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THE ROLE OF VASCULAR GROWTH FACTORS IN HYPEROXIA-INDUCED INJURY TO THE DEVELOPING LUNG

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1. ABSTRACT

Normal pulmonary vascular development is the result of a complex interplay of growth factors, including vascular endothelial growth factor (VEGF) and the angiopoietins. Injury to the developing lung, whether due to hyperoxia or mechanical ventilation, results in disordered vascular development, ranging from an apparent arrest of microvascular development in milder injury to extensive microvascular derangement in more severe injury. Alterations in vascular growth factors may participate in these injuries. During injury to the developing animal lung, VEGF abundance is markedly decreased. In models of post-injury recovery, up-regulation of VEGF accompanies the re-establishment of normal vasculature. Alterations in lung VEGF levels in human premature infants are less clear cut. However, among humans premature newborns who later go on to develop bronchopulmonary dysplasia (BPD), VEGF production is decreased in comparison to those newborns who recover. Other angiogenic factors, such as the CXC ELR+ chemokines, are also altered in injury to the developing lung, but their specific roles in vascular injury are less clear. Strategies that enhance microvascular integrity, whether through attenuating alterations in vascular growth factors or by other means, also improve the outcome of lung injury. Such therapies may eventually offer hope in human BPD.

2. INTRODUCTION

Bronchopulmonary dysplasia (BPD), the chronic lung disease of newborn infants, is characterized by pulmonary inflammation, fibrosis and architectural changes, including disordered vascular development (1,2). The pathogenesis of the disease is complex and multifactorial. Increasing immaturity, more aggressive mechanical ventilation and higher inspired concentrations of oxygen all predispose to the condition. Exposure of newborn animals to hyperoxia recapitulates many of the findings found in human BPD (3). This review will focus on the vascular abnormalities seen in animal models of BPD, and describe the changes in vascular growth factors accompanying these alterations.

3. VASCULAR CHANGES IN NEONATAL LUNG INJURY

Fetal and early neonatal life are times of rapid angiogenesis. This is particularly true of the lung. During the first 6 weeks after birth, lung endothelial cell mass in the rat increases six-fold (4). Pulmonary injury during this critical period of pulmonary vascular development can have far-reaching consequences (5,6). Newborn rats exposed to 100% oxygen for six days show markedly decreased capillary density when compared to air-breathing controls (7). If these animals are allowed to recover in air for 1-2 weeks, they develop normal to increased numbers of capillaries. Extending similar 80%-100% oxygen exposures in rats for 8 days to 2 weeks makes the decrease in pulmonary vascularity irreversible, even if the animals are allowed to recover thereafter in air (8,9). Neonatal mice exposed to 100% oxygen for 6 weeks also have decreased capillary surface area (10).

Premature lambs delivered at 120-130 days of a 147 day gestation (80-85% of gestation) and exposed to mechanical ventilation and a fractional concentration of inspired oxygen (FiO_2) of 0.40-0.50 for 3 weeks develop a chronic lung disease similar to human BPD (11). These animals show decreased small arteries and veins when compared to term newborn lambs or 3 week old lambs. In addition, the capillary surface density of the animals is decreased in comparison to controls, while the epithelial surface density is unchanged.

Premature baboons delivered at 140 days (75% of a 185 day gestation, roughly equivalent to 30 weeks gestation in a human) and exposed to high FiO_2 and mechanical ventilation develop a pulmonary lesion very similar to the "classic," fibrotic human BPD (1). These animals display decreased endothelial cell densities in areas of damaged lung when compared to less aggressively ventilated infant baboons (12). Extremely premature baboons, delivered at 125 days (approximately 67% of gestation, roughly equivalent to a human gestation of 26-27 weeks), develop chronic pulmonary changes even if treated with exogenous surfactant and managed with the minimum oxygen and positive pressure ventilation possible (13). Although these animals display less fibrosis than aggressively ventilated more mature animals, they still have markedly deranged architecture, a finding typical of the "new" BPD seen in extremely premature human infants (14). Extremely premature baboons treated in this manner and sacrificed at 1-2 months of age display severe capillary hypoplasia, with decreased volume density of capillaries when compared to term animals or 2-month-old animals born at term (13). Adult baboons exposed to mechanical ventilation and 100% oxygen for 11 days also manifest decreased endothelial cell numbers (15).

Human infants who die as a result of BPD also have disordered pulmonary vasculature. Much of the work in humans has focused on abnormalities in larger vessels, with mixed results (16). One analysis of a small number of patients dying of BPD showed increased arterial concentrations in two patients (17). Another study evaluated premature infants dying either during their initial neonatal illnesses or 4-15 months after having apparently recovered from their neonatal illnesses (2). The lungs of both latter sets of infants showed normal arterial-to-alveolar ratios, but reduced alveolar numbers and reduced overall numbers of arteries.

Functionally, human newborns with severe BPD often display evidence of pulmonary hypertension, although it is unclear how this relates to the pathologic microvascular findings in these infants (18,19). In one study in which both pre-mortem cardiac catheterization data and post-mortem morphometric data were available on 3 children, the infants had high pulmonary vascular resistance and raised intrapulmonary shunts during life (18). Medial thickness in the small pulmonary arteries was increased at post-mortem, and, in one child, some vessels were completely obliterated by fibrosis. Although alveolar numbers were decreased, no comment was made on the microvasculature in these children. Another group of investigators followed 10 children with BPD who resided at an altitude of about 1500 m (5,000 feet) through the age of about 6 years. In the four children who underwent repeat cardiac catheterization, pulmonary artery pressure and pulmonary vasculature resistance, although improved, continued to be elevated throughout follow-up (20).

4. VASCULAR DEVELOPMENT AND ITS CONTROL

4.1. Processes of vascular development

New blood vessel creation and growth occurs by two processes. The first of these, termed vasculogenesis, is primarily limited to early embryonic life (21). In vasculogenesis, endothelial cell precursors known as angioblasts differentiate and form tubes in the absence of pre-existing vessels (Figure 1). Tissues of endodermal origin, including the lung, heart tube and major vessels, are among those vascularized by vasculogenesis (22). Angiogenesis describes the formation of small vessels and capillaries by budding or sprouting from existing larger vessels (23). New vessels can be formed by the growth of an interstitial column of cells that eventually divides a vessel lumen into two parallel lumens (a process known as intussusception) or by a new vessel sprouting at an angle from an existing vessel (known as sprouting angiogenesis).

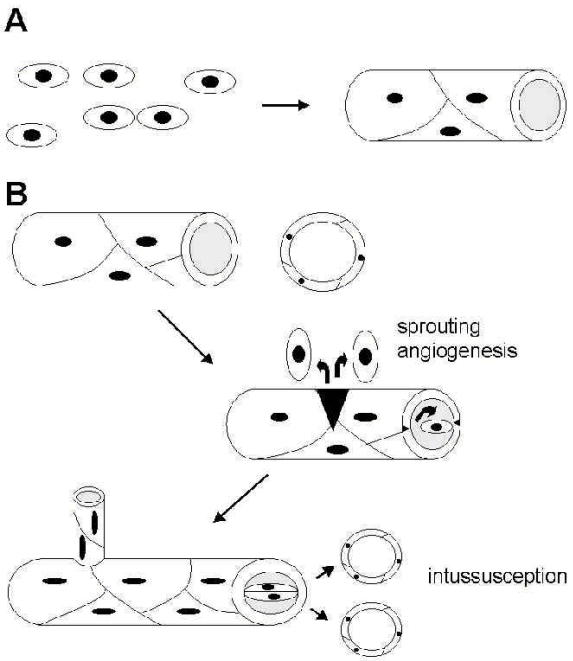

Figure 1. In vasculogenesis (A), tubes form de novo from organizing mesenchymal cells (angioblasts). This process is largely limited to early embryonic development. In angiogenesis (B), endothelial cells within existing vessels dissociate, migrate and proliferate, either to form a connecting tube (sprouting angiogenesis) or to divide an existing tube (intussusception).

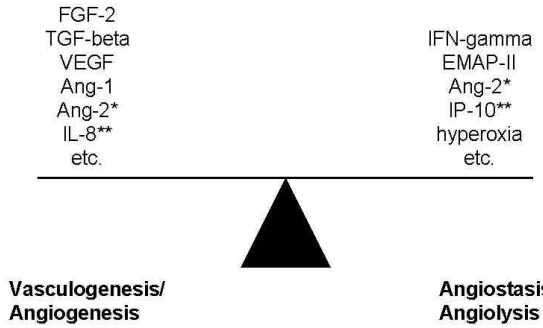

Figure 2. The net potential for new vessel growth and development is the product of a balance between factors promoting vasculogenesis and/or angiogenesis and those inhibiting this activity. Angiopoietin-2 (*) lyses existing vessels, but results in a milieu conducive to new vessel formation, and hence is listed in both areas. The CXC chemokines (**) may also have either angiogenic or angiostatic effects, depending on the presence (as exemplified by IL-8) or absence (as exemplified by IP-10) of a Glu-Leu-Arg ("ELR") amino acid motif.

Sprouting angiogenesis entails an initial lysis of the basement membrane of the original vessel and of the surrounding extracellular matrix, followed by the migration of endothelial cells into the resulting space, proliferation and tube formation by the endothelial cells, and the formation of loops by anastomoses of growing sprouts.

Nascent vessels formed by sprouting angiogenesis are then "stabilized" by the re-formation of a basement membrane and the enclosing of the vessel by pericytes (for a capillary) or smooth muscle cells (for a vessel). Immature capillaries that are not stabilized experience rapid apoptosis of the endothelium and regress (24). Angiogenesis is the primary mode of vascularization in ectoderm- and mesoderm-derived tissues (25). Unlike vasculogenesis, angiogenesis continues throughout fetal development, and plays an important role in such normal processes of postnatal life as wound healing and the female reproductive cycle.

During development and throughout life, vessels are also subject to remodeling. Many vascular beds begin as a plexus of relatively undifferentiated capillary tubes. As development progresses, the vessels in these networks acquire the differing lumen sizes and wall thicknesses that characterize the mature, arboreal pattern of vessels seen in the adult (25). Remodeling also involves a dynamic balance between the growth of new vessels and the regression of others. In some circumstances, such as the female reproductive cycle, this results in marked alterations in vessel number over time.

4.2. Vasculogenic, angiogenic and angiostatic factors

As is common in biological systems, the net potential for new vessel growth and development is the product of a balance between factors promoting vasculogenesis and/or angiogenesis and those inhibiting or reversing this activity (Figure 2). These include a wide variety of molecules responsible for matrix resorption and reconstitution, cellular chemoattraction, cellular proliferation, cellular differentiation, and cell adhesion, as well as for the blockade or downregulation of each of these processes. These factors have been extensively reviewed elsewhere (21,25,26). This review will focus primarily on several of the vascular growth factors regulating endothelial cell function in angiogenesis.

4.2.1. Vasculogenic growth factors

The control of vasculogenesis is, as yet, poorly understood. Fibroblast growth factor-2 (FGF-2, bFGF) is member of a large family of heparin-binding, 18-30 kDa growth factors (27). In numerous *in vitro* and *in vivo* models, FGF-2 is capable of stimulating angiogenic activity. *In vitro*, it also causes primitive mesodermal cells to differentiate into hemangioblast-like cells, implying involvement in the earliest steps of vasculogenesis (28). However, FGF-2 deficient mice develop without any evident early defects in vasculogenesis or angiogenesis (29). The transforming growth factor beta (TGF-beta) family contains a number of homodimeric peptides involved in a wide variety of cellular growth and differentiation processes (30). TGF-beta1-deficient mice die in early embryogenesis with extensive derangement or absence of vasculogenesis (31). This may represent an indirect effect of TGF-beta through its regulation of matrix accumulation or integrin expression (25).

The vascular endothelial growth factor (VEGF) system, described in detail below, also has an apparent role

in vasculogenesis. Mice deficient in VEGF die early in gestation with disordered vasculogenesis, even if only a single VEGF allele is missing (32,33). Mice lacking the VEGF receptor 2 (VEGFR-2) have defects in early angioblast formation (34), while those lacking VEGFR-1 form angioblasts but do not develop usable blood vessels (35). Both receptor knockout animals die in early embryogenesis.

4.2.2. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF or VEGF-A) is the prototype and best-described member of a family of endothelial-specific, heparin-binding, angiogenic growth factors. The family also contains four closely related proteins labeled VEGF-B, -C, and -D, and placental growth factor (PlGF). VEGF itself undergoes alternative mRNA splicing of exons 6 and 7 of the gene to produce 5 isoforms varying from 121 to 206 amino acids in length (and varying slightly in size among species). The relative abundance of the alternatively spliced variants of VEGF mRNA presumably affects the species of VEGF protein present in a tissue. $VEGF_{121}$ lacks a heparin binding domain and is soluble, while the other isoforms are increasingly heparin bound with increasing size. $VEGF_{165}$ is the predominant isoform in most tissues. VEGF stimulates endothelial cells to degrade extracellular matrix, proliferate, migrate and form tubes, and may act as an endothelial cell survival factor. *In vivo*, VEGF also increases vascular permeability, leading to its alternative name, vascular permeability factor (VPF) (36).

VEGF mRNA transcription is upregulated by a number of other growth factors, including platelet derived growth factor (PDGF), epidermal growth factor (EGF), and TGF-beta, and the cytokines tumor necrosis factor alpha (TNF-alpha), and interleukin-1 beta (IL-1 beta) (37-39). In addition, VEGF abundance is increased by hypoxia and decreased by normoxia. The effect of hypoxia is modulated in part through the hypoxia-sensing transcription factor hypoxia inducible factor-1 (HIF-1), which binds to a site in the VEGF promoter (40). Hypoxia also stabilizes VEGF mRNA (41).

The direct effects of hyperoxia on VEGF mRNA expression are somewhat unclear. Although VEGF mRNA expression following hyperoxia exposure is generally decreased in the lung (as detailed in section 5.2.1. below) and retina (42,43), hyperbaric hyperoxia exposure has been associated with increased VEGF protein levels in a rat wound model (44).

VEGF interacts primarily with two receptors, VEGFR-1 (also known as flt-1) and VEGFR-2 (also known as flk-1 or KDR). Binding of VEGF to VEGFR-1 stimulates endothelial cell migration, and may mediate vascular organization (35). VEGFR-2 activation stimulates endothelial cell differentiation, proliferation and migration, and has been implicated in angioblast differentiation (34). A related receptor, VEGFR-3 (flt-4), binds VEGF-C and VEGF-D. The cell surface proteins neuropilin-1 and neuropilin-2 also bind the $VEGF_{165}$ isoform, but these proteins have short intracellular domains, and it is unclear whether they act independently to transduce any signal (45). In the embryo, VEGFR-1, VEGFR-2 and VEGFR-3 are expressed in developing vessels and capillaries throughout vascular maturation (46,47).

4.2.3. The angiopoietin system

The angiopoietins, angiopoietin-1 (Ang-1) and Ang-2, are ligands for the endothelial cell-specific receptor TIE (tyrosine kinase with immunoglobulin- and EGF-like domains)-2. TIE-2 and a closely related receptor, TIE-1, were described several years before the angiopoietins were found to be the ligands for TIE-2. At present, the ligand of TIE-1 remains unknown. Ang-1 interacts with TIE-2 to transduce a signal, while Ang-2 occupies the receptor without signal transduction, acting as an antagonist of Ang-1 (48). Ang-1 mediates vascular remodeling and endothelial cell interactions with supporting cells, making it an important factor in vascular stabilization following angiogenic sprouting (48). The balance between Ang-1 and Ang-2 determines the degree of vascular stability (49). In quiescent vasculature, Ang-1 is bound to TIE-2, and vessels are stabilized. In hypoxia or following VEGF stimulation, Ang-2 is upregulated and displaces Ang-1 from TIE-2 (50). As a result, endothelial cells lose their association with pericytes and the matrix. In the presence an angiogenic factor such as VEGF, endothelial mitogenesis and migration ensues, resulting in new vessel formation. In the absence of such a factor, endothelial apoptosis and vascular regression occur (49).

Mice lacking either Ang-1 or TIE-2 die in early embryogenesis with vascular abnormalities (48,51,52). The embryos have extensive vascular disorganization. The vascular plexuses remain immature without progression to a normal network of large and small vessels. The hearts remain immature, and in some TIE-2 knockout animals the aorta is completely disrupted (52). These findings all support the importance of the angiopoietin system in completing the work of angiogenesis.

4.2.4. Chemokines

The chemokines are a superfamily of heparin binding cytokines originally described as having chemotactic and activating effects on leukocytes (53). Members of the superfamily share a highly conserved pattern of 4 cysteine residues crucial to the secondary structure of the molecules. Members of the CXC subfamily of chemokines, so called due to an interposed amino acid between the first two conserved cysteine residues, also have apparent effects on angiogenesis (53). Among CXC chemokines, some members contain a three-amino-acid pattern of Glu-Leu-Arg ("ELR") immediately N-terminal to the CXC motif. These members of the CXC family (CXC ELR+) display angiogenic capacity. Other CXC members lack the three amino acid pattern (CXC ELR-) and possess angiostatic properties.

The CXC ELR+ chemokines include interleukin-8 (IL-8) and the growth related proteins (GRO alpha, beta and gamma). CXC ELR+ proteins induce endothelial cell chemotaxis and mitogenesis *in vitro* (54). They also cause angiogenesis in a variety of *in vivo* systems in a manner

that appears divorced from their ability to cause inflammation (53,55). CXC ELR+ chemokines are expressed by a variety of tumor cells, and depletion or blocking of the CXC ELR+ chemokines limits the angiogenic activity of the tumors (56). Although the receptor that mediates the angiogenic activity is unknown, the CXC chemokine receptor 2 (CXCR2) is a possible candidate. Mice deficient in the CXCR2, which binds all CXC ELR+ chemokines, have been generated. These mice do not display angiogenesis in response to the exogenous administration of CXC ELR+ cytokines, implying that the CXCR2 is responsible for transducing the angiogenic signal of these chemokines (57). However, other than decreased neovascularization during wound healing, CXCR2 deficient mice do not display alterations in normal angiogenesis (58). Whether this is due to the extensive duplication in the chemokine-chemokine receptor pathways or whether this implies that CXC ELR+ chemokines have limited roles in developmental angiogenesis is unknown.

The CXC ELR- chemokines include several chemokines induced by interferon gamma, such as monokine induced by interferon gamma (MIG) and interferon-gamma-inducible protein (IP-10). These interferon-inducible CXC ELR- chemokines block the angiogenic effects of CXC ELR+ chemokines and other angiogenic factors, including VEGF, in a variety of *in vitro* and *in vivo* models (53). In tumor models, CXC ELR- chemokines limit tumor growth by reducing tumor-associated angiogenesis (59,60). The angiostatic effects attributed to interferon gamma (IFN gamma) may be mediated through its induction of CXC ELR- chemokines. Evidence accumulated with the CXC ELR- chemokine platelet factor 4 (PF4) suggests that some of its angiostatic effects may be due to its binding to $VEGF_{165}$ and FGF-2, which prevents their association with their receptors (61,62).

The CXC ELR- chemokine stromal cell-derived factor (SDF-1) has been reported to have both angiostatic (63) and angiogenic (64) activities *in vitro* and *in vivo.* However, deficiency of the receptor for SDF-1, CXCR4, in mice leads to defective vascular development, hematopoiesis and cardiogenesis, and to death *in utero* (65). This implies that the contribution to angiogenesis by CXC chemokines may have complexities beyond our current models of the process.

4.2.5. Angiostatic factors

Several cytokines and growth factors have been credited with being angiostatic. As discussed above, the CXC ELR- chemokines prevent new vessel growth. Ang-2, by virtue of its capacity for disrupting endothelial cell interactions, can result in vascular destruction if its actions are unaccompanied by those of an angiogenic factor. Endothelial monocyte activating peptide II (EMAP-II) is a recently described cytokine with angiostatic properties both *in vitro* and *in vivo* (66,67). It apparently operates by specifically causing endothelial cell apoptosis (66). In the developing mouse lung, EMAP II markedly inhibits neovascularization, and also inhibits alveolar type II cell development (67). It is unclear whether the alterations of lung epithelial morphogenesis are primary or secondary effects of EMAP II.

4.3. Vascular growth factors in normal lung development

4.3.1. Lung vascular development

The normal early development of the lung vasculature involves three processes (68). The central pulmonary vessels branch from the existing systemic vasculature. Smaller vessels originate by vasculogenesis within the lung mesenchyme. Finally, connections between the two systems are formed by lysis of some existing vessel walls and fusion of the lumens. In the human embryo, the first pulmonary vascular structures to form are vascular lakes of hematopoietic cells surrounded by mesenchyme, which are present by about 4-5 weeks of gestation (69). Central pulmonary vessels are present and beginning to branch into the lung mesenchyme by 7-8 weeks of gestation. The pulmonary venous network forms earlier than the pulmonary arterial tree, with central pulmonary veins having connections with the intrapulmonary vasculature by 7-8 weeks of gestation. The pace of venous development exceeds the pace of branching of the developing airways. By contrast, the formation of the pulmonary arterial circulation lags behind the branching of the developing airways. By the close of the pseudoglandular phase of lung development (16 weeks), both pulmonary arterial and pulmonary venous branches accompany all airways. However, the process of fusion of the central arteries to the existing venous and capillary networks continues throughout the canalicular phase (16-23 weeks). At the close of the canalicular stage, capillaries remain centrally located in the relatively thick septa of the distal airways (70). As the septa thin during the saccular phase of lung development at 24 weeks and beyond, the capillaries become closely apposed to the developing alveolar epithelium.

4.3.2. VEGF in lung development

VEGF is expressed abundantly in the developing lung of humans and animals, and appears to be important in the genesis and maintenance of the developing vasculature (71-74). The lung is one of the chief organs expressing VEGF mRNA in the mid-trimester human fetus (73). VEGF is expressed in the developing mouse lung, as well as other tissues, by the early third trimester of murine fetal development at embryonic day 14 (E14) and E17 (of a 20 day gestation) (75). VEGF-D, however, is expressed almost exclusively in the lung, and does not appear until the late third trimester (E17) (76). VEGF-B and VEGF-C do not appear to be expressed in the mouse lung during fetal development (75). In normal lung development in the mouse, VEGF and VEGFR-2 mRNAs undergo coordinate, 3-fold increases in expression during the canalicular and saccular stages of lung development between E13 and E18, and a further 2-to-3-fold increase by two weeks following delivery (77). Both messages increase twofold again by adulthood. VEGF mRNA expression appears localized primarily to the alveolar epithelium from E18 onward, with specific expression in alveolar type II epithelial cells by 8 days postnatal age. VEGF protein immunostaining is more

diffuse, with protein detected in epithelial, mesenchymal and vascular smooth muscle cells (77).

In the developing baboon, mRNA expression for VEGF and its receptors VEGFR-1 and VEGFR-2 increase throughout the third trimester of pregnancy, as the lung progresses from saccular to alveolar stages (78). VEGF mRNA expression reaches adult levels by shortly before term, and VEGFR-1 mRNA expression in late gestation exceeds adult levels. VEGF mRNA is expressed in a scattered fashion throughout the septa in the distal airways in the early third trimester baboon fetus, and becomes more localized to the alveolar epithelium as gestation progresses. The less soluble $VEGF_{189}$ splice variant becomes progressively more expressed in the lung during the third trimester, while the more soluble $VEGF_{121}$ variant experiences a relative decrease. A similar pattern of increasing $VEGF_{189}$ to $VEGF_{121}$ ratio during the third trimester is present in the lung of the developing rabbit fetus (79).

Explanted embryonic mouse lungs display a pattern of lung vascular development similar to the *in utero* murine fetus, including the progressive organization and extension of originally mesenchymal vascular plexes to surround the developing airways (80). In the explanted mouse lung, VEGF is diffusely distributed in the airway epithelium and subepithelial matrix during the initial vascular-lake phase of vessel development at E11.5. As the vessels organize and surround the airways during E13.5-E15.5, VEGF expression becomes limited to the branching tips of distal lung airways. Similarly, the VEGF receptor, VEGFR-2 is present in the lung mesenchyme immediately underlying the developing epithelium in the rat as soon as the lung evaginates from the foregut, and remains present in clusters of cells immediately under the epithelium throughout lung vascular development (81). If lung mesenchyme is removed from proximity to the epithelium, VEGFR-2 expression rapidly fades. However, if mesenchyme and epithelium are explanted together, the normal *in vivo* pattern of VEGFR-2 expression persists (81).

Newborn rabbits express VEGF mRNA in the alveolar epithelium from birth through 5 weeks of age (72). Pulmonary alveolar epithelial cells are major VEGF-expressing cells in both newborn and adult rabbit lung (72,82). As a result, VEGF is also present in alveolar fluid from these animals (79). VEGF is also apparently necessary even when active angiogenesis is not taking place, since moderate levels of VEGF mRNA are expressed in pulmonary alveolar epithelial cells of normal adult rats and rabbits, and VEGF mRNA is expressed diffusely in adult mouse lungs (75,82,83).

Lungs from human fetuses of 16-20 weeks' gestation display VEGF mRNA and protein expression localized primarily to distal airway epithelial cells, with some expression in connective tissue (84). Over the brief age span covered in the study, the authors did not note an effect of gestational age on VEGF expression. In a single study of VEGF protein in lung fluid collected on the first day after birth from newborn humans born at 24-33 weeks' gestation, we have documented a trend of decreasing VEGF with increasing gestational age (85). Since the VEGF in lung lavage fluid is likely to consist predominantly of the more soluble $VEGF_{121}$ isoform, this finding may be more reflective of changes in isoform ratios (the proportion of $VEGF_{121}$ falls in most animals during gestation) than of overall lung VEGF levels (which tend to rise in animals during gestation).

VEGF has also been examined immunohistochemically in human newborns dying in the first 48 hours after birth from pulmonary hypoplasia due to congenital diaphragmatic hernia or from non-pulmonary causes (86). In lungs from control infants, VEGF immunostaining is present in bronchial epithelial cells and in arterial medial smooth muscle cells. In the hypoplastic lungs, the immunostaining in the bronchial epithelium and smooth muscle is more intense. In addition, VEGF is detected in the media of smaller diameter arteries and in the arterial endothelium in hypoplastic lungs.

4.3.3. Angiopoietins in lung development

Ang-1 and Ang-2 are both expressed in the developing mouse lung throughout its prenatal development from E9.5 until term (87). However, spatial co-localization of these growth factors with the developing lung vasculature has not yet been confirmed. In the developing baboon fetus, mRNA expression for both Ang-1 and its receptor TIE-2 each increase about two-fold in a coordinate fashion between 125 days gestation (equivalent to about 27 weeks gestation in the human fetus) and term (185 days) (78). Unlike VEGF and the VEGF receptors, there does not appear to be a specific cellular localization for Ang-1 and TIE-2 mRNAs. Messenger RNA expression for the orphan receptor, TIE-1, appears to peak in late gestation (175 days), and to decrease after birth in the baboon.

5. VASCULAR GROWTH FACTORS IN LUNG INJURY

5.1. Endothelial alterations in animal lung injury

Hyperoxia is toxic to isolated endothelial cells, although the specific mechanisms of oxygen-induced toxicity in these cells remain unclear. Hyperoxia and the resulting free radicals can induce direct biochemical injury through such mechanisms as lipid peroxidation, protein sulfhydryl oxidation, enzyme inactivation, mitochondrial injury and DNA damage (88). In isolated endothelial cells, DNA synthesis is decreased by as little as 24 hours in 100% oxygen (88). Hyperoxia also mediates changes in multiple growth factors and cytokines expressed by endothelial cells, including such factors as heme oxygenase I and TGF beta (88,89). In the hyperoxia-exposed animal, endothelial cells may induce secondary damage to themselves by upregulation of such leukocyte adhesion factors as intracellular adhesion molecule-1 (90).

In the adult mouse lung, overall DNA synthesis is markedly decreased by 72 hours of 100% oxygen exposure (91). Lung microvascular endothelial cells show swelling and disruption following 90 hours of hyperoxia. The total

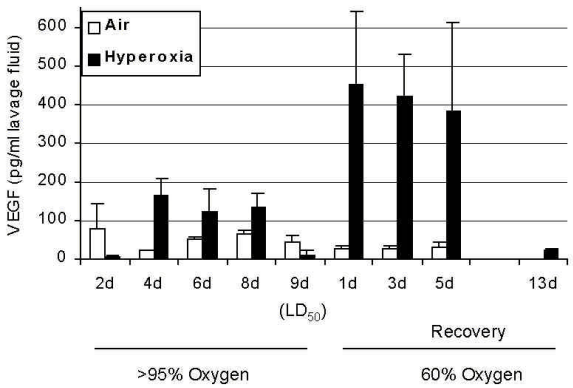

Figure 3. Vascular endothelial growth factor (VEGF) protein in lung lavage fluid increases during recovery from hyperoxic injury in neonatal rabbits. Concentrations of immunoreactive VEGF were measured by enzyme-linked immunosorbent assay (ELISA) in lung lavage from air-exposed controls, oxygen-injured (>95% oxygen) and recovering (60% oxygen) newborn rabbits. Data are ± standard error (SE) for 2-3 animals at each time point. Amount of VEGF in lavage fluid during injury increased twofold compared with age-matched controls, dropped to barely detectable levels at the 50% lethal dose (LD50) time point, and increased by 8-fold compared with control levels during the first 5 days of recovery. By 2 weeks of recovery, VEGF concentrations returned to the normal range. Adapted with permission from Watkins *et al.* (79).

mass of endothelial cells in the lung falls progressively during a 90 hour hyperoxia exposure. In the adult rat, there is electron microscopic evidence of endothelial cell apoptosis as early as 24 hours into a >95% oxygen exposure (92). Successful recovery from hyperoxia-induced lung injury depends, in part, on the successful proliferation of endothelial cells to repopulate the denuded capillary basement membrane (93).

In the premature baboon delivered at 125 days gestation and managed with the minimum of mechanical ventilation and increased fraction of inspired oxygen necessary to maintain normal partial pressures of O_2 and CO_2 in arterial blood, significant lung injury still supervenes (13). Levels of protein and mRNA expression of the endothelial marker platelet endothelial cell adhesion molecule (PECAM)-1 in the lung normally rise progressively during the third trimester of baboon gestation (78). In animals delivered at 125 days, PECAM-1 protein levels and mRNA expression cease rising following delivery, and following 14 days of ventilation (139 days equivalent gestational age) fall significantly below the levels found *in utero* in animals at 140 days' gestation. Histologically, there appears to be a delay in maturation of the capillary network of the lung. Capillary numbers are decreased, and the capillaries remain centrally located in the developing septa, a pattern more consistent with the early saccular pattern of development of the 125 day fetus than the subepithelial capillary pattern becoming apparent in the late saccular lung of the 140 day fetus. This delay in maturation is apparent after as little as 6 days of *ex utero* ventilation.

5.2. VEGF and the angiopoietins in lung injury

5.2.1. VEGF in animal lung injury

Acute lung injury depresses pulmonary VEGF mRNA expression in adult animals. Adult rats exposed to polytetrafluoroethylene (PTFE) fumes experience a fall in VEGF mRNA expression to 20% of control levels within 4 hours (94). In adult rats exposed to >95% oxygen, VEGF mRNA expression falls to about 70% of control levels by 24 hours of exposure, and to 50% of control levels by 48 hours (92). VEGF mRNA expression in adult rats appears localized to alveolar type II epithelial cells. VEGF mRNA expression in these cells is markedly decreased following 24 hours of hyperoxia, and is virtually absent by 48 hours of hyperoxia. VEGF protein is more diffusely present in the bronchial and alveolar epithelium, but undergoes a similar pattern of decrease by 48 hours of hyperoxia. The mRNAs for the receptors VEGFR-1 and VEGFR-2 also decrease to approximately half control levels during a 48-hour hyperoxia exposure (92). Adult rabbits exposed to 100% oxygen for 64 hours experience a marked depression of pulmonary VEGF mRNA expression (82). Again, VEGF expression appears to be limited largely to the type II alveolar epithelial cell and to disappear with hyperoxia exposure. If 64-hour exposed rabbits are allowed to recover from hyperoxic injury, their type II epithelial cells express supranormal levels of VEGF mRNA during the first 3-5 days after their return to room air. Secreted VEGF protein, measured in lung lavage from oxygen-injured adult rabbits, is mildly elevated during a 64-hour hyperoxia exposure, but falls at the close of the exposure, before undergoing a 40-fold rise during recovery (79). Other investigators have also reported an increase in bronchoalveolar lavage VEGF from adult mice exposed to hyperoxia for 72 hours, but do not have data for recovering mice (95).

In the adult rat lung, the relative abundances of mRNA for the splice variants $VEGF_{188}$ and $VEGF_{164}$, which code for heavily and intermediately heparin-bound isoforms of the protein, do not change over a 48 hour hyperoxia exposure (92). In the adult rabbit lung, however, the relative abundance of $VEGF_{189}$ mRNA (homologous to the 188 amino acid protein in the rat) decreases by half during a 64-hour hyperoxia exposure, while the relative expression of the mRNA for the soluble $VEGF_{121}$ species increases twofold. The expression of $VEGF_{189}$ does not return to control levels until 5-7 days following hyperoxic lung injury.

Newborn animals of many species are more resistant to hyperoxia than adults, but eventually succumb to oxygen-induced lung injury (3). Hyperoxia-exposed newborn rabbits exhibit changes in VEGF expression similar to the adult, albeit with a longer time frame. In the newborn rabbit, pulmonary VEGF mRNA expression is depressed during an acute, 9-day, 100% oxygen exposure and returns toward normal during a recovery period in 60% oxygen (72). In addition, VEGF protein in lung lavage from newborn rabbits, although mildly increased early in hyperoxia exposure, falls as the inflammation peaks (Figure 3). VEGF protein in lavage then increases more than 8-

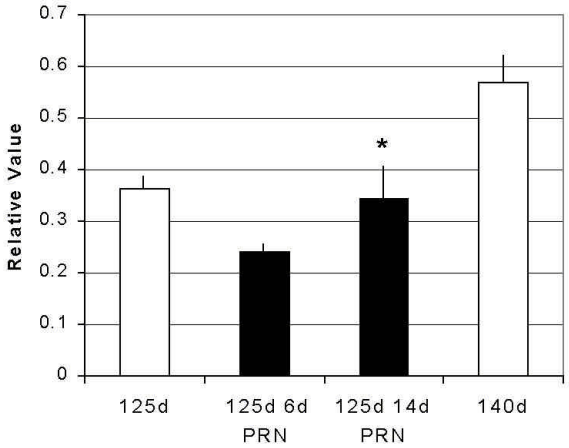

Figure 4. VEGF mRNA decreases in chronic lung disease. Quantification of ribonuclease protection assay for VEGF mRNA showed that the message did not increase in newborn baboons delivered at 125 days gestation and treated with appropriate oxygen and ventilation ("PRN"). The 125 day + 14 day PRN animals had VEGF mRNA levels significantly lower than the 140 day gestational controls (* indicates $p = 0.025$). Data are means ± SE of 4-6 animals at each point. See text for full details of animal model. Reprinted with permission from Maniscalco *et al*. (78).

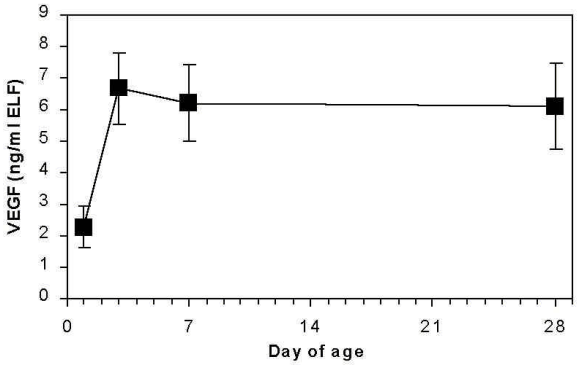

Figure 5. VEGF levels in pulmonary epithelial lining fluid obtained by bronchoalveolar lavage from intubated human premature newborns. VEGF was measured by ELISA. VEGF rose above day 1 (within 24 hours of birth) levels by day 3 ($p < 0.05$ by paired t test), and continued to be elevated through day 28 in those infants who remained intubated. Data are shown as mean ± SE. Number of samples, day 1: 26; day 3: 22; day 7: 19; day 28: 10. Adapted from data in D'Angio *et al*. (85).

fold over control levels during recovery from the lung injury (79). A pattern of decreased $VEGF_{189}$ and increased $VEGF_{121}$, similar to the adult, occurs following 9 days of hyperoxia exposure. $VEGF_{189}$ does not return to control levels until 5 days of recovery (79).

In the fetal baboon, VEGF mRNA expression progresses from a diffuse pattern throughout the alveolar septa at 125 days gestation to a more localized expression in cells at the alveolar surface by 140 days gestation (78). If the animal is delivered at 125 days gestation, and treated with oxygen and mechanical ventilation as needed for 14 days, overall expression of VEGF mRNA is markedly decreased (Figure 4), and the localized epithelial pattern of VEGF expression does not develop. A similar decrease in overall abundance and inhibited pattern of maturation is seen if immunoreactive VEGF protein is measured. Expression of mRNA for VEGFR-1 also decreases 30-40% in treated animals compared to 140-day-gestation controls, while the VEGFR-2 mRNA expression remains unchanged. The decreases in VEGF and its receptor parallel the stunted capillary development in these animals (78).

5.2.2. The angiopoietin system in animal lung injury

In contrast to the findings with VEGF, premature baboon lung injury does not alter the normal gestational pattern of increasing mRNA expression for the vascular stabilization factor Ang-1 and its receptor, TIE-2, in the lung (78). However, the mRNA expression for the orphan TIE-1 receptor is decreased about 40% in the same injury. Ang-1 and TIE-2 alterations have not yet been examined in other newborn lung injuries.

5.2.3. VEGF in human BPD

Several studies have evaluated levels of VEGF in lung fluid obtained from intubated human premature newborns born at 23-33 weeks' gestation (85,96,97). Although the methods varied somewhat between studies, all investigators found that VEGF protein measured in lung fluid rose at least 3-to-4-fold over the first 3-10 days after birth (Figure 5). These findings appear to be at odds with information gathered in animals, particularly that gathered in the roughly comparable premature baboon (section 5.2.1.). In the baboon, lung VEGF mRNA expression falls about 30% in the six days following delivery at 125 days' gestation (approximately equivalent to 26-27 weeks human gestation), and barely returns to 125-day levels by 14 days following delivery (Figure 4). Lung VEGF protein appears to follow suit. One potential explanation of the discrepancy may lie in the differences between the compartments sampled in the baboon and the human. The human studies all depended upon measurement of VEGF protein in lung lavage fluid. The VEGF measured in this fluid may over-represent soluble $VEGF_{121}$. As detailed above in section 5.2.1., this splice variant of VEGF is increased relative to other VEGF isoforms during lung injury in adult and newborn rabbits. In addition, measurements of lavage VEGF in both adult and newborn animals show initial increases during hyperoxia, culminating only later in decreases as inflammation reaches critical levels (79,95). The measurements available in human newborns may reflect results from a specific compartment during a relatively mild, early injury.

More consonant with the animal findings are the later patterns of VEGF expression in human newborns with more severe lung injury, i.e. those who go on to develop BPD. Lassus and colleagues (96), in a study of 44 intubated premature infants with a mean gestational age of 27 weeks, found that although tracheal aspirate fluid VEGF levels rose over the first week of life, infants destined to develop BPD had depressed VEGF levels between days 4 and 7, when compared to those infants who would recover

without BPD. In a study of 40 premature infants born at 24-33 weeks' gestation, we were unable to delineate an association between VEGF levels at any time over the first week after birth with the subsequent development of BPD (85). There was a trend, however, toward gradual elevation of VEGF over time in children who recovered without BPD, while children who did develop BPD or died appeared to have a decrease in VEGF with age. This suggests that the subset of human infants who develop BPD may have lung VEGF alterations similar to those in the premature baboon model, where VEGF is markedly depressed over the first week following delivery.

The confusing picture of lavage VEGF measurements in humans may also be due in part to additional variables not present in animal models. In premature humans, elevated levels of lung fluid VEGF are associated with prolonged or premature rupture of the membranes, and with chorioamnionitis, factors which predispose to the later development of BPD (85,96). Corticosteroid administration may also be a factor. Our cohort of infants was drawn from a trial comparing the administration of dexamethasone at 12 and 24 hours of age to placebo for the prevention of BPD (98). There were no differences in VEGF levels in lung fluid between dexamethasone-treated and placebo groups at 1 or 3 days after birth, but dexamethasone subjects had increased VEGF levels at 7 days after birth. Although experiments in isolated cells (99,100) have indicated that VEGF expression is decreased following glucocorticoid administration, animal data suggest otherwise. In neonatal mice, VEGF mRNA is increased in a dose-dependent fashion by a 3-day course of dexamethasone, with a 2-fold increase at the highest dose administered (5 mg/kg/day) (77). VEGF protein, however, shows no corresponding increase over that time frame. It may be that the increase in VEGF protein seen in our human subjects followed an earlier increase in mRNA expression akin to that seen in the animal.

5.3. Chemokines in lung injury

5.3.1. Angiogenic chemokines in animal lung injury

Although IL-8 and other CXC ELR+ chemokines have been implicated in pulmonary angiogenesis (101), it is difficult in observational studies of pulmonary injury to dissociate the angiogenic effects of these molecules from their pro-inflammatory functions. A number of investigators have evaluated the role of CXC ELR+ chemokines in newborn hyperoxia-induced lung injury. In a newborn rabbit model of >95% oxygen exposure for 8-9 days, followed by recovery in 60% oxygen until 5 weeks of age, histologic evidence of acute lung injury and neutrophil infiltration peak at the close of the >95% oxygen exposure, and recede thereafter (102,103). Signs of chronic lung injury, including simplification of alveolar architecture, persist through 3-5 weeks of age. IL-8 mRNA expression coincides very closely with the temporal pattern of neutrophil infiltration, and is largely limited to neutrophils. IL-8 mRNA expression returns to levels similar to those in air-exposed, age-matched controls during the period of recovery and repair. In a similar model of 8-day hyperoxia exposure in the newborn rat, Auten and colleagues have shown 10-fold increases in the CXC ELR+ chemokines cytokine-induced neutrophil chemoattractant protein-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) by the close of exposure (104). These increases are accompanied by inflammatory cell infiltrates, septal thickening and decreases in alveolar volume and surface density (104,105). Treatment with anti-CINC-1 or anti-MIP-2 antibodies or with a CXCR2 blocker prevents many of these hyperoxia-related changes (104-106). Perhaps most importantly for the purposes of this discussion, anti-CINC-1 antibody therapy decreases CINC-1 levels to those of air-exposed controls and preserves normal alveolar architecture (105). The close correlation of CXC ELR+ chemokines to neutrophil infiltration and the preservation of normal alveolar architecture in their absence argue that their major roles in hyperoxic lung injury are proinflammatory rather than angiogenic. If their roles were indeed angiogenic, one might expect them to be present during recovery, and for their absence to lead to disordered alveolar repair. However, neither the complementary network of chemokines and chemokine receptors nor the possible roles of anti-angiogenic CXC ELR- chemokines have been adequately addressed in neonatal hyperoxic lung injury. Further study may reveal as-yet-unanticipated effects of chemokines upon the pulmonary vasculature during hyperoxia.

5.3.2. Interleukin-8 in human BPD

Premature infants who later develop bronchopulmonary dysplasia display elevated airway fluid levels of several proinflammatory cytokines. Elevations in the CXC ELR+ chemokine IL-8 in infants who subsequently develop BPD have been reported in a number of studies (107-110). Peak levels of IL-8 appear to be present at 1-2 weeks of age (107,108,111), a time at which clinical signs heralding BPD are beginning to emerge. A small number of studies have suggested that changes in IL-8 within the first few days of life may also predict the development of BPD (110,112,113). The pattern of IL-8 elevation in human newborns appears to be the inverse of that described for VEGF. This implies that, as in the animal, IL-8 may be less important in vascular growth or repair than in inflammation. The absence of either therapeutic trials or extensive histology in humans makes any definite inference difficult, however.

6. THERAPY FOR ENDOTHELIAL LOSS DURING LUNG INJURY

Several recent publications have presented fascinating glimpses of the potential for endothelial preservation during hyperoxic lung injury. Keratinocyte growth factor (KGF, also known as FGF-7) administered intratracheally prior to hyperoxia exposure protects adult mice against lung injury, with both prevention of histologic injury and improvement in survival (91,114). KGF has well-described mitogenic effects on pulmonary epithelial cells, and much research has focused on these effects. However, a recent study of prophylactic KGF administration in the rat also showed significant preservation of capillary endothelial structure and of total endothelial volume during a 90-hour hyperoxia exposure

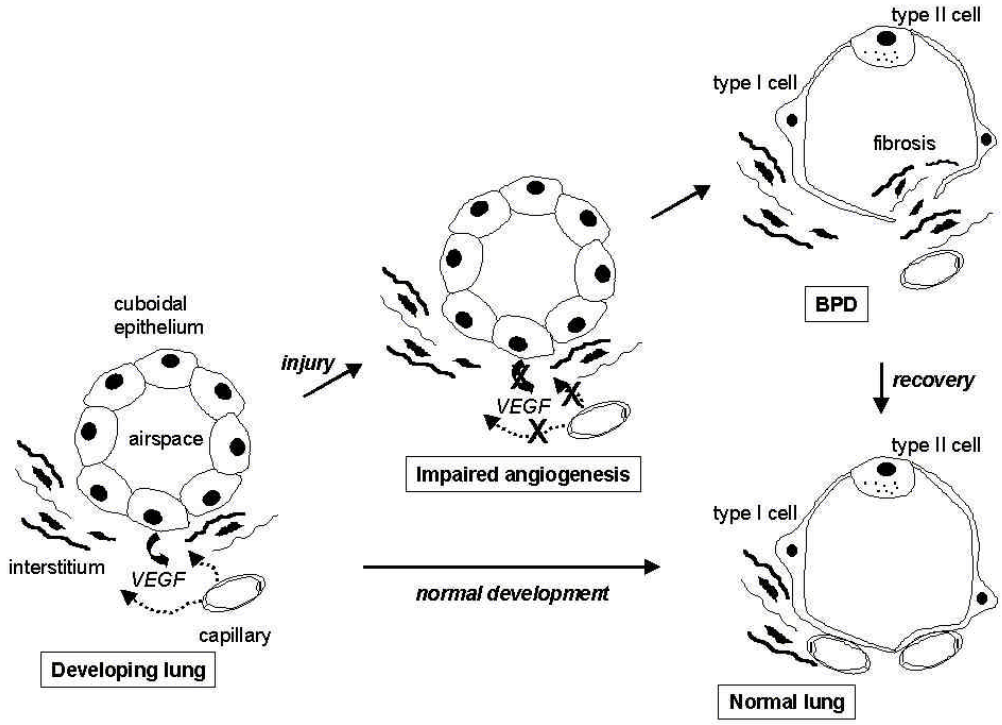

Figure 6. Impaired angiogenesis in bronchopulmonary dysplasia (BPD). In the fetal lung, capillary growth and apposition to the developing alveolar epithelium is controlled by angiogenic factors such as VEGF, resulting in a thin air-blood barrier in the normal newborn lung. Lung injury during development decreases VEGF secretion by the epithelium, resulting in lack of endothelial proliferation and migration. Capillary growth remains stunted and the architecture immature, resulting in a widened air-blood barrier in BPD. Recovery from BPD, in part, involves the completion of normal vascular development.

(91). It is unclear whether this effect is a direct effect of KGF on the endothelium, an indirect effect through the epithelium, or a less-specific effect of KGF-mediated attenuation of pro-apoptotic factors such as bax and bcl-x (91).

Interleukin-13 (IL-13) is a cytokine with a variety of pro- and anti-inflammatory properties that also has mitogenic effects on endothelial cells (95,115). Adult transgenic mice that overexpress IL-13 under the control of the Clara cell 10-kDa protein (CC10) promoter are markedly protected during hyperoxia, with a tripling of survival time (95). A more modest survival advantage is incurred by intranasal administration of exogenous IL-13. The IL-13 transgenic mice have increased amounts of VEGF in bronchoalveolar lavage, with immunohistochemical evidence of VEGF protein in airway and alveolar epithelium, smooth muscle, alveolar macrophages and blood vessels. This increase is further augmented in hyperoxia, with VEGF levels following 72 hours of hyperoxia some fourfold higher than in non-transgenic, hyperoxia-exposed mice. While the predominant VEGF isoform in either air- or hyperoxia-exposed wild type mice is $VEGF_{164}$, hyperoxia-exposed transgenic mice express increased amounts of $VEGF_{120}$ and $VEGF_{188}$. Neutralization of VEGF using anti-VEGF antibody shifts the survival in hyperoxia of IL-13 transgenic mice back toward that of wild type mice, implying that a significant portion of the IL-13 survival effect is mediated through VEGF. The specific effect of increased VEGF on the capillary endothelium in this model remains unknown.

Taken together, the effects of these therapeutic trials imply that preservation of the capillary endothelium, perhaps through a VEGF-mediated mechanism, may be a promising avenue of investigation for the prevention of oxygen-induced lung injury in humans. Further research will be necessary to determine whether such an approach would be useful in the multi-factorial injury that leads to BPD in immature humans.

7. SUMMARY AND PERSPECTIVE

Multiple vascular growth factors, including VEGF and the angiopoietins, interact to induce normal vasculogenesis and angiogenesis in the developing lung. Normal patterns of vascular development are disrupted by early delivery and the attendant lung injury that leads to bronchopulmonary dysplasia (figure 6). Hyperoxia exposure and/or mechanical ventilation of newborn animals, which mimics BPD, leads to an apparent arrest of microvascular development in milder injury or extensive microvascular derangement in more severe injury. Some of

these alterations may be due to disruption of the elaboration of vascular growth factors. Of these factors, VEGF has been examined the most extensively. In most adult, newborn or premature animal models of hyperoxic or ventilator-associated injury, VEGF mRNA expression and protein production are markedly attenuated by the injury. In models of post-injury recovery, there appears to be an up-regulation of VEGF that accompanies the re-establishment of normal vasculature. The direction of changes in VEGF secretion in human BPD is less certain. VEGF, as measured in bronchoalveolar lavage, appears to rise in the first few days following premature delivery in newborn humans, a finding difficult to reconcile with the decreases over the same time period in most animal models. Some of the differences, however, may be due to the different compartment (lung fluid) sampled in human studies. Several animal studies have documented similar increases in lavage VEGF early in hyperoxia exposure, although levels tend to fall as injury worsens. In human premature newborns who later go on to develop BPD, there is evidence that VEGF production may be decreased in comparison to those newborns who recover. Overall, the data imply that in human BPD, as in animal lung injuries, decreases in vascular growth factors such as VEGF may be associated with poorer vascular development and poorer outcome. Preliminary evidence from animal studies suggests that strategies that enhance microvascular integrity also improve the outcome of lung injury. This gives hope that such therapies may eventually prove useful for the prevention or treatment of human BPD.

8. ACKNOWLEDGMENTS

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