

# Angiogenesis in Lung Development, Injury and Repair: Implications for Chronic Lung Disease of Prematurity

Bernard Thébaud

Vascular Biology Group, Division of Neonatology, Department of Pediatrics, University of Alberta, Edmonton, Alta., Canada

## Key Words

Newborn · Lung development · Injury and repair · Oxygen · Angiogenesis

## Abstract

Since the initial description of bronchopulmonary dysplasia (BPD) 40 years ago, advances in perinatal care have allowed the survival of infants that are more immature. The disease has not disappeared, but it now affects infants with undeveloped distal airspaces, resulting in an arrest of alveolar development. The histological changes that occur during normal lung development are well described, but little is known about the signaling mechanisms that regulate *saccular* and *alveolar* development. Understanding how alveoli and the underlying capillary network develop and how these mechanisms are disrupted in preterm infants with BPD is critical to develop efficient and effective therapies for lung diseases characterized by alveolar damage. This brief review focuses on the recently recognized role of angiogenic growth factors during normal alveolar development, injury and repair with a particular emphasis on the vascular endothelial growth factor.

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## Chronic Lung Disease of Prematurity: An Arrest in Alveolar Development

In 1889, Alexander Graham Bell, when he presented a negative pressure body type respirator for use in newborn infants, suggested: *‘Many children, especially those prematurely born, die from inability to expand their lungs sufficiently when they take their first breath. I have no doubt that in many of those cases, lives could be saved by starting the respiration artificially by means of apparatus operating in the manner described above.’* It was not until the mid-1960s that attempts were made to provide mechanical ventilation for infants with severe respiratory distress. Less than a decade later, bronchopulmonary dysplasia (BPD) was born: in 1967, Northway et al. [1] described *‘a new chronic pulmonary syndrome that is associated with the use of intermittent-positive pressure respirators and high oxygen...’* Survivors had a mean gestational age of 34 weeks, and non-survivors had a mean gestational age of 31 weeks. In 1969, a conference on assisted ventilation was organized in Paris by Professor Alex Minkowski (the proceedings of which were later published in *Biology of the Neonate*, the journal established by Prof. Minkowski) and the big question was whether it was appropriate to continue to push forward with assisted ventilation [2]. Since then, advances in perinatal medicine – including major breakthroughs such as antenatal

steroids and postnatal surfactant – have allowed the survival of infants that are more and more immature, but the disease has not disappeared. In 2007, marking the 40th anniversary of Northway's seminal description, infants at risk for BPD are born at <28 weeks' gestation when they are just beginning the parallel processes of alveolarization of the distal lung saccules and development of the alveolar capillary bed [3]. Although exogenous surfactant treats the biochemical immaturity of the premature lung, it does not overcome its underlying structural immaturity. Indeed, perinatal lung injury in neonates born during the late canalicular stage disrupts the normal sequence of lung development, resulting in the histological pattern of alveolar simplification (larger but fewer alveoli with decreased septation), and decreased capillary density [4, 5]. Similar structural abnormalities have been demonstrated in various animal models of BPD [4]. Consequently, besides improving the use of current available therapies [6, 7], a better understanding on how sacculi and alveoli and the underlying capillary network develop and how these mechanisms are disrupted in BPD is critical to develop efficient and effective therapies to prevent lung injury or regenerate established lung injury, not only in premature infants.

### **The Mystery of Secondary Crest Outgrowth, the Anatomical Substratum of Alveolar Development**

The development of the lung can be divided into two periods: (1) the initial creation of an air-conducting system and (2) the later evolution of the respiratory portion of the lung. In contrast to the large amount of information on early lung branching morphogenesis, much less is known about the mechanisms that regulate secondary crest outgrowth (or septation). Much of our understanding about the genetic control of the dichotomous division of the *conducting airways* in mammals derives from studies of the respiratory system in *Drosophila* [8, 9]. Conversely, the mechanisms that regulate *alveolar* development remain poorly understood, mainly because of the lack of alveoli in *Drosophila* [10, 11]. Furthermore, the conducting airways grow into the surrounding mesenchyme whereas alveolar septae evaginate inwards into the airspace [12]. What determines septal outgrowth and what is the driving force of this process? How do distal lung cells (i.e. alveolar epithelial cells, endothelial cells, and myofibroblasts) interact during septal outgrowth? What is the respective role of growth factors and the mechanical forces and lung fluid in utero?

One interesting theory is that a driving force pushes the septa inwards [13]. One such driving force for septal outgrowth, besides elastin and platelet-derived growth factor A [14], could be provided by the developing blood vessels in the distal lung parenchyma, a mechanism that might be referred to as 'capillary erection' by analogy to the early description of increased pulmonary blood flow during the transition from fetal to newborn life [15]. Indeed, recent evidence suggests that blood vessels in the lung actively promote normal alveolar development [16, 17] and contribute to maintenance of alveolar structures throughout life [18]. The fact that interactions between airways and blood vessels are critical for normal lung development, and that combined abnormalities in the vasculature and airways occur in BPD suggests that (i) angiogenesis may be important for normal alveolarization and (ii) impaired angiogenesis during BPD may lead to decreased alveolarization; (iii) therefore, enhancing angiogenesis may promote alveolarization and reverse established experimental BPD. This review will focus on the crucial role of the angiogenic growth factors during alveolar development with a particular emphasis on the vascular endothelial growth factor (VEGF).

### **Role of Angiogenesis and Angiogenic Growth Factors during Alveolar Development, Injury and Repair**

#### *VEGF and Its Receptors Are Pivotal for the Proper Formation of Blood Vessels*

VEGF is a highly specific mitogen and survival factor for vascular endothelial cells. VEGF binds to transmembrane tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed on vascular endothelium [19]. The absolute requirement of VEGF for development of the embryonic vasculature in mice has been demonstrated by inactivation studies of VEGF alleles [20, 21] and knockouts of VEGFR-1 [22] and VEGFR-2 [23]. In each of these studies, inactivation of the target genes resulted in lethal phenotypes characterized by deficient organization of endothelial cells. Inducible Cre-*loxP*-mediated gene targeting or administration of a soluble VEGF receptor chimeric protein (mFlt (1-3)-IgG) to inactivate VEGF in early postnatal life results in increased mortality, stunted body growth, and impaired organ development [24]. VEGF inhibition resulted in less significant alterations as the animal matured, and the dependence on VEGF is lost around the fourth postnatal week. Interestingly, this period coincides with the

end of alveolarization and microvasculature maturation in the lung.

#### *VEGF Promotes Normal Alveolar Development*

The spatial relationship between receptor and ligand suggests that VEGF plays a role in the development of the alveolar capillary bed. Indeed, VEGF mRNA and protein are localized to distal airway epithelial cells and the basement membrane subjacent to the airway epithelial cells [25]. This suggests that translocation of VEGF protein occurs after its synthesis in the epithelium. VEGFR-1 and VEGFR-2 mRNA expression also increases during normal mouse lung development [26, 27] and is localized to the pulmonary endothelial cells closely apposed to the developing epithelium [28]. VEGF<sub>120</sub>, VEGF<sub>164</sub> and VEGF<sub>188</sub> are present in alveolar type II cells in the developing mouse lung, and their expression peaks during the canalicular stage, when most of the vessel growth occurs in the lung, then decreases towards until day 10 postnatal (P10) when it increases to levels that are maintained through adulthood [28]. Targeted exon deletion of the VEGF gene reveals that mice that lack the heparin-binding isoforms VEGF<sub>164</sub> and VEGF<sub>188</sub> display a variety of vascular defects, including a significant reduction in the formation of airspaces and capillaries, resulting in distended and underdeveloped alveoli [29]. Likewise, pharmacological and genetic VEGF inhibition during alveolar development decreases alveolarization and pulmonary arterial density, features encountered in clinical BPD [17, 29–31]. Chronic treatment of adult rats with the VEGFR-1 and -2 blocker SU5416 leads to enlargement of the airspaces, indicative of emphysema [18], suggesting that VEGF is required for the formation, but also the maintenance of the pulmonary vasculature and alveolar structures throughout adulthood. Conversely, lung overexpression of VEGF during normal lung development disrupts the lung architecture [32, 33]. The data discussed above suggest that inhibition of vascular growth itself may directly impair alveolarization.

#### *VEGF Signaling Is Disrupted in BPD; Link between Angiogenesis and BPD*

The proposed link between alveolarization and angiogenesis is suggested by the secondary abnormalities that occur in one process when the other is primarily affected. Alveolar hypoplasia and dysmorphic changes of the lung vasculature are consistent findings in BPD. The first evidence that abnormal vascular development may contribute to postnatal lung disease came from autopsy studies showing reduced pulmonary microvascularization in in-

fants dying from BPD [34]. A more recent post-mortem study of newborns dying after short and prolonged durations of mechanical ventilation also quantified lung microvascular growth [35]. This study confirmed the reduction in vascular branching arteries, but interestingly lung PECAM-1 protein content (a marker of endothelial cells) was decreased in infants dying after brief ventilation, but was increased after prolonged ventilation [35]. These findings suggest a transient decrease in endothelial proliferation, followed by a brisk proliferative response, despite a reduction in vessel number. This observation suggests that dysmorphic lung vascular growth in BPD may not necessarily result simply from a reduction in the number of endothelial cells, suggesting the need to better discern distinct mechanisms regulating endothelial cell survival, proliferation, migration, vessel formation and maturation, especially in response to injury. These findings further emphasize the need for more extensive studies in animal models of BPD to better define the mechanisms that underlie early and the time-dependent sequence of events that precede the development of impaired distal lung structure.

Various animal models of impaired alveolar development also display abnormal lung vascular development [36–40]. Accordingly, animal and human studies show decreased expression of VEGF and its receptors in chronic newborn lung injury. Decreased VEGF expression is found in alveolar type II cells of newborn rabbits exposed to 100% O<sub>2</sub> for 9 days [41] or in newborn rat lungs [30]. In the preterm baboon model of BPD, arrested platelet endothelial cellular adhesion molecule-1 (PECAM-1, a distal lung endothelial cell marker) expression and reduced capillary density are associated with lower VEGF and VEGFR-1 mRNA and protein expression seen at 125 vs. 140 day term animals [42]. Premature sheep with antenatal endotoxin exposure also display decreased lung VEGF expression [43]. In humans, similar observations have been made in some [44–46], but not all [47] studies. Infants developing BPD have lower VEGF levels than those surviving without BPD [46]. VEGF may participate in pulmonary repair after acute lung injury. In lung tissue from infants who died from BPD, the typical patterns of alveolar simplification with ‘dysmorphic’ microvasculature are associated with reduced lung VEGF and VEGFR-1 mRNA and protein expression [44]. Likewise, there are lower VEGF levels in the tracheal aspirates of preterm infants dying from severe respiratory distress syndrome than in survivors and in infants subsequently developing BPD, as compared to premature infants surviving without pulmonary complications [44]. Hypoxia is a major

stimulator of VEGF expression [48]. Premature exposure of the developing lung to a hyperoxic environment may downregulate VEGF expression. Even ambient O<sub>2</sub> levels (21%), i.e. premature birth per se, may interfere with normal lung vascular development [49].

*Perturbation of Other Angiogenic Growth Factors Provides Further Evidence for a Link between Angiogenesis and BPD*

*Nitric Oxide (NO).* The role of the endothelium-derived relaxing factor NO in the regulation of the pulmonary vascular tone in the perinatal period is well established [50]; however, little was known about its potential role in the structural development of the pulmonary vasculature. Recent studies suggest that VEGF-induced lung angiogenesis is in part mediated by NO. Neonatal treatment with the VEGF inhibitor, SU5416, downregulates eNOS protein and NO production, and treatment with inhaled NO improves vascular and alveolar growth in this model of BPD [51]. Lungs of late fetal and neonatal eNOS-deficient mice can have a paucity of distal arteries and reduced alveolarization [52], and are more susceptible to failed vascular and alveolar growth after exposure to mild hypoxia and hyperoxia [53]. Finally, chronically ventilated preterm lambs have decreased lung eNOS expression [54], suggesting that NO deficiency may contribute to the decreased alveolarization seen in this model of BPD.

*PECAM-1.* Administration of an anti-PECAM-1 antibody that inhibits endothelial cell migration, but not proliferation or survival in vitro, also impairs septation in neonatal rats, without reducing endothelial cell content [55]. Overall, these data suggest that the loss of PECAM-1 function compromises postnatal lung development and provides evidence that inhibition of endothelial cell function, in contrast to loss of viable endothelial cells, inhibits alveolarization.

*Angiopoietin (Ang).* Despite its central role in vascular formation, VEGF works in concert with other factors, notably Ang to stabilize the vascular wall. Unlike mouse embryos lacking VEGF or VEGFR-2, embryos lacking Ang1 or its receptor Tie2 develop a rather normal primary vasculature [56–58]. However, endothelial cells fail to associate appropriately with underlying support cells, which are the cells that provide the Ang1 protein that acts on endothelial Tie2 receptors [59]. While VEGF leads to immature, leaky and hemorrhagic vessels, Ang1 generates vessels that are resistant to leaking, suggesting that Ang1 maximizes interactions between endothelial cells and their surrounding support cells and matrix [60].

These findings indicate that Ang1 is complementary to VEGF during vessel formation, acting at a later stage of angiogenesis to elicit vessel maturation and integrity. This suggests multiple therapeutic possibilities (i.e. combined administration of both factors) in order to promote angiogenesis (see below). However, little is known about the role of Ang during alveolarization. In the baboon lung, Ang1 is mainly expressed in the septal mesenchymal cells, and Ang1 and its receptor Tie2 increases during lung development. Conversely, Tie-2 expression is decreased in lungs of ventilated baboons [42] and humans with BPD [61]. The role of Ang2 during angiogenesis is less clear. Ang2 displays similarly high affinity for Tie2 and may act as a Tie2 antagonist. In lung epithelial cells, Ang2 expression is induced during hyperoxia [62]. Hyperoxia-induced oxidant injury, cell death, inflammation, permeability and mortality are ameliorated in Ang2 knockout mice and in Ang2 siRNA-treated mice. Finally, Ang2 tracheal aspirate levels are increased in newborns that develop BPD.

*Antiangiogenic Factors.* Endothelial monocyte activating polypeptide II (EMAP-II) and endostatin are potent natural angiogenesis inhibitors. The role of EMAP-II has been explored only at earlier stages of lung development, but its activation decreases neovascularization, interrupts lung branching morphogenesis and decreases lung surfactant [63]. Endostatin was recently measured in tracheal aspirate fluid, and higher endostatin concentrations correlated with parameters reflecting lower lung maturity [64]. It is very likely that other pro- and antiangiogenic growth factors play a role during lung angiogenesis and the balance between the two types of growth factors are crucial for the coordinated assembly and remodeling of blood vessels during alveolar development.

In summary, these data provide strong evidence that angiogenesis in general, and VEGF in particular, is necessary for alveolarization during normal lung development, and that inhibition of VEGF during a critical period of growth contributes to the late sequelae of BPD. These observations also provide the rationale for testing the therapeutic potential of VEGF to promote alveolar development in experimental BPD.

*Therapeutic Potential of Angiogenic Growth Factors to Prevent Alveolar Injury in BPD*

Recombinant human VEGF (rhVEGF) treatment of newborn rats during or after exposure to hyperoxia enhances vessel growth and improves alveolarization [65, 66]. Likewise, postnatal intratracheal adenovirus-mediated VEGF gene therapy improves survival, promotes

lung capillary formation, preserves alveolar development and regenerates new alveoli in this same model of irreversible lung injury [30]. In both animal studies, VEGF induced immature and leaky capillaries and lung edema. However, combining lung VEGF and Ang1 (which promotes vascular maturation) gene transfer preserves alveolarization and enhances angiogenesis with more mature capillaries that are less permeable, reducing the vascular leakage seen in VEGF-induced capillaries [30].

These observations highlight the tightly orchestrated process of angiogenesis and points towards the need to closely recapitulate this process to warrant efficient and safe angiogenesis. Hypoxia-inducible factor (HIF) is a master transcription factor modulating O<sub>2</sub>-sensitive gene expression (including VEGF and Ang1) and vessel growth [48]. HIF is activated in hypoxia and inhibited by increased O<sub>2</sub> levels [67, 68]. However, because HIF deficiency is embryonically or immediately postnatally lethal, the role of HIF during alveolarization remains unknown. Nonetheless, HIF activation via inhibition of prolyl hydroxylase domain-containing proteins prevents lung injury in the premature baboon model of BPD and supports further a potential role for angiogenic growth factor in promoting alveolar development [69].

Given that VEGF-induced angiogenesis is in part mediated by NO, some of these findings may explain the beneficial effects of early and prolonged low-dose inhaled NO seen in three recent randomized controlled trials to prevent BPD [70–72]. Factors acting downstream of NO may exert similar beneficial effects. Accordingly, the phosphodiesterase type 5 inhibitor sildenafil, which acts downstream to enhance NO-induced cGMP levels, prevents hyperoxia-induced alterations of lung structure in neonatal rats [73].

### Conclusions and Future Directions

These observations provide proof of concept for the crucial role of the lung vasculature in what is traditionally thought of as an airspace disease and open new therapeutic avenues to protect or regenerate new alveoli. However, much more needs to be learned about the morphological changes and the mechanisms that regulate lung vascular development and saccular and alveolar growth. This is emphasized by the recent description of a new mode of blood vessel formation: intussusceptive microvascular growth, i.e. the internal division of the pre-existing capillary plexus (insertion of transcapillary tissue pillars) without sprouting [74]. Intussusceptive mi-

crovascular growth may promote alveolar development and remodeling throughout adult life and thus be amenable to therapeutic modulation for lung regeneration if we can unravel its regulatory mechanisms. Likewise, it will be crucial to recapitulate the tightly orchestrated process of angiogenesis (appropriate factor or combination of factors, dosing and timing) if this mechanism is to be exploited therapeutically. Finally, the recent excitement in stem cell biology has sparked the interest in the reparative potential of endothelial progenitor cells. If angiogenic growth factors contribute to alveolar homeostasis, then vascular progenitor cells are appealing candidate cells likely to be involved in the same mechanisms. Recent observations, including the developing lung [75], suggest that endothelial progenitor cells migrate from the bone marrow to the peripheral circulation and the lung where they contribute to the repair of injured endothelium and help restore lung integrity. These are exciting perspectives unraveling the therapeutic potential of promoting angiogenesis to restore lung damage. However, BPD is a multifactorial disease and it will be the translation of bench-side acquired knowledge about the basic mechanisms underlying lung development, injury and repair combined with the improved use of currently available therapies that will one day make this disease disappear.

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