaminants. 20-50 multiplicity of infection adenoviruses were used to infect 70% confluent HeLa cells. The infection was carried out in serum-free media containing 0.2% BSA for 1 h. After infection the cells were incubated in Modified Eagle's Media containing fetal calf serum for a period of 36 h. After incubation the conditioned media was collected and used for analysis.

2.5.4 Immunoprecipitation and western blotting

Protein secretion of stably transfected 293T cell lines as well as protein secretion from HeLa cells infected with the adenoviral vectors was verified as follows. Immunoprecipitation from the conditioned media was collected with anti-VEGF-C serum and analyzed by polyacrylamide gel electrophoresis under reducing conditions followed by western blotting with anti-VEGF-C serum. Results were then visualized by horse-radish peroxidase based chemiluminescence reaction.

2.5.5 Generation of recombinant proteins

Recombinant human VEGF-C and recombinant human ΔNΔC -VEGF-C proteins were generated as follows. The cDNA encoding the VEGF homology domain of human VEGF-C (ΔNΔC; nucleotides 658−996) and full-length human VEGF-C (nucleotides 445−1608) were cloned into a baculoviral transfer vector between the sequences encoding the honeybee melittin signal peptide and the hexahistidine tag. Because of its dimeric nature, VEGF-C has two histidine tags per molecule and thus allows for very stringent washing conditions in the affinity chromatography. The recombinant protein was isolated from serum-free conditioned supernatant of HighFive cells infected with recombinant baculovirus with nickel nitriloacetic acid−affinity chromatography.

2.6 Cord plasma (III)

In study III, 140 blood samples (2.0 mL) were collected at birth into EDTA tubes from the cord vein. After the tubes were centrifuged (3000 rpm for 10 min) the plasma was stored at -20°C until analysis. Endostatin concentrations were measured with the Human Endostatin Immunoassay Kit (R&D Systems Inc.).

2.7 Statistical analyses

StatView 5.1 (SAS Institute, Inc, Cary, NY) was used for statistical comparisons in all studies. For patient data values represent mean ± standard deviation (SD) whereas for experimental results values represent mean ± standard error of the mean (SEM). Frequency is used to express categorical variables. If variables exhibited skewed distribution they were Log_{10} transformed before analysis. However, values in text and tables are expressed in a non-transformed manner. Results were considered statistically significant when the p-value was less than 0.05.

For immunohistochemistry, data was analyzed by contingency table test and T-test.

For analysis of TAF, in study I nonparametric methods were used in analyses of TAF because of smaller sample size. Analyses included the Mann-Whitney U-test and Kruskal-Wallis test. In addition simple regression analysis was used to calculate correlations. In studies II and IV student's t-test was used to test differences between unpaired items. One-way ANOVA with Bonferroni post-hoc test was used to compare differences between groups. To compare frequency distributions between groups the chi-square test was used. Simple and multiple regression analyses were used to further analyze correlations.

In study III the same analyses as for TAF in studies II and IV were used. In addition, multiple regression analysis was used to analyze factors explaining endostatin concentrations and logistic regression analysis was used to analyze associations between the development of BPD and endostatin concentration.

Results

1. Growth factors and receptors in the perinatal period

1.1 Immunohistochemistry (I, II and IV)

Gender or post-mortem time to autopsy did not influence the results in immunohictochemistry analyses in any of the studies.

1.1.1 PlGF (I)

PlGF staining was seen throughout the perinatal period in bronchial epithelium. Both larger bronchi as well as smaller bronchioli displayed positivity. Apical and cytoplasmic parts of the cell both stained uniformly. Staining in alveolar epithelium was largely restricted to fetuses and preterm infants. Indeed, distal airway positivity for PlGF associated both with lower GA (p=0.0003) as well as lower weight at time of abortion or lower BW (p=0.0060). Distribution patterns within the cells differed between fetuses and preterm infants; staining in fetuses was seen apically whereas staining in preterm infants was more evenly distributed (I).

Some macrophage positivity was seen in PlGF throughout the perinatal period (I).

1.1.2 Endostatin (II)

Positively staining ECs were seen throughout the perinatal period. Positively staining cuboidal epithelial cells were seen in 7 out of 9 fetuses with preterm or term controls exhibiting no epithelial positivity. No positively staining macrophages were seen in fetuses, but 2 preterm and 2 term controls stained positive (II).

1.1.3 VEGF-C (IV)

VEGF-C staining was seen in bronchial epithelium throughout the perinatal period. In most cases, positivity was observed in all bronchial structures in which most of the bronchial cells stained positively. Uniform and strong intensity staining was seen apically and less intense and more scattered staining in areas not adjacent to lumen. In 8 fetuses VEGF-C staining was observed in alveolar epithelium. During later stages of development no alveolar epithelial staining was seen. In most of the positive cases, staining was visible in most of the alveoli in which staining was seen in type-II pneumocyte resembling cells. Staining of VEGF-C in macrophages remained negative throughout the perinatal period (IV).

1.1.4 VEGFR-2 (I)

VEGFR-2 staining was seen in vascular endothelium in 18 of the 21 cases perinatally. A pattern for VEGFR-2 staining was evident; lower GA (p=0.024) and lower weight at time of abortion or BW (p=0.024) associated with positively staining venous endothelium, whereas higher GA (p=0.021) and higher weight at time of abortion or BW (p=0.019) associated with positively staining arterial endothelium. VEGFR-2 staining was seen additionally in lymphatic resembling structures in 7 of the 21 cases. In addition to 2 term control samples being positive, positive lymphatic endothelial staining was seen mostly in fetuses, with 5 out of 9 fetal samples being positive (I).

1.1.5 VEGFR-3 (IV)

Staining for VEGFR-3 was observed in all samples in lymphatic endothelium adjacent to vascular endothelium (IV).

1.2 TAF (I, II and IV)

The mean concentration of each growth factor during the first postnatal week was used for statistical analysis in TAF studies.

1.2.1 Growth factor concentrations postnatally

Mean PlGF concentration during the first postnatal week was stable (I). Mean endostatin concentration decreased during the first postnatal week. During week 2 it increased slightly, and decreased again during postnatal weeks 3-5 (II). Mean VEGF-C concentration decreased during the first postnatal week. VEGF-C protein expression then stabilized during week 2, and again decreased during weeks 3 to 5 (IV). VEGF-A and VEGF-D were analyzed in conjunction with VEGF-C concentrations. VEGF-D concentrations in the samples were below the measurement threshold. Also, no correlation between VEGF-A and VEGF-C concentrations were found (p=0.28).

1.2.2 Association of growth factor concentrations to parameters reflecting lung development

All studied antenatal, perinatal parameters as well as parameters reflecting maturity are shown in Table 3. Delivery by C-section correlated with higher PlGF levels (p=0.049). However, no correlations were found between BW, GA, pH or base excess (BE) and PlGF. Higher endostatin concentration associated with delivery by C-section (p=0.012), and also correlated with low BW (p=0.043) but not GA, pH or BE (Table 3). Higher concentrations

of VEGF-C correlated with lower BW, (p=0.004), as well as higher umbilical cord pH (p=0.038), but not GA or BE (Table 3, IV).

1.3 Cord plasma (III)

In the healthy term infant group none of the clinical parameters presented in Table 2 correlated with endostatin concentrations. Endostatin concentration in VLBW infants was lower than in healthy term infants (p<0.0001) (Figure 5).

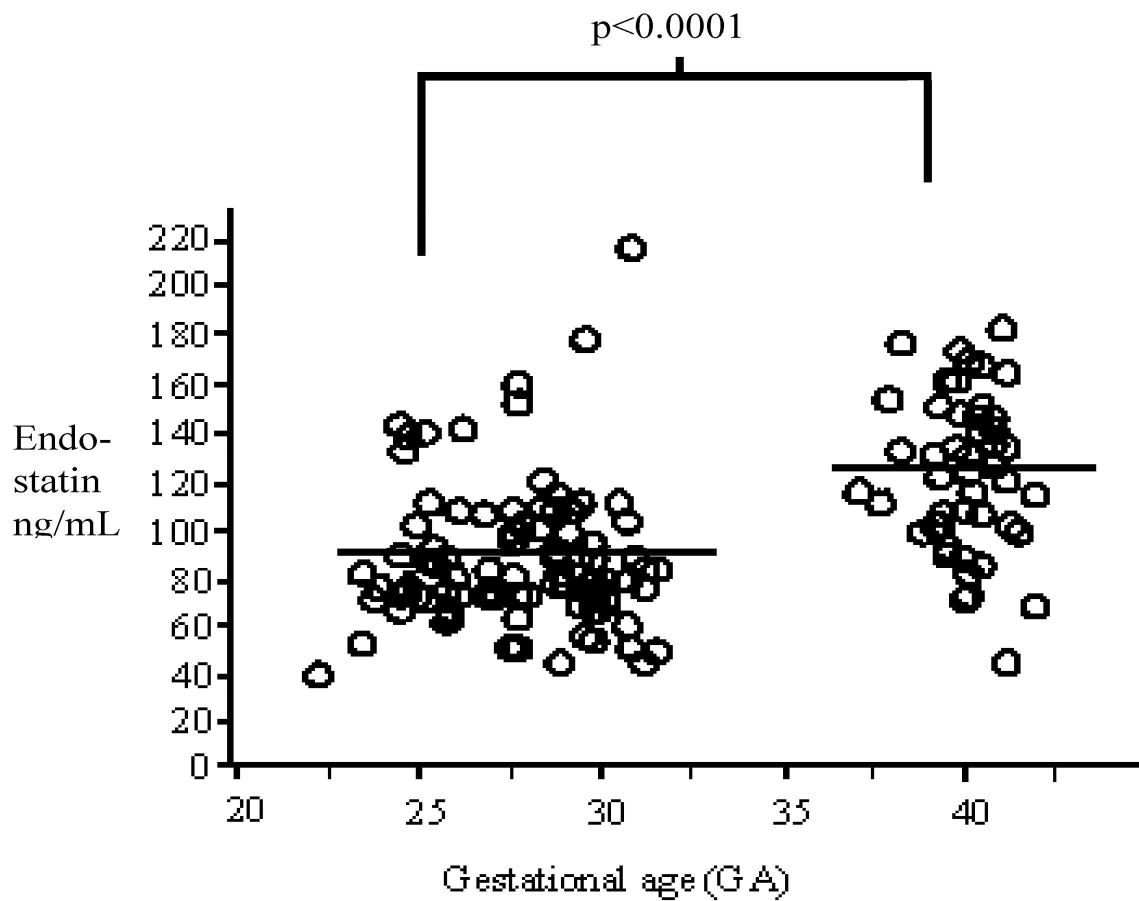

Figure 5. *Endostatin concentration in cord plasma is significantly higher in term than in VLBW infants. Horizontal lines indicate mean. Janér et al. Pediatrics 2009; in press, reprinted with permission of the copyright holder.*

Table 3. *Results of ELISA of growth factors and association to clinical parameters (I-IV)*

	I *PLGF*	*II* *ES*	*III* *ES cord*	*IV* *VEGF-C*
	N=20	N=59	N=92	N=54
Antenatal parameters				
Betamethasone	NS	↑, *	NS	↑, *
number of doses (1 vs. 2 doses)	NS	NS	NS	↑, **
time of administration pre delivery (days)	NS	NS	NS	↓, *
Antenatal dg.[1]				
Chorioamnionitis vs. preeclampsia	NA	↓, **	NA	↓, *
Chorioamnionitis vs. no ant. dg.	↓, *	↓, ***	NS	↓, *
Preeclampsia vs. no ant. dg.	NS	NS	NS	NS
C-section + vs. -	↑, *	↑, *	NS	NS
Parameters reflecting maturity				
Gestational age (weeks)	NS	NS	NS	NS
Birth weight (g)	NS	↓, *	NS	↓, **
Birth weight (standard deviation)	NS	NS	NS	NS
L/S ratio (0-10) †	NS	↓, *	NA	NS
Perinatal parameters				
Apgar score at 1 min	NS	NS	NS	NS
Indomethacin	NS	NS	NS	↓, *
LC †	↓, *	↓, *	NA	↓, **
Umbilical cord arterial pH	NS	NS	↓, *	↑, *
Umbilical cord arterial BE	NS	NS	↓, **	NS
Respiratory distress parameters				
aA ratio	NS	NS	NA	NS
Surfactant	NS	NS	NS	NS
Extubation (days)	NS	NS	NS	NS
FiO_2 (%)	NS	↑, *	↑, *	NS
Development of BPD	NS	NS	↑, *	NS

Continuous data given as mean±SD. Definition of abbreviations: **:** *ES = endostatin, NA = not applied, NS = not significant, chorioamnionitis = clinical signs, leukocytosis (B-leuk>14x10^9/L), or c-reactive protein concentration in plasma > 50mg/L, L/S = lecithin/sphingomyelin, †: fetal lung maturity was measured from a tracheal aspirate sample within 3 hours after birth, aA = arterial alveolar, p<0.05 = *, p<0.01 = **, p<0.001 = ***, ↑ = associating or correlating with higher concentration, ↓ = associating or correlating with lower concentration. [1]In studies III and IV chorioamnionitis and preeclampsia were analyzed as follows: chorioamnionitis vs. preeclampsia and no antenatal dg. and preeclampsia vs. chorioamnionitis and no antenatal dg, STUDY I: PlGF, II: endostatin, III: endostatin in cord plasma (VLBW group), IV: VEGF-C.*

1.3.1 Association of endostatin concentrations to parameters reflecting lung development

In VLBW infants endostatin concentrations showed no correlation with GA (p=0.91), BW (p=0.49) or relative BW (p=0.80). Higher endostatin concentration associated with both lower umbilical cord pH (p=0.022) as well as lower BE (p=0.0039). In multiple regression analysis after adjustment for GA, relative BW and impaired umbilical artery flow the associations between pH and BE and endostatin remained statistically significant.

2. Growth factors and receptors in lung injury in preterm infants

2.1 Immunohistochemistry (I, II and IV)

2.1.1 PlGF (I)

PlGF staining was seen evenly in epithelium as well as in macrophages in the RDS and BPD groups. More staining was observed in bronchial than in alveolar epithelium. However, no statistically significant differences in staining existed between the groups (I).

2.1.2 Endostatin (II)

Endostatin positivity was observed in ECs in all RDS and BPD cases. In early RDS neither epithelial cell nor macrophage staining was observed. However, in late RDS positive staining bronchiolar epithelial cells (2/7) as well as alveolar epithelial cells (2/7) in addition to alveolar macrophages (5/7) were seen. In BPD, positivity was observed in alveolar epithelial cells (3/8) as well as alveolar macrophages (5/8) (II).

2.1.3 VEGF-C (IV)

VEGF-C staining was seen in bronchial epithelium in all samples of preterm infants in the RDS and BPD groups. Positivity was observed in the same manner as previously mentioned during the perinatal period. Staining in alveolar epithelium was seen in late RDS in 1 case and in BPD in 2 cases.

Additionally staining was seen in alveolar macrophages in 2 late RDS cases and in 4 BPD cases. In these samples, macrophages were found in clusters and in each positive case more than 50% of the macrophages stained positively for VEGF-C (IV).

2.1.4 VEGFR-2 (I)

VEGFR-2 staining between the groups revealed that overall capillary positivity was over 60%, except in the BPD group, where positivity was only 33%. Subsequently capillary VEGFR-2 positivity associated with lower postnatal age of death (p=0.0034) (I).

2.1.5 VEGFR-3 (IV)

No difference between the groups was seen for VEGFR-3 staining.

2.2 TAF (I, II and IV)

Subsequent development of BPD did not associate with VEGF-C, PlGF or endostatin concentrations directly.

2.2.1 PlGF (I)

Lower levels of PlGF correlated with chorioamnionitis (p=0.015), as well as LC positivity (p=0.027) (Table 3).

2.2.2 Endostatin (II)

Higher endostatin associated with administration of antenatal betamethasone (p=0.019), well as a higher FiO_2 (p=0.024). LC positivity (p=0.029), higher lecithin sphingomyelin ratio, a measure of lung maturity, (p=0.055) and chorioamnionitis on the other hand associated with lower endostatin concentrations. The association of chorioamnionitis and endostatin was statistically significant both when chorioamnionitis was compared only to preeclampsia (p=0.010) in addition to cases with preeclampsia as well as no antenatal diagnosis (p=0.001) (Table 3).

2.2.3 VEGF-C (IV)

Higher VEGF-C concentrations associated with administration of antenatal betamethasone (p=0.046), a higher number of antenatal betamethasone doses (p=0.01) as well as administration of betamethasone closer to birth (p=0.046). LC positivity (p=0.002) as well as indomethacin treatment (p=0.016) both associated with lower VEGF-C concentrations (Table 3). Multiple regression analysis for VEGF-C was performed by inclusion of all significant parameters after which all nonsignificant parameters were withdrawn. Three significant parameters remained: time elapsed between antenatal betamethasone administration and birth (p=0.013), presence of LC (p=0.047), and treatment with indomethacin (p=0.007).

2.3 Cord plasma (III)

2.3.1 Association of endostatin concentrations to the development of BPD

Higher endostatin concentration associated with a higher FiO_2 (p=0.027) and also directly with the development of BPD (p=0.029) (Figure 6). We assessed whether endostatin concentrations would predict the development of BPD by performing a logistic regression analysis in the VLBW group. A 1-SD higher endostatin concentration was associated with a 1.76-fold higher OR (95% confidence interval 1.04-2.99) for BPD. GA was a strong predictor of the development of BPD: a 1-week higher GA was associated with an OR of 0.40 (95% confidence interval 0.26-0.61). When we adjusted the analysis of endostatin concentrations for GA, the OR for BPD was higher: a 1-SD higher endostatin concentration was associated with a 5.54-fold OR (95% confidence interval 1.82-16.91). This association remained statistically significant after further adjustment for gender, umbilical cord pH, relative BW (SD), impaired umbilical artery flow, postnatal sepsis, patent ductus arteriosus and RDS.

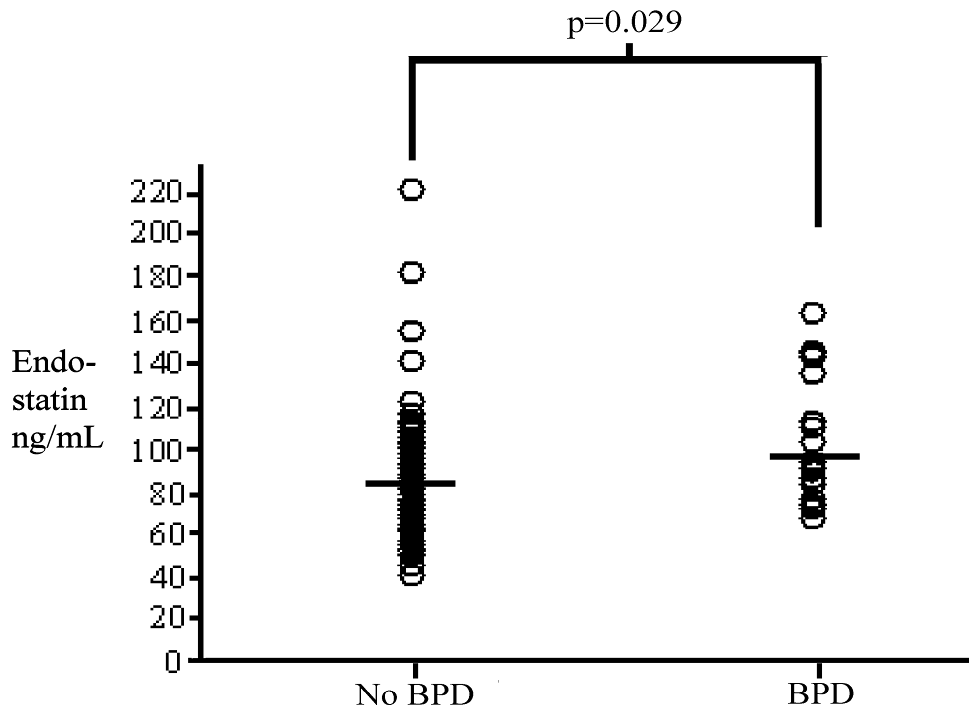

Figure 6. *Endostatin concentration in cord plasma of VLBW infants. Higher endostatin concenentration associates with an increased risk to later develop BPD. Horizontal lines indicate mean. Janér et al. Pediatrics 2009; in press, reprinted with permission of the copyright holder.*

Discussion

It has become accepted that the arrest of development of vascular structures because of premature birth is an important factor in the development of BPD (Jobe, 1999; Abman, 2001). The lung at this stage normally acts to extend, shape and multiply the developing alveoli and associating capillaries, but is forced into a new dynamic environment. Instead of continuing normal development, the lung has to adapt to become a gas-exchanging organ prematurely. This situation changes the surroundings of the preterm lung, and it comes into contact with factors that it is not equipped to cope with, and which are commonly associated with the development of BPD, such as inflammation and distension of the airways in addition to oxygen.

Since the development of BPD is considered to be a consequence of the arrest of the development of the vascular structures of the lung, it seems logical to assume that a disturbance in the balance of proangiogenic and antiangiogenic factors as well as lymphangiogenic factors might exist in developing BPD. We wanted to study such growth factors to increase our understanding of normal lung development as well as the development of BPD.

Our material consisted of samples gathered from patients receiving treatment at the Hospital for Children and Adolescents in the Helsinki University Central Hospital. The advantage of having a human material was that we were able to draw accurate conclusions about the presence and distribution of each growth factor during human lung development. Also, since accurate and complete patient journals were at hand we could assess the effect of a broad range of clinical parameters associated with BPD on growth factor concentrations. The limitations of the clinical material were that it was fairly small and can never be as controlled as an animal model. For TAF, no comparison with healthy controls could be made since healthy term patients are not intubated for several days postpartum.

1. Growth factors during lung development

PlGF and VEGFR-2 are both known to act in a proangiogenic fashion. PlGF was seen throughout development in lung epithelium as well as at a constant concentration during the first postnatal week in TAF (I). Distal staining in alveolar and cuboidal epithelium correlated with lower GA (I). The expression pattern for PlGF was to be expected due to the fact that VEGFR-1, the main receptor for PlGF, has been shown to be expressed in a similar way (Lassus et al, 2001). This suggests that at least during later stages of development PlGF asserts its effects mainly on conducting airways. There was no correlation between lower GA and higher PlGF concentration in TAF (I). $PlGF^{-/-}$ mice have been shown to exhibit normal vascular development (Carmeliet et al, 2001) and it seems despite clearly playing some part that PlGF might not be critical for normal lung development.

Previously, no study on VEGFR-2 expression in the human lung had been published. We were able to show that VEGFR-2 staining between different types of endothelium varied during development (I). Interestingly, we detected some VEGFR-2

expression in lymphatic endothelium, mostly in fetuses. Additionally, in immature lungs more venous staining whereas in mature lungs more arterial staining was seen (I). VEGFR-2 plays a role early during development in EC commitment as well as being the main receptor for VEGF-A mediated proangiogenic action (Shalaby et al, 1997; Hidaka et al, 1999; Schuh et al, 1999,). We interpreted the switch of VEGFR-2 staining between lymphatic and venous endothelium early in development to arterial endothelium later in development as a reflection of the ongoing differentiation and activity of different endothelia. In fact, during early development there seems to be a certain overlapping of vasculo-, angio- and lymphangiogenesis; in mice, VEGFR-2 has an essential role in the development of hematopoietic cells and ECs (Shalaby et al, 1995) and has previously been detected on lymphatic ECs (Makinen et al, 2001a).

VEGF-C is recognized as a lymphangiogenic growth factor. VEGF-C$^{+/-}$ heterozygous knockout mice develop cutaneous lymphatic hypoplasia and lymphedema, and VEGF-C$^{-/-}$ homozygous knockout mice do not survive until birth and exhibit chylous fluid accumulation in tissues (Karkkainen et al, 2004). However, during early development VEGF-C binds VEGFR-2 in addition to VEGFR-3 (Joukov et al, 1996), and may play a role in the development of the vascular tree (Dumont et al, 1998). Also, VEGFR-3, which later in development is strictly lymphangiogenic, is vital to venous development in mice lung (Dumont et al, 1998).

Similarily to PlGF staining, all fetuses and infants exhibited VEGF-C staining in bronchial epithelium, whereas alveolar staining was seen mostly in fetuses (IV). Staining for VEGFR-3 protein was observed in all samples from fetuses to term infants in lymphatic endothelium adjacent to vascular structures, however, no vascular staining was seen (IV). The fetuses included in the study were of GA 18.5±2.1 weeks and it seems if any VEGFR-3 vascular affinity does occur, it would occur at an earlier period of development. The commercial ELISA used was initially assayed for the ability to recognize only full length VEGF-C, not spliced forms of the protein. The concentration of VEGF-C in TAF was highest during the first 2 postnatal days, after which VEGF-C levels decreased during the first postnatal week (IV). Further, in cord plasma VEGF-C concentrations at birth were significantly higher in term than in preterm infants (Janér, et al, unpublished results). Since VEGF-C staining was seen throughout development in epithelium, the decrease in VEGF-C protein levels in the lungs of preterm infants postnatally could be interpreted to have adverse effects on the developing lymphatic vasculature.

Endostatin acts in concert with proangiogenic growth factors such as VEGF-A, and seems to model and guide the developing vasculature (Abdollahi et al, 2004; Jia et al, 2004). Mouse models indicate that while endostatin does play a role in physiological angiogenesis, it is not critical for survival. Endostatin knockout mice have a normal lifespan, and apart from ocular abnormalities, exhibit no major vascular abnormalities. A more proangiogenic environment in these mice is suggested by the fact that aortic explants show a twofold increase in the number and length of microvessels (Li & Olsen, 2004). Immunohistochemical staining of endostatin exhibited a pattern reminiscent to that of VEGF-C and PlGF; staining was more abundant during earlier stages of development; fetuses exhibited endostatin staining in endothelium as well as in epithelium, whereas at term no positively endostatin staining epithelium was seen (II). Endostatin protein was

found consistently in TAF in preterm infants. As with VEGF-C, the concentration was highest immediately postpartum decreasing during the first postnatal week (II) and in cord plasma endostatin concentrations at birth were significantly higher in term than in preterm infants (III). On the basis of these findings it seems that endostatin plays a role in normal angiogenesis during all stages of lung development. Previously the expression of EMAP-II, an antiangiogenic factor, was shown to vary greatly in mice during lung vascularization (Schwarz et al, 1999a). Similarly, endostatin seems to exert its action at different times during development. We cannot draw any conclusions on endostatin activity during development, but the fact that concentrations increase closer to term might indicate that endostatin has a time of action near term and possibly after birth in normal vascular development. This timepoint coincides with the period of alveolarization, in which precise angiogenic growth is important.

Thus, it seems that VEGF-C, endostatin and to a lesser extent PlGF are important growth factors during lymphangiogenesis and angiogenesis in normal lung development. In addition, VEGFR-2 seems to have a strong role in the developing endothelium to direct the proangiogenic action of VEGF-A. VEGFR-3 was seen to mediate lymphangiogenic action exclusively. A disturbance in the expression or action of any one of these growth factors or receptors could have adverse effects on the developing human lung.

2. The role of growth factors in lung injury

BPD is a multifactorial disorder. Several clinical parameters known to be associated with BPD were analyzed in the TAF and umbilical cord studies in addition to BPD (I, II, IV).

Prepartum and postpartum inflammation is associated with BPD. Initiation of ventilation causes injury in the lung parenchyma, which leads to further release of proinflammatory cytokines. The cytokines in turn activate and attract neutrophiles and phagocytes to the site of injury (Jobe & Bancalari, 2001; Turunen et al, 2006). PlGF is also associated with recruitment of inflammatory cells (Khurana et al, 2005) and has been shown to induce pathologic angiogenesis in the adult by assisting VEGF-A (Carmeliet et al, 2001; Kumazaki et al, 2002). In addition, VEGF-A expression is increased in the lung prenatally in response to inflammation (Lassus et al, 1999). Also, a higher PlGF concentration in cord plasma of preterm infants has been shown to be associated with the development of BPD (Tsao et al, 2004). Considering this background we expected to find an elevation in PlGF concentrations in preterm infants later developing BPD. Be that as it may, such an elevation in concentration amongst our study group was not seen. In fact, infants born from pregnancies complicated by chorioamnionitis or preterm premature rupture of the membranes, and those who were positive for LC had lower PlGF in TAF during the first postnatal week (I). Thus, we could not find evidence that PlGF assists VEGF-A in angiogenesis in the human perinatal lung even during inflammation as we would have expected. On the basis of our findings it seems that the role of PlGF in the development of BPD is marginal. However, this issue remains open for further investigation.

VEGF-C immunohistochemistry revealed that macrophages stained positively only in late RDS and BPD (IV). This is in accordance with experimental data demonstrating that lymphangiogenesis seems to be driven by VEGF-C and VEGF-D derived from inflammatory cells that migrate into the airways (Baluk et al, 2005). Maternal chorioamnionitis and the presence of LC in TAF both associated with lower, whereas treatment with antenatal glucocorticoids was associated with higher VEGF-C levels in TAF (IV). In the preterm infant administration of glucocorticoids prior to birth induces lung maturation (Jobe, 2001). Prenatal inflammation *in utero* has also been associated with accelerated lung development (Hallman et al, 1989; Bry & Lappalainen, 2001). However, when inflammation continues postnatally it is associated with an impairment in lung development and may possibly lead to the development of BPD (DeSa, 1969; Watterberg et al, 1996; Speer, 2006). Thus, we conclude that the increase in VEGF-C levels seen in association with glucocorticoid treatment (IV) may be part of the accelerated maturation process of the lung. However, the lower TAF concentration of VEGF-C that associated with prenatal inflammation (IV) is interpreted as harmful for continued postnatal lymphatic development. This may lead to hypoplastic lymphatic structures, impairing the ability of the preterm lung to clear excess fluid accumulated either due to the treatment of the clinical symptoms of the patient or the development of BPD or both. It may be that the increased VEGF-C positivity seen in macrophages in the late RDS and BPD groups (IV) is a reflection of the preterm lung trying to increase lymphatic development to try to correct the situation.

Lower endostatin concentration in TAF was found in cases with maternal chorioamnionitis as well as associated with LC positivity (II). As discussed above, inflammation accelerates lung maturation, but also disrupts the continued physiological vascular development of the lung. We consider the decrease in endostatin concentrations in association with inflammation to be a reflection of the maturation process of the lung. In addition, inflammation seems to tip the angiogenic environment of the lung towards a more proangiogenic surrounding as seen by the decrease in endostatin concentrations and the previously reported increase in VEGF-A concentrations in TAF (Lassus et al, 1999).

The need for higher FiO_2 was found to correlate with higher endostatin concentration in TAF (II). This same correlation also existed in cord plasma in preterm infants (III). Previously higher oxygenation has been found to associate with BPD (STOP-ROP trial, 2000), and mechanical ventilation in addition to the direct effects of oxygen are part of the pathogenesis of BPD (Coalson et al, 1999; Charles et al, 2004; Chu et al, 2004). It is also known that endostatin downregulates several important signalling pathways in human microvascular endothelium associated with proangiogenic activity (Abdollahi et al, 2004). In cord plasma, a higher concentration of endostatin was associated with the development of BPD (III). In a logistic regression analysis this association remained statistically significant after adjustment for factors known to be associated with BPD (III). In immunohistochemistry, late RDS and BPD cases displayed positive endostatin staining in epithelial cells as well as in macrophages. This pattern of protein expression was unique for these groups and could be seen neither in controls nor in early RDS cases (II). It seems that with the progression of lung injury the protein expression of endostatin is upregulated and appears in cells normally dormant for endostatin expression at that point in time. When

comparing the endostatin expression pattern with that seen in VEGFR-2, almost a reversal of the results is seen. VEGFR-2 staining was fairly constant throughout the groups (I). However, staining of capillary and septal endothelium was markedly decreased in BPD (I). VEGFR-2 expression is guided by VEGF-A (Gerhardt et al, 2003; Hiratsuka et al, 2005) and lower levels of VEGF-A in TAF during the latter part of the first postnatal week correlates to the development of BPD (Lassus et al, 1999; Bhandari et al, 2008). VEGFR-2 deficient mice fail to develop blood islands resulting in severely impaired vasculogenesis (Shalaby et al, 1995). In addition, in rats, hyperoxia leads to decreased mRNA and later protein expression of VEGF-A and VEGFR-2, and results in diminished alveolarization (Hosford & Olson, 2003). VEGFR-2 is the primary receptor through which VEGF-A mediates its proangiogenic action. We feel that the decreased VEGFR-2 expression in septal capillaries is a sign of the dysregulation of vascular development that is characteristic of BPD.

In conclusion, the pattern of VEGFR-2 and endostatin protein expression illustrates the unbalanced situation that exists between proangiogenic and antiangiogenic growth factors in the development of BPD. Further, a higher concentration of endostatin in the preterm lung might play a role in the pathogenesis of BPD. This is strengthened by the fact that a higher endostatin concentration in cord plasma of preterm infants predicts the development of BPD.

Conclusions

All studied growth factors; PlGF, endostatin and VEGF-C, as well as receptors VEGFR-2 and VEGFR-3 were seen consistently throughout human lung development. In addition PlGF, endostatin and VEGF-C were found postnatally in TAF and cord plasma and the concentrations in term infants were higher than in preterm infants for endostatin. This supports the role of endostatin and VEGF-C and to a lesser extent PlGF in normal vascular development of the lung. VEGFR-2 was shown for the first time to be expressed in the human lung during development. VEGFR-2 seems to have a strong role in the developing endothelium as a proangiogenic mediator of VEGF-A activity. VEGFR-3 was seen to mediate lymphangiogenic action exclusively. A disturbance in the expression or action of any one of these growth factors or receptors could have adverse effects on the developing human lung.

We found no connection between PlGF, VEGF-C, and VEGFR-3 and the development of BPD. On the other hand, both endostatin and VEGFR-2 seem to be important in the development of BPD. This was illustrated by the disruption of VEGFR-2 staining in capillary and septal endothelium seen in the BPD group, as well as the increase in endostatin concentrations both in TAF and cord plasma that associated with BPD. This is in agreement with previous studies where preterm infants developing BPD had lower concentrations of VEGF-A in TAF postnatally. There thus seems to be a shift in the angiogenic balance towards a more antiangiogenic environment in BPD. These findings underline the vascular hypothesis of BPD, which states that the disorder is caused by an arrest in the vascular development of the preterm lung.

In summary, endostatin, VEGFR-2 and to a lesser extent PlGF play a role in physiological angiogenesis, whereas VEGF-C, VEGFR-3 and early during development VEGFR-2 play a role in lymphangiogenesis in the human lung. Additionally endostatin and VEGFR-2 participate in the pathogenesis of BPD, reflecting the impairment in vascular development that is characteristic for the disorder.

Yhteenveto (Finnish summary)

Raskausviikolla 24-28 syntyneen keskosen keuhkojen kypsymättömyys altistaa keuhkovaurioon, bronkopulmonaaliseen dysplasiaan (BPD), joka kehittyy n. 30 prosentille pikkukeskosista. BPD:n kehittymiseen vaikuttavat osaltaan ainakin infektiot, tulehdus, hengitysilman hapen suora toksisuus ja sisäänhengityksen liian korkean paineen aiheuttama mekaaninen vaurio. BPD johtaa keuhkojen kehityksen merkittävään hidastumiseen.

Monet kasvutekijät vaikuttavat keuhkojen verisuonituksen kehitykseen ja BPD:ssa kasvua säätelevien tekijöiden ja niiden vastavaikuttajien välillä vallitsee epätasapaino. Tärkein verisuonten kasvuun vaikuttava tähän asti löydetty kasvutekijä on vascular endothelial growth factor A (VEGF-A). VEGF-A on elintärkeä normaalille verisuonikehitykselle, sillä jo yksi pistemutaatio VEGF-A-geenissä johtaa sikiöaikaiseen kuolemaan. Keuhkojen VEGF-A-pitoisuus on korkea syntymän jälkeen, mutta matalampi ensimmäisen syntymänjälkeisen viikon loppupuolella niillä keskosilla, joille myöhemmin kehittyy BPD. Tätä taustaa vasten väitöskirjatyön tavoitteena oli tutkia VEGF-A:n liittyvien kasvutekijöiden placental growth factor:n (PlGF), endostatiinin sekä VEGF-C:n yhteyttä keuhkojen kehitykseen ja BPD:n.

Työ koostuu kolmesta osa-analyysista. Ensimmäistä osa-analyysia varten kerättiin menehtyneiden ihmissikiöiden ja lasten keuhkokudosnäytteitä, Helsingin Yliopiston Lasten ja Nuorten Sairaalassa vuosina 1991-2000 hoidetuista potilaista. Näytteistä normaalia keuhkojen kehitystä edustivat keskenmenneiden raskauksien sikiöt (raskausviikot 16-20), keskosina syntyneet (raskausviikot 24-32) ja täysiaikaisina syntyneet (raskausviikot 38-42). Kaikilla potilailla oli kuolinhetkellä normaalisti kehittyneet keuhkot ja keuhkokudos oli näytteissä normaalia. Lisäksi näytteitä kerättiin keuhkovaurioon menehtyneiltä pieniltä keskosilta. Sairaiden keskosten osalta ryhmät jaettiin akuuttiin (aikainen RDS, kuolinaika 0-2 päivää syntymästä), subakuuttiin (myöhäinen RDS, kuolinaika 3-10 päivää syntymästä) sekä krooniseen keuhkovaurioon (BPD).

Toista osa-analyysia varten määritettiin kasvutekijäpitoisuuksia pikkukeskosten imulimanäytteistä ensimmäisen viikon aikana syntymän jälkeen ELISA-menetelmällä. Aineisto valittiin niin, että noin puolelle potilaista myöhemmin kehittyi BPD.

Kolmatta osa-analyysia varten endostatiinipitoisuus määritettiin napaverestä ELISA-menetelmällä 92 pikkukeskosesta ja 48 täysiaikaisena syntyneestä lapsesta syntymän yhteydessä.

Ensimmäisessä osa-analyysissa kaikki tutkitut kasvutekijät ja reseptorit ilmenivät kaikissa keuhkokehityksen vaiheissa. Keuhkokehityksen aikaisemmassa vaiheessa epiteelisoluissa (PlGF, endostatiini ja VEGF-C) ja endoteelisoluissa (endostatiini) havaittiin värjäytymistä kauttaaltaan keuhkoissa, kun taas kehityksen myöhemmässä vaiheessa värjäytyminen rajoittui keuhkojen ylempiin osiin (PlGF ja VEGF-C). VEGFR-3:ta havaittiin keuhkokehityksen kaikissa vaiheissa ja ainoastaan imusuonirakenteissa. Keuhkokehityksen aikaisemmassa vaiheessa VEGFR-2:ta havaittiin imusuonten- ja laskimoiden endoteelisoluissa, kun taas kehityksen myöhemmässä vaiheessa VEGFR-2:n ekspressio rajoittui valtimoiden endoteelisoluihin. Myöhäisessä RDS- ja BPD-ryhmässä joka toisessa näytteessä havaittiin endostatiinia keuhkorakkuloiden epiteeli- ja makrofagisoluissa. VEGF-C:tä havaittiin keuhkorakkuloiden makrofageissa ainoastaan

myöhäisessä RDS- ja BPD-ryhmissä. VEGFR-2:ta havaittiin tasaisesti ryhmien välillä. Poikkeuksena BPD-ryhmän hiussuonten endoteelisoluissa VEGFR-2 puuttui joka toisesta näytteestä.

Toisessa osa-analyysissa pikkukeskosten imulimanäytteiden PlGF-pitoisuus pysyi vakaana, kun taas endostatiini- ja VEGF-C-pitoisuudet vähenivät ensimmäisen elinviikon aikana. Korkeammat endostatiini- ja VEGF-C-pitoisuudet liittyivät matalampaan syntymäpainoon, sekä ennen syntymää toteutettuun kortisonihoitoon. Gestaatioiällä ei ollut yhteyttä imulimanäytteiden kasvutekijäpitoisuuksien kanssa. Kohdunsisäistä tulehdusta heijastavat kliiniset tekijät olivat yhteydessä matalampiin PlGF-, endostatiini- ja VEGF-C-pitoisuuksiin. Sisäänhengitysilman korkeampi happiosapaine (FiO_2) ensimmäisen elinviikon aikana liittyi korkeampiin imulimanäytteiden endostatiinipitoisuuksiin.

Kolmannessa osa-analyysissa täysiaikaisilla vastasyntyneillä tavattiin korkeampia napaveren endostatiinipitoisuuksia kuin pikkukeskosilla. Keskosryhmässä korkeampi endostatiinipitoisuus oli suoraan yhteydessä BPD:an. Tämä yhteys säilyi merkitsevänä logistisessa regressioanalyysissa, jossa huomioitiin tulosta mahdollisesti sekoittavia kliinisiä tekijöitä, joiden tiedetään lisäävän BPD:n riskiä.

PlGF:n, endostatiinin sekä VEGF-C:n tasainen esiintyminen keuhkokehityksen kaikissa vaiheissa sekä johdonmukainen esiintyminen pikkukeskosilla syntymän jälkeen imulimassa painottaa kasvutekijöiden merkitystä normaalissa keuhkokehityksessä. Tämä yhteys korostuu endostatiinin osalta, sillä endostatiinipitoisuus napaveressä oli korkeampi täysiaikaisilla vastasyntyneillä kuin pikkukeskosilla. VEGFR-2:n värjäytymismalli saattaa heijastaa erityyppisten endoteelisolujen erilaistumista kehityksen aikana.

Imulimassa havaittiin kaikkien kasvutekijöiden osalta, että matalammat kasvutekijäpitoisuudet ovat yhteydessä kohdunsisäiseen tulehdukseen liittyviin kliinisiin tekijöihin. Kohdunsisäinen tulehdus saattaa näin ollen aiheuttaa verisuonikehityksen vähenemistä. Verisuonikehityksen hidastumiseen liittyen VEGFR-2:n värjäytymisen väheneminen keuhkorakkuloiden hiussuoniendoteelisoluissa BPD-ryhmässä sopii vaskulaariseen hypoteesiin, jonka mukaan BPD:n kehittymisen syynä on keuhkojen verisuonikehityksen pysähtyminen. Korkeampi FiO_2-paine oli pikkukeskosilla yhteydessä imuliman ja napaveren korkeampaan endostatiinipitoisuuteen. Tämän lisäksi napaveren korkeampi endostatiinipitoisuus oli suoraan yhteydessä BPD:an. Nämä tulokset viittaavat siihen, että korkea endostatiinipitoisuus on haitallinen pikkukeskosen keuhkokehityksessä ja lisää pikkukeskosen riskiä sairastua BPD:n.

Sammandrag (Swedish summary)

Framsteg inom vården av prematurt födda barn har möjliggjort överlevnaden av barn med en födelsevikt på 500-1000 g. I detta skede är lungorna inte fullt utvecklade, vilket utsätter barnet för kronisk lungskada, bronkopulmonär dysplasi (BPD). BPD utvecklas då utvecklingen av blodkärl i lungan störs till följd av en störning i balansen mellan tillväxtfaktorer som styr blodkärlens utveckling. Målsättningen för avhandlingen var att undersöka rollen av placental growth factor (PlGF), endostatin och vascular endothelial growth factor (VEGF) -C, samt deras receptorer VEGFR-2 och VEGFR-3 i människolungans fysiologiska utveckling och i utvecklingen av BPD. De undersökta tillväxtfaktorerna valdes på basen av deras samband med VEGF-A, som har en viktig blodkärlsstimulerande roll i lungans fysiologiska utveckling. Hos små prematurer finns ett samband mellan en lägre koncentration av VEGF-A och en högre risk för BPD.

Avhandlingen består av tre delanalyser. I den första delanalysen färgades lungbiopsier immunohistokemiskt. Proverna samlades från avlidna patienter som vårdats på Barn- och ungdomssjukhuset i Helsingfors under åren 1991-2000. Lungbiopsier togs från foster (graviditetsvecka 16-20), små prematurer (graviditetsvecka 24-32) och fullgångna nyfödda (graviditetsvecka 38-42) utan lungskada, samt från små prematurer med akut (tidig respiratory distress syndrome (RDS), avliden vid ålder 0-2 dagar), subakut (sen RDS, avliden vid ålder 3-10 dagar) eller kronisk lungskada (BPD). Proven färgades med antikroppar för PlGF, endostatin, VEGF-C, VEGFR-2 samt VEGFR-3. Färgningsmönstret analyserades både inom och mellan de olika grupperna. I den andra delanalysen bestod patientmaterialet av 54-59 små prematurer, som vårdats på intensivvårdsavdelningen på Barn- och ungdomssjukhuset i Helsingfors under åren 1993-2002. Materialet valdes så att hälften senare insjuknade i BPD, medan hälften klarade sig utan BPD. Trakealaspirat samlades dagligen genom en standardiserad metod under de första fem veckorna efter födseln. PlGF-, endostatin- och VEGF-C-koncentrationer mättes med ELISA-analys. I den tredje delanalysen samlades navelblod från såväl fullgångna barn (n=48) som små prematurer (n=92) i samband med förlossningen. Endostatinkoncentrationer mättes med ELISA-analys och jämförelser gjordes både inom och mellan grupperna.

Samtliga tillväxtfaktorer och receptorer kunde påvisas i de olika stadierna av lungans utveckling från och med gestationsåldern 16 veckor och framöver. Under tidigare utvecklingsstadier sågs positiv färgning i epitelceller (PlGF, endostatin, VEGF-C) och endotelceller (endostatin) i hela lungan. I senare utvecklingsstadier sågs färgning endast i epitelceller i övre delar av lungan (PlGF, VEGF-C). VEGFR-3 förekom i lymfatiska strukturer under alla stadier av lungans utveckling. VEGFR-2 förekom i tidigare skeden av utvecklingen både i lymfatiska och venösa endotelceller och senare i arteriella endotelceller. Alveolära makrofager färgades positivt för VEGF-C endast i grupperna för sen RDS och BPD. För endostatin fanns positivitet i alveolära epitelceller och makrofager i grupperna för sen RDS och BPD i ungefär hälften av fallen. I de övriga grupperna sågs ingen färgning. VEGFR-2-färgningen var relativt jämn mellan grupperna, med undantag av att färgning av kapillära endotelceller var nedsatt i BPD-gruppen.

I trakealaspirat var PlGF-koncentrationen jämn, medan endostatin och VEGF-C-koncentrationerna minskade under först veckan efter födseln. En högre endostatin- och

VEGF-C-koncentration korrelerade med en lägre födelsevikt, såväl som med administration av kortikosteroider före födseln. Ingen korrelation kunde påvisas mellan tillväxtfaktorerna och gestationsåldern. Kliniska parametrar som reflekterar inflammation före födseln korrelerade med lägre PlGF-, endostatin- och VEGF-C-koncentrationer i trakealaspirat. En högre koncentration av inandat syre under de första två veckorna efter födseln (FiO_2) korrelerade med en högre endostatinkoncentration i trakealaspirat.

I navelblod var endostatinkoncentrationen högre hos fullgångna än hos prematura barn. Bland prematurer hade en högre endostatinkoncentration samband med en högre risk för BPD. Associationen förblev signifikant efter logistisk regressionsanalys med korrigering för faktorer som hör ihop med en högre risk för BPD.

Den jämna expressionen av PlGF, endostatin och VEGF-C i immunohistokemi samt i trakealaspirat stöder tanken om att alla undersökta tillväxtfaktorer spelar en roll i lungans fysiologiska utveckling. För endostatin stärks sambandet ytterligare genom att fullgångna nyfödda har högre endostatinkoncentrationer i navelblod än prematurer. VEGFR-2-expressionsmönstret verkar avspegla den fortsatta differentieringen av endotelceller under fosterutvecklingen.

En lägre koncentration av alla de undersökta tillväxtfaktorerna i trakealaspirat kunde associeras med kliniska parametrar för inflammation före födseln. Detta kan reflektera en dämpning av blodkärlsutveckling under inflammatoriska tillstånd. I BPD-gruppen var VEGFR-2-färgningen i kapillära endotelceller betydligt lägre än i de andra grupperna. Utvecklingen av BPD är associerad med nedsatt angiogenes, vilket VEGFR-2-färgningen i BPD-gruppen verkar återspegla. Högre FiO_2 kunde associeras med en högre endostatinkoncentration både i trakealaspirat och i navelblod. En högre endostatinkoncentration i navelblod av prematurer kunde direkt associeras med utvecklingen av BPD och associationen förblev signifikant även efter logistisk regressionsanalys. Tillsammans tyder resultaten på att en högre endostatinkoncentration hos prematurer försämrar den fysiologiska lungutvecklingen och ökar risken för BPD.

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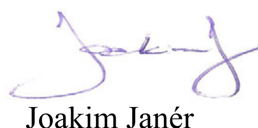

Joakim Janér

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