LUNG VASCULAR DEVELOPMENT: Implications for the Pathogenesis of Bronchopulmonary Dysplasia

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Abstract  Past studies have primarily focused on how altered lung vascular growth and development contribute to pulmonary hypertension. Recently, basic studies of vascular growth have led to novel insights into mechanisms underlying development of the normal pulmonary circulation and the essential relationship of vascular growth to lung alveolar development. These observations have led to new concepts underlying the pathobiology of developmental lung disease, especially the inhibition of lung growth that characterizes bronchopulmonary dysplasia (BPD). We speculate that understanding basic mechanisms that regulate and determine vascular growth will lead to new clinical strategies to improve the long-term outcome of premature babies with BPD.

INTRODUCTION

The lung is a complex organ system whose basic physiologic function is to perform gas exchange across a thin blood-gas interface. The lung has the largest epithelial surface area of any mammalian organ and is capable of supporting a systemic oxygen consumption of between 250 ml/min at rest to 5500 ml/min during exercise (1). To create such a large, diffusible interface with the circulation, the lung epithelium must not only undergo a series of proliferative, branching, and morphogenetic steps, but also must interact continuously and in a well-coordinated manner with mesenchymal tissue to ensure the development of functioning vascular and lymphatic systems. The lung originates as a pair of invaginations from foregut endoderm. These endodermal buds branch and differentiate within the surrounding mesoderm, ultimately giving rise to the airways, alveoli, blood vessels, and lymphatics of the mature lung. Lung branching morphogenesis and epithelial development and differentiation have been the subject of intense investigation over the past 50 years, and the processes involved have been the subject of many comprehensive recent reviews (2–9). However, studies of the mechanisms that
regulate lung vascular and lymphatic development and that link capillary growth with alveolarization are relatively recent and limited in scope.

Until recently, most of the information regarding pulmonary vascular development has been largely descriptive in nature and often based on model systems and methodologic techniques that may preclude accurate assessment of the diverse processes governing pulmonary vascular development. This is especially true with regard to the origin, differentiation, and maturation of the various cell types within the vascular wall of lung blood vessels. This fact is important because it has become increasingly clear that airway and vascular development are closely interactive processes and that disruption of one system may have catastrophic consequences on the development of the other and, ultimately, of lung structure and function.

Developmental abnormalities of the pulmonary circulation contribute to the pathogenesis of several neonatal cardiopulmonary disorders, including diseases associated with persistent pulmonary hypertension of the newborn (10–12). More recently, however, there has been growing recognition that the importance of understanding basic mechanisms of lung vascular growth in the context of human disease may be best highlighted in the setting of bronchopulmonary dysplasia (BPD) (13, 14). Premature birth with injury to the immature lung disrupts normal lung growth and causes severe chronic lung disease, or BPD, which remains a major cause of late morbidity and mortality of premature newborns. Histologically, BPD is characterized by arrested lung growth, with decreased alveolarization and a dysmorphic vasculature. Recent studies suggest that disruption of normal lung vascular growth may play a central role in the pathogenesis of BPD; however, little is known about basic mechanisms of pulmonary vascular injury in the immature lung, the impact of this injury on subsequent growth and development of either the pulmonary or bronchial circulations, and the contribution of abnormalities of pulmonary and/or bronchial vascular growth to either the pathogenesis of BPD or functional abnormalities that characterize the disease. The purpose of this review is to examine lung vascular development in the context of understanding how growth of the pulmonary circulation and lung structure is impaired in BPD, with an eye toward restoring the injured lung to normal.

LUNG BLOOD VESSELS: NORMAL STRUCTURE AND FUNCTION OF THE PULMONARY AND BRONCHIAL CIRCULATIONS

The vascular system of the lung is divided into the pulmonary and bronchial systems. The pulmonary arteries supply the intrapulmonary structures and ultimately regulate gas exchange; vessels branch with the airways but branch into an extensive capillary network only at the level of respiratory bronchioles and alveoli. The bronchial system is the nutrient supply to the lung and perfuses the capillary bed within the bronchial wall and the structures of the perihilar region, including lymph
nodes and the adventitia of elastic and large muscular pulmonary vessels. All intrapulmonary structures drain to the pulmonary veins, whereas the hilar structures drain to the so-called true bronchial veins and then to the azygos system (15, 18).

The pulmonary artery accompanies the airways but gives off many more branches than the airway. In fact, two main types of pulmonary arterial vessels have been described: (a) conventional vessels, which are the long pulmonary artery branches that run within an airway, dividing as the airway divides and finally distributing to the capillary bed beyond the level of the terminal bronchioles; (b) supernumerary vessels, which are additional branches that arise from the pulmonary artery between the conventional branches, run a short course and supply the capillary bed of alveoli immediately around the pulmonary artery (9, 16, 17). Supernumerary arteries are thought to be a prominent component of the mature lung with the ratio of conventional to supernumerary vessels in the order of 1:2 in the prelobular region and 1:13 in the pre- and intra-acinar region (16). There appear to be distinct functional differences between supernumerary and conventional arteries. For instance, serotonin-induced vasoconstriction in supernumerary vessels is 30 times more potent than in conventional vessels (18). Others have suggested that plexiform lesions selectively develop in supernumerary vessels (19).

The pattern of veins in the lung resembles the arteries in that there are many more venous tributaries than airway branches (9, 15, 16). Similarly, at least two types of veins can be identified. Conventional veins arise from the points of division of an airway, pass to the periphery of a given lung unit, and combine to form increasingly large venous tributaries. Tributaries to the pulmonary veins also arise from the pleura and connective tissue septa. Within the lung, the anatomic distribution of the arteries and veins is characteristic. The broncho-arterial bundle includes the airway with the bronchial artery capillary bed and the adjacent pulmonary artery and lymphatic vessels, all within a single adventitial sheath. The veins always run at the periphery of any unit, whether it is the acinus, lobule, or segment. At the hilum, the veins join to form superior/inferior veins that drain to left atrium, giving them a distribution within the mediastinum different from that of the pulmonary artery.

A systemic blood supply to the lung was suggested at least 500 years ago by Galen. Its presence was confirmed by Ruysch in 1732, who designated the lung’s systemic vessel as the bronchial artery (15). It is now known that the bronchial arteries may arise from the descending aorta, intercostals, subclavian, or internal mammary arteries. The bronchial arteries may be classified as extrapulmonary or intrapulmonary. The extrapulmonary artery gives off small branches to the esophagus, to mediastinal tissues, to hilar lymph nodes, and the lobar bronchus. The intrapulmonary bronchial artery distributes to the supporting tissue and structures of the intralobal bronchi (mucous membrane, muscle, perichondrium, secretory glands), to pulmonary pleura, to lymph nodes, to walls of the pulmonary artery, and to veins and nerves. Bronchial arteries on the walls of the pulmonary artery and vein are functionally vasa vasorum. They are not as dense as on the bronchial walls and cannot be easily observed on the walls of small vessels. The extrapulmonary
bronchial artery blood drains into extrapulmonary bronchial veins and connect to the azygous or hemiazygous veins, which ultimately drain to the right atrium. The intrapulmonary bronchial artery drains into intrapulmonary bronchial veins and/or pulmonary veins, which then connect to the left atrium.

There are close and important relationships between the pulmonary and bronchial vascular systems, which become most evident in the diseased lung or in the setting of aberrant cardiovascular development (9, 15). For example, enlargement of the bronchial circulation is especially striking in association with congenital heart diseases, such as pulmonary atresia or transposition of the great vessels. Interestingly, ligation of the pulmonary arteries produces enlargement and dilation of the bronchial artery resulting in the increase of precapillary anastomosis and the development of various abnormal flow routes (20, 21). Similarly, severe pulmonary thromboembolic disease can result in proliferation of bronchial vessels in and around the obstructed pulmonary artery (15, 22). In bronchiectasis, a marked proliferation of bronchial vessels, which anastomosis with the pulmonary vascular system via precapillary, capillary, or postcapillary networks, is observed (23, 24). In emphysema, the normal structure of the alveolar capillary networks often disappears and enlarged branches of bronchial vessels are observed to occupy those areas, a situation obviously unfavorable to gas exchange (15). A similar proliferation of bronchial vessels is noted in the setting of chronic atelectasis (24). Thus many observations would support the idea that conditions leading to the narrowing or stenosis of blood vessels of the pulmonary vascular system are generally related to a proliferation and dilation of vessels of the bronchial vascular system. This relationship will be examined in the setting of BPD (see below).

Because the primary function of the lung is gas exchange, it is not surprising that keeping the airspaces and lung interstitium free of excess fluid is a critical component of normal lung function. The lymphatic system plays a critical role in fluid clearance, as well as in host defense, and in clearing solid particles and cells from the lung (26–28). It is also involved in the genesis of diseases such as tuberculosis and the metastasis of lung cancer. The lymph vessels form a closed circulatory system lined by endothelium. According to size, they are called lymph capillaries, lymph vessels, and collecting lymph vessels (15, 26). Lymph vessels are clearly evident at the level of the alveolar ducts (none is observed in direct relation to alveoli) and extend proximally (15, 26). Interestingly, the caliber of lymph vessels does not necessarily increase as they move toward the central lung, with lymph capillaries sometimes being larger than collecting lymph vessels. The critical nature of the lung lymphatic system is illustrated in human cases in which there is a primary developmental defect of the lung lymphatics, such as pulmonary lymphangiectasia. In the majority of neonatal cases, effective respiration is never established at birth, and the infants are stillborn or die in the first weeks of life despite aggressive support (29). In patients with bilateral pulmonary lymphangiectasia who survive, many develop chronic respiratory disease with pulmonary hypertension and cor pulmonale. Thus an understanding of the development of the lung and lung vasculature must take into account lymphatic development.
MORPHOGENESIS OF THE PULMONARY AND BRONCHIAL CIRCULATIONS

The most proximal part of the pulmonary circulation, the pulmonary trunk, is derived from the truncus arteriosus, which becomes divided into the aorta and pulmonary trunk by 8 weeks of gestation in humans by growth of the spiral aorticopulmonary septum (30). The pulmonary trunk connects to the pulmonary arch arteries, which are derived from the sixth branchial arch arteries, the most caudal of the brachial arteries, by 7-weeks gestation in humans (31). There is no consensus as to the mode of development of the sixth branchial arch artery, but recent reappraisal suggests that these pulmonary arch arteries originate from a strand of endothelial precursors that connect the ventral wall of the dorsal aortae to the pulmonary trunk (32, 33). These strands become lumenized, initially at the sites of connection with pulmonary trunk and dorsal aortae, then in between these sites of attachment. The origin of the intrapulmonary arteries has been variously described by early investigators as endothelial sprouts from either the aortic sac (31) or from the dorsal aortae (34), or as originating from a network of capillaries around the foregut (35, 36). The latter observation comes closest to the results of more recent studies suggesting that the pulmonary artery and vein develop in situ within the splanchnic mesoderm surrounding the foregut via at least two processes that likely occur concurrently: (a) vasculogenesis, in which new blood vessels form in situ from angioblasts and (b) angiogenesis, which involves sprouting of new vessels from existing ones.

The idea that some blood vessels arise de novo in the mesoderm surrounding the protruding endoderm may actually not be so new because it was initially raised some 70 years ago by Chang (37). Strong support for this idea has come from recent studies using molecular markers of endothelial progenitor cells, as well as endothelial differentiation markers (38–42). Cells expressing primitive endothelial markers appear in the mesoderm at early stages of lung development (embryonic and early pseudoglandular), which proceeds any documented connection with the established circulatory system. Lung vascular development continues throughout lung development, and requires epithelial-mesenchymal cross talk at each stage of development (8). Studies combining light and transmission electron microscopy with scanning electron microscopy of mercox vascular casts further suggest that large pulmonary arteries originate via the process of angiogenesis of central vessels, whereas distal vascular development requires vasculogenic mechanisms within the lung mesenchyme (43, 44). In addition to angiogenesis and vasculogenesis, a third process, fusion, is necessary to ultimately connect the angiogenic and vasculogenic vessels and expand the vascular network (43, 45).

At least two studies suggest similar mechanisms apply to human lung vascular development. DeMello et al. studied human embryos from the Carnegie Collection of Human Embryos and presented evidence to support the idea that both vasculogenesis and arteriogenesis operate cooperatively to form the pulmonary vessels (45). Using different techniques, Hall et al. also proposed that vasculogenesis is a
primary mechanism for intrapulmonary vascular development (46). Both investigations suggested that the venous circulation develops in a fashion similar to the arterial circulation and, importantly, both showed that the venous circulation is the first to be established.

On the other hand, this concept has recently been challenged by the suggestion that the lung vasculature is formed by distal angiogenesis, a process in which the formation of new capillaries from pre-existing ones occurs at the periphery of the lung (47). In this model epithelial/endothelial interactions are decisive in inducing angiogenesis and ensure the coordinate expansion of a vascular network as branching proceeds. Newly formed vessels remodel dynamically to form the afferent (arterial) and efferent (venous) vascular systems. Therefore, questions remain regarding the process that may be related to methodologic approaches and/or semantics. Future studies using new and improved techniques will likely resolve these issues.

At the end of 16 weeks of gestation in humans, all preacinar bronchi are in place and are accompanied by the pulmonary and bronchial arteries (48, 49). However, although the conducting airways and arteries are formed by the end of this so-called pseudoglandular stage (5–17 weeks) of lung development, the development of the gas-exchanging surface of the lung has just begun. The canalicular stage (17–26 weeks) encompasses the early development of the pulmonary parenchyma during which there is a great increase in the number of lung capillaries (9, 50). The capillaries begin to arrange themselves around the air spaces and come into close apposition with the overlying cuboidal epithelium. At sites of apposition, thinning of the epithelium occurs, to form what will be the first air-blood barrier. During the saccular stage (24 weeks to birth), the airways end in clusters of thin-walled saccules and by term have formed the last generations of airways, the alveolar ducts. Capillaries form a bilayer within the relatively broad and cellular intersaccular septa at this stage (9, 50). The period of alveolarization is largely a postnatal phenomenon, with more than 90% of all alveoli being formed after birth in humans (6, 9). During the period of alveolarization the initially thick interalveolar septa are attenuated and the double capillary layer fuses to form a single layer adult form. Secondary septae initially form as low ridges that protrude into primitive airspaces to increase their surface area. During this time, the lung is undergoing marked microvascular growth and development as well (51). Multiple stimuli contribute to alveolar and vascular growth and development, and in part, involve cross talk of paracrine signals between epithelium and mesenchyme (52). For example, vascular endothelial growth factor (VEGF) is expressed in developing lung epithelium, whereas VEGF receptor-2 (VEGFR-2) is localized to angioblasts within the embryonic mesenchyme (53–57). Diffusion of VEGF to precursors of vascular endothelial cells within the mesenchyme leads to angiogenesis by stimulating endothelial proliferation, a key step in vessel development. In addition, the process of septation involves alternate upfolding and growth of capillary layers within primary septa (54). This mechanism of alveolarization suggests that the failure of capillary network formation and growth, or disruption of the infolding of the
double capillary network, could potentially cause failed alveolarization. Less is known of the mechanisms contributing to development of the bronchial circulation. However, molecular marker studies suggest that intrapulmonary bronchial arteries also form in situ from the mesenchyme surrounding the epithelial buds (32, 33). Then, early in development (12 weeks in humans) connections to the systemic circulation are made. Presently, it is unclear as to what directs the proximal and distal connections between the systemic versus pulmonary vascular beds, especially when in early lung development the intrapulmonary vascular plexus are virtually indistinguishable. The question is of obvious importance because the behavior of these two circulations is vastly different. Current studies provide little insight into how the marked differences in endothelial cell and smooth muscle phenotype and function arise within the lung and its two circulations. Further, they do not adequately address how heterogeneity in phenotype and function of endothelial and smooth muscle cells arise even along the longitudinal axis of the respective circulations.

ROLE OF GROWTH AND TRANSCRIPTION FACTOR SIGNALING IN CONTROL OF LUNG VASCULAR GROWTH

Tremendous advances in our understanding of blood vessel formation within the embryo and various organs and tissues have taken place over the past 10 years. Many of the molecules involved seem to be highly conserved both across species and among various organs (Figure 1, Table 1). However, there may be subtle differences, and a complete understanding of the signaling pathways that regulate and coordinate vessel development and differentiation in a cell-specific fashion in the lung will be critical to understanding the unique functions and responses that the pulmonary and bronchial circulations exhibit in response to many pathophysiologic stimuli. Molecular programs important for the development of the lung vasculature have only recently begun to be elucidated. Among the different regulators of vessel formation, those that are specifically expressed in the endothelial lineage are best characterized during vascular development. This is the case for the ligand-receptor pairs VEGF/VEGFR, angiopoietin/TIE, ephrins/eph receptors, and notch/jagged.

Several studies have documented the appearance of VEGFR-2 (Flk-1) at very early time points of lung development in the mesenchyme (38, 58–60). VEGF also appears early in the developing epithelium and mesenchyme (58–63). It is known that VEGF-A is absolutely required for embryonic vascular development. Loss of even a single VEGF allele results in early embryonic death, before embryonic day 9.5. VEGF-A expression and function is tightly regulated during lung development. At least in the mouse, VEGF-A exists as three predominant isoforms, VEGF-A120, 164, and 188. Each isoform has differing properties, including affinity for the heparin sulfate component of the extracellular matrix, as well as for VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). VEGF-120 is highly diffusible because of lack of binding to heparin sulfate and probably serves a key early role in vascular formation.
Figure 1  A proposed model for the development of a mature vessel wall. (A) In this model, early expansion of mesoderm, perhaps driven by bFGF, is followed by an emergence of angioblasts or endothelial precursor cells within this mesenchyme as identified by Flt-1 and Flk-1. VEGFA is critical in the expansion of this cell population. (B) In the lung, epithelial-mesenchymal interactions are critical for the development of the vasculature, which involves diverse molecules [adapted from Ng et al. (88)]. Recruitment of mural cells follows endothelial tube formation. Angiopoietin, produced by undifferentiated mesenchymal cells, binds to and activates the TIE2 receptor on endothelial cells (C), resulting in release of a recruitment/chemotactic signal (e.g., PDGF, HB-EGF) for mesenchymal cells. Migration of mesenchymal cells to the developing endothelial tube and subsequent contact with the endothelium may activate signals (D) (e.g., TGF-β) that are necessary for the commitment of mesenchymal cells to SMC-specific lineages. The developing vessel then undergoes structural assembly (E) that includes inhibition of endothelial cell proliferation, rapid SMC proliferation, and extracellular matrix deposition. Throughout this period, differentiation to a more mature cell occurs as SMC gradually accumulate contractile and cytoskeletal proteins. Importantly, the vessel remains surrounded by mesenchymal cells or fibroblasts, which may serve as a continued source for mural cells. It is possible that progenitor cells reside in even the mature adventitia. Heterogeneity in both SMC and fibroblast populations is established and, as the mature vessel morphology is achieved (F), SMC become quiescent, respond poorly to mitogenic stimulation, and produce minimal matrix proteins. The adventitia may provide a reservoir of cells for vessel repair after injury in postnatal life.
TABLE 1  Factors likely to be involved at different stages of blood vessel formation

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Localization</th>
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<tbody>
<tr>
<td>Mesoderm formation (Stage A)</td>
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<tr>
<td>VEGF-A</td>
<td>Endoderm</td>
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<tr>
<td>BFGF</td>
<td>Endoderm</td>
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<tr>
<td>VEGFR-2, VEGFR-1</td>
<td>Endoderm</td>
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<tr>
<td>FGFR(s)</td>
<td>Endoderm</td>
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<tr>
<td>Fibronectin</td>
<td>Extracellular matrix</td>
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<tr>
<td>Aggregation of angioblasts (Stage A)</td>
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<tr>
<td>VEGFR-2</td>
<td>Angioblasts</td>
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<tr>
<td>VEGFR-1</td>
<td>Angioblasts</td>
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<tr>
<td>VE-cadherin</td>
<td>Angioblasts</td>
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<tr>
<td>tie-2/tek</td>
<td>Angioblasts</td>
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<tr>
<td>ets-1</td>
<td>Angioblasts</td>
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<tr>
<td>PECAM-1</td>
<td>Angioblasts</td>
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<tr>
<td>Fibronectin</td>
<td>Extracellular matrix</td>
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<tr>
<td>VEGF</td>
<td>Endoderm</td>
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<tr>
<td>Endothelial differentiation and formation of primary capillary plexus (Stage B)</td>
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<tr>
<td>VEGFR-2</td>
<td>Endothelial cells</td>
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<tr>
<td>VEGFR-1 endothelial cells</td>
<td>Endothelial cells</td>
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<td>VE-cadherin</td>
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<td>tie-2/tek</td>
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<td>tie-1</td>
<td>Endothelial cells</td>
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<td>E-selectin</td>
<td>Endothelial cells</td>
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<td>ets-1</td>
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<td>PECAM-1</td>
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<td>Integrins (e.g., αVβ3)</td>
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<tr>
<td>Notch</td>
<td>Endothelial cells</td>
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<td>delta/jagged</td>
<td>Endothelial cells</td>
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<td>Ephrins</td>
<td>Endothelial cells</td>
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<td>Ephs</td>
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<td>Collagen IV</td>
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<td>VEGF-A</td>
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<tr>
<td>Smooth muscle cell recruitment and differentiation (Stages C, D)</td>
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<tr>
<td>tie-2</td>
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<tr>
<td>PDGF-BB</td>
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<td>PDGF-AA</td>
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<tr>
<td>HB-EGF</td>
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<tr>
<td>BFGF</td>
<td>Endothelial cells</td>
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<tr>
<td>Thromboxane A2</td>
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<tr>
<td>Angiotensin II</td>
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<td>Endothelin</td>
<td>Endothelial cells</td>
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TABLE 1 (Continued)

<table>
<thead>
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<th>Molecules</th>
<th>Localization</th>
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<td>Leukotrienes</td>
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<tr>
<td>Serotonin</td>
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<tr>
<td>Angiopoietin</td>
<td>Endothelial cells</td>
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<td>TGF-β</td>
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<tr>
<td>IFG-II</td>
<td>Mesenchyme</td>
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<tr>
<td>Tissue factor</td>
<td>Mesenchyme</td>
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<tr>
<td>WNT</td>
<td>Mesenchyme</td>
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<tr>
<td>Laminin</td>
<td>Basement membrane</td>
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<tr>
<td>Type IV collagen</td>
<td>Basement membrane</td>
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<tr>
<td>Perlecan</td>
<td>Basement membrane</td>
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<tr>
<td>Heparan sulfate</td>
<td>Basement membrane</td>
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</table>

*Stages correspond to those illustrated in Figure 1.

through driving endothelial commitment and expansion (61). The importance of VEGF-164 and 188 isoforms was demonstrated in mice engineered to express only the VEGF-120 isoform. VEGF-120 only animals had fewer air-blood barriers and decreased airspace to parenchyma ratios compared with that of wild-type littermates. Thus as development proceeds, the pattern of VEGF isoform expression becomes more restrictive, and the VEGF isoforms serve different roles (64).

VEGF has other family members including VEGF-C and -D. These VEGFs have different affinities for specific VEGF receptors with both VEGF-C and -D demonstrating an ability to bind to VEGFR-3 (65–67). It is now recognized that VEGF-C and -D(?)/VEGFR-3 interactions are probably crucial for development of the lymphatic vascular system (65, 66). Recent studies have evaluated temporal and spatial pattern of VEGF-D expression during mouse lung development (62). The pattern of expression is distinct from that of VEGF-A, suggesting a unique function for each VEGF during lung development. In addition, the finding of VEGF-D expression in the mesenchyme by cells distinctly different from endothelial cells and smooth muscle cells (SMC), i.e., fibroblasts, suggests that mesenchymal cells can influence endothelial phenotype during lung development through expression of either VEGF-A or VEGF-D (and possibly VEGF-C). Thus it is likely that subsets of cells within the mesenchyme must have specific roles pertaining to inductive events between mesenchymal and endothelial compartments and therefore may also exert influence on the generation of endothelial heterogeneity described above.

The angiopoietins (Ang) and their major receptor Tie-2 (tyrosine kinase with immunoglobulin and EGF-like domains) are critical for normal vascular development because Ang 1−/− or Tie 2−/− mice are embryonic lethal owing to the failure of vascular integrity (68, 69). Ang 1 is produced by lung mesenchyme and smooth muscle, whereas Tie 2, its receptor, is restricted to endothelial expression (69–71).
Ang 1 binding to Tie 2 causes receptor tyrosine phosphorylation and downstream signals for endothelial cell survival through phosphatidylinositol 3-kinase PI3K/Akt signaling (72, 73). Angiogenic actions of Ang 1 require endothelium-derived NO (74). Ang 1 promotes interactions between endothelial cells, extracellular matrix, and pericytes that are required for vessel maturation (68). Ang 1-VEGF interactions are critical for normal vascular maturation, but their interactive effects are complex and dependent upon multiple factors. In some settings, Ang1 treatment attenuates VEGF-induced angiogenesis by increasing intercellular endothelial cell junctions, but Ang 1 also makes vessels resistant to VEGF withdrawal (70, 75, 76). Ang 2 also binds Tie 2, but blocks the function of Ang 1 (72). Ang 2 is expressed only at sites of vessel remodeling where it destabilizes vessels, perhaps via Ang 1 inhibition, which may increase endothelial cell responses to VEGF. The combination of low Ang 2 and low VEGF may enhance endothelial cell death (72). Low Ang 1 to Ang 2 ratios favor decreased Tie 2 phosphorylation, which leads to weaker endothelial connections and perhaps increased responsiveness in VEGF. Little is known about Ang expression during development, especially in models of lung hypoplasia, but it is increased in nitrofen model of congenital diaphragmatic hernia (77). In addition, the role of Ang 1 in pulmonary hypertension has been extremely controversial (78). Overexpression of Ang 1 causes severe pulmonary hypertension and is increased in lungs from human patients with pulmonary hypertension (79). However, cell-based gene transfer studies with Ang 1 protected against experimental pulmonary hypertension caused by monocrotaline (80). These differences may be related to the mixed effects of Ang 1 on isolated cells: Ang 1 stimulates smooth muscle cell proliferation but inhibits endothelial cell apoptosis.

Less is known about the other ligand-receptor pairs with regard to lung vascular development. The notched/jagged pathways seem to lie downstream from VEGF signaling and be important during embryonic vascular development (81). Taichman et al. performed a systematic evaluation of notch-1/jagged-1 gene and protein expression in the developing mouse lung and found that the mRNA transcripts for notch-1 and jagged-1 increased progressively from early to later lung development, accompanied by simultaneous rise in endothelial cell-specific gene expression, a pattern somewhat unique to the lung (82). Notch-1 and jagged-1 appeared initially on well-formed larger vessels within the embryonic lung and were progressively expressed on smaller developing vascular networks (82). Notch signaling may mediate its effects, at least in part, through the forkhead box (Fox) f-1 transcription factors (81). That this transcription factor family plays a critical role in development of the pulmonary vasculature was recently shown in studies demonstrating that Fox-f1 haploinsufficiency is capable of disrupting pulmonary expression of genes in the notch-2 signaling pathway resulting in abnormal development of the lung microvasculature (83).

Another autocrine/paracrine pathway shown to be important in lung vascular development is Wnt. Wnt proteins are homologs of the Drosophila wingless gene and have been shown to play important roles in regulating cell differentiation,
proliferation, and polarity (84, 85). Several Wnt genes have been shown to be expressed in the developing lung including Wnt-2, Wnt-2b, Wnt-7b, Wnt-5a, and Wnt-11. Of those only Wnt-7b is expressed at high levels exclusively in the developing airway epithelium during early lung development (86). Morrisey et al. have recently reported the generation and characterization of a Wnt-7b (lacZ) knock-in mouse (87). These mice exhibit severe pulmonary hypoplasia and lung-specific vascular defects. The vascular defects include dilation of large blood vessels in the lung with subsequent cell death and degradation of the vascular SMC layer leading to pulmonary hemorrhage. Defects in the lungs of Wnt-7b (lacZ−/−) embryos, including early defects in mesodermal proliferation, suggest that Wnt-7b is required for lung mesodermal development. It has been demonstrated that the transcription factor Fox-f2, which is expressed in the developing lung mesoderm, is down-regulated in Wnt-7b (lacZ−/−) lung tissue. Because Fox-f2 is related to Fox-f1, which, as mentioned above, has been shown through loss-of-function experiments to regulate lung vascular development (83), this suggests that Wnt signaling through forkhead box transcription factors is critical for development of vascular SMC from the lung mesoderm and for mesodermal thickening. These studies are among the first to specifically evaluate the factors involved in SMC recruitment/differentiation during lung vascular development.

Other factors have been shown to be involved in regulating the recruitment of mural cells (pericytes or SMC) to the vessel wall. However, few of these studies have been specifically performed in the developing lung. It is known that proliferating endothelial cells secrete platelet-derived growth factor-b (PDGF-b), which acts as a chemoattractant and mitogen for mural cell precursors derived from the mesenchyme surrounding the endothelial tubes (88). Other molecules thought to be involved in mural cell recruitment include angiopoietic tissue factor, notch/jagged 1, ephrin/eph, and COUP-TFI1 (89, 89a). Because the molecules involved in mural cell recruitment across organs appear similar, it has been assumed by many that organ-specific mesodermal cells contribute to the mural or smooth muscle layers of developing vessels, which likely results in tissue-specific functional and regulatory properties of vascular SMC and pericytes. The anatomical position of developing vessels may also contribute to mural cell heterogeneity. SMC in the pulmonary trunk appear to be largely derived from neural crest tissues, whereas mural cells in the parenchymal lung blood vessel appear to have a distinct mesenchymal origin. However, because bronchial and pulmonary vessels are apparently derived from similar (identical?) mesenchyme and have similar anatomic locations within the lung, the cell- and tissue-specific factors that lead to the emergence of SMCs around the pulmonary and bronchial arteries with distinct functional phenotypes are unclear. Even more intriguing is how the differences in functional phenotype between SMC of conventional versus supranumery arteries arise.

Upon recruitment to the endothelial tube, newly recruited mesenchymal progenitor cells are induced toward a smooth muscle fate. This process seems to be mediated, at least in part, by the activation of transforming growth factor-β (TGF-β). Although the exact mechanisms of how TGF-β is activated and signals to cause
mural cell differentiation are unknown, it is clear that this signaling system plays a critical role in vascular development (90, 91). TGF-β response elements have been identified in the promoter regions of smooth muscle (SM) genes, such as SM-α-actin, SM-22, and calponin (92). TGF-β can also induce differentiation via the up-regulation of the transcription factor serum response factor (SRF) (93). SRF binds the serum response elements in the promoter regions of mural cell–specific genes, including SM-α-actin, SM-γ-actin, SM-22α, and calponin and induces their coordinated expression (94). The acquisition of differentiated contractile SM or SM-like cells is critical not only to stabilize and maintain the integrity of the developing endothelial tubes but also to control blood flow through these tubes. This may be particularly important in the pulmonary circulation where early intense vasoconstriction capabilities must be acquired to limit blood flow to the developing lung. The mechanisms involved in this mural cell/endothelial cell interaction, which results in the acquisition of SMC with properties differing from systemic vascular smooth muscle and critical for lung function, remain to be elucidated.

Knockout studies of gap junction proteins, including CX43 and CX45, suggest that gap junction communication is required for endothelial-induced mural cell differentiation and function (95, 96). To our knowledge, developmentally regulated expression of these genes in the lung circulation has not been studied. Other requirements include cell-cell adhesion and appropriate cell-matrix interactions mediated via integrin interactions. Unfortunately, very little is known about the regulation of integrin expression, either by endothelial cells or mural cells, during pulmonary vascular development.

Several studies have suggested that homeobox genes may be involved in early vessel development and mural cell recruitment. Homeobox genes, including Hex, are important in early vasculogenic or angiogenic processes (97, 98). Other homeobox genes, such as the paired-related homeobox gene, PRX-1, may be involved in cell differentiation and stabilization of the vessel wall (99, 100). In the developing systemic vasculature, Prx-1 appears early and is highly evident in prospective connective tissues, including the endocardio-cushions, the epicardium, and the walls of great arteries and veins (99). In the chick embryo, Prx-1 mRNA expression is first evident within the primary vessel wall of coronary and pulmonary arteries. As the vessels mature and thicken, the pattern of expression becomes restricted to nonmuscle cells in the adventitial and outer medial regions (100). These data suggest that Prx-1 may control the expression of genes involved not only in early differentiation of endothelial cells but also in the assembly and segregation of different cell types within the differentiating blood vessel wall.

In addition to proangiogenic stimuli, normal vascular development may also require the expression of angiostatic molecules. Schwartz et al. have described the presence in the lung of a protein, termed endothelial monocyte-activating peptide-2 (EMAP-2), with potent angiostatic properties both in vitro and in vivo (101, 102). It appears to act by specifically causing endothelial cell apoptosis. Studies suggest that its presence may balance and therefore help shape the pulmonary vasculature in the developing mouse lung. Overexpression of EMAP-2
markedly inhibits neovascularization and also inhibits alveolar type II cell development. Other angiostatic proteins, including Ang-2, and monokine-induced by interferon-γ (MIG) and interferon-γ inducible protein (IP-10), have been identified and shown to block the angiogenic effects of angiogenic factors including VEGF. The role of these chemokines in normal lung vascular development remains to be determined.

ROLE OF OXYGEN TENSION IN THE REGULATION OF LUNG VASCULAR MORPHOGENESIS

Oxygen tension appears to be an important physiologic mediator of embryonic and fetal development, and hypoxia is known to be an important regulator of both vasculogenesis and angiogenesis. As such, it is not surprising that studies of mammalian development in vitro demonstrate that proper embryonic development is dependent on low oxygen tension (3–5%) and that even short exposures to normoxic environments (20%) can be detrimental to embryonic development. The critical role of oxygen tension in lung development is strongly supported by observations regarding the development of the tracheal system of Drosophila (103). Initiation of tubulogenesis in Drosophila depends on the expression of two basic helix-loop-helix PAS transcription factors, tracheless and single-minded (trh and sim), which dimerize with the tango (tgo) gene product to direct expression of genes that govern tracheal invagination (103). These Drosophila genes share high sequence and functional homology with members of the mammalian HIF-1α/ARNT family where both trh and sim act as transcriptional initiators homologues to HIF-1α, which depends on an ARNT homolog, (tgo), for nuclear transport and the formation of the transcriptional complex (104). Experimental knockout of the complex results in complete failure of tracheal system development in Drosophila. Null mutation of HIF-1α in mice has no effect until embryonic day 9, a period that coincides with the initiation of lung development and vasculogenesis. These mice display apparently enlarged vascular structures, fail to initiate lung morphogenesis fully, and die by E 10.5 (105). In addition, there is a regulated loss of mesenchymal tissue suggesting a role for HIF-1α in regulating the survival and differentiation of mesenchymal progenitor cells into vascular structures (105, 106).

Recent work utilizing mammalian fetal lung explants also provides evidence that the process of lung budding and airway bifurcation is oxygen dependent (106–108). In these studies, budding and bifurcation is accelerated at PO2S that mimic those in the fetal environment. Interestingly, the increases in branching morphogenesis appear to be confined to the periphery in explant culture, suggesting that the locus of hypoxia dependence is within the region of active airway bifurcation and vascular cell proliferation (106, 108). This effect is reversible, a finding that provides insight into the changes in gene expression and lung structure that occur at the time of birth when the lung becomes immediately exposed to alveolar (very high compared with the fetal lung) oxygen concentrations. Embryonic
lung explants maintained at alveolar PO2s show traits of alveolarization (i.e., mesenchymal thinning, epithelial flattening) and saccularization, and also express the surfactant protein C gene. Removal of these explants to fetal PO2 for as little as 24 h results in the rapid loss of surfactant protein C expression and regrowth of airways into the saccular spaces and an increase in airway surface complexity (108). This is consistent with observations showing that neonatal mice or rats exposed to moderate hypoxia exhibit arrested postnatal lung maturation and alveologenesis, as well as surfactant protein expression and the perinatal increase in epithelial sodium transport (109–111). Thus O2 tension may modulate both physiologic and structural characteristics of lung vascular and airway development.

Several oxygen-regulated genes appear to be involved in this process. Fibroblast growth factor (FGF)-9 expression is increased at fetal PO2, whereas FGF-10, which is also known to be involved in branching morphogenesis, demonstrates no such O2 dependency (108). The FGF-9 promoter has a putative binding site for C/EBP-β. Importantly, studies demonstrate that low oxygen tension results in the nuclear translocation and DNA binding of C/EBP-β. Thus O2 regulation of FGF-9 via C/EBP-β may be a critical mediator of lung and lung vascular development.

Other factors, including members of the hepatocyte nuclear factor (HFN) family, a homolog of Drosophila forkhead gene, may play roles in determining epithelial cell lineage fates by acting as dimerization partners for crucial developmental transcription factors such as NKX-2.1 (113, 114). The role of HNFs in hypoxic responses is demonstrated by their involvement in erythropoietin gene expression, suggesting that interactions, at least between HNF-4 and HIF-1α are necessary for regulation of erythropoietin gene expression. Similarly, Sp1 transcription factors, known to be activated by hypoxia, are dimerization partners for HNF-3. Thus HNF transcription factors may integrate the association between oxygen availability and lung development (106).

TGF family genes (TGFβ-1, -2, -3) and their receptors (TGF-βR1, 2, 3) promote proliferation of mesenchyme tissue and thereby inhibit the rate of differentiation of the lung into branched airway structures (116). TGFβ-3 is a HIF-1α−regulated gene that mediates mesenchymal proliferation under many conditions, both developmental and pathophysiologic. In embryonic lung explants, HIF-1α and TGFβ-3 appear to be up-regulated, thus supporting a role for both low oxygen concentrations and TGFβ-3 in lung development. Neutralizing antibody against TGFβ-3 results in mesenchymal thinning and arrest of airway bifurcation. On the other hand, and very importantly, exposure of neonatal rats to reduced oxygen concentrations (9.5%) results in a dramatic increase in TGFβ signaling and TGFβ-R1 receptor expression that collectively act to diminish alveolarization (117). Other groups have reported that TGFβ-1 is an effective inhibitor of stress-invoked inducible nitric oxide synthase (iNOS) activity (118). Nitric oxide generated endogenously and exogenously can increase branching morphogenesis by as much as twofold (119). All three NOS isoforms are expressed in fetal pulmonary tissues with NOS expression increasing dramatically within the final trimester (120). Thus it is possible that endogenous NO production is required for normal airway growth and
development both pre- and postnatally. Although hypoxia is known to evoke and sustain NO synthesis and release, it is possible that the simultaneous augmentation of the TGF\(\beta\) pathway results in inhibition of airway growth and epithelial maturation through direct repression of iNOS activity and expression.

Recent studies have begun to elucidate the signaling pathways through which hypoxia might act to modulate vascular cell proliferation and angiogenesis. With regard to hypoxia-induced angiogenesis, it is clear that PI3K activity is essential and that downstream targets, independent of Akt, are important (121). Activation of mTOR appears essential for hypoxia-mediated amplification of cell proliferation and angiogenesis and activation may occur independently of Akt activation under hypoxic conditions (122). This observation demonstrates a unique signaling aspect of hypoxia compared with that of other mitogenic stimuli. mTOR can act as an important upstream regulator of both HIF-1\(\alpha\) and C/EBP\(\beta\) and thus may represent a key point of convergence in the sensing pathways that regulate cell cycle progression under hypoxic conditions (123).

In addition, recent studies also suggest that purinergic signaling is critical in hypoxia-induced fibroblast proliferation and differentiation as well as in SMC migration and endothelial proliferation (124). This purinergic signaling loop may be autocrine in nature because hypoxia induces the release of ATP from vascular wall cells. In addition, sympathetic or sensory nerves may be an additional source of purine nucleotides and thus may exert significant trophic effects on both the nascent and the mature vasculature. Recent studies have clearly demonstrated the role of the nervous system in directing vascular development (125). However, whether this is true in the lung or just for proximal lung vessels as opposed to distal lung vessels is currently unclear.

Thus it appears unequivocal that hypoxia activates unique cellular signaling pathways critical for growth and differentiation of cells within the pulmonary vasculature. Studies in the future need to be directed at utilizing the effects of oxygen to study lung vascular development. Furthermore, we need to utilize this information to better understand the response of the premature lung, which is removed from this hypoxic environment to a hyperoxic environment. That this transition can inhibit lung development as well as vascularization often leading to significant cardiopulmonary dysfunction is clear. How the effects are mediated is not.

### IMPAIRED VASCULAR GROWTH IN BRONCHOPULMONARY DYSPLASIA

Perhaps the most relevant example of how disruption of lung vascular development contributes to human disease lies in the clinical problem of BPD. Abnormalities of the pulmonary circulation, especially the development of pulmonary hypertension, have long been recognized as playing an important role in the pathophysiology and clinical outcomes of premature infants with BPD (126, 134). More recent data from animal and clinical studies suggest that impaired vascular growth may also
contribute to abnormalities of lung architecture, especially decreased alveolarization, and may play a critical role in the pathogenesis of BPD. In the following section, we provide a brief overview of problems related to abnormalities of the pulmonary circulation in BPD and review recent data from animal models and clinical studies of BPD that may provide insight into how normal developmental processes are interrupted by premature delivery.

**BPD—the Clinical Problem**

BPD is the chronic lung disease of infancy that follows mechanical ventilation and oxygen therapy for acute respiratory distress after birth in premature newborns (14, 135, 136). As first characterized by Northway and colleagues in 1967, BPD has traditionally been defined by the presence of persistent respiratory signs and symptoms, the need for supplemental oxygen to treat hypoxemia, and an abnormal chest radiograph at 36-weeks corrected age (135) (Figure 2). Despite improvements in perinatal care, chronic lung disease after premature birth remains a major clinical problem. With increasing survival of extremely premature newborns, BPD remains as one of the most significant sequelae of neonatal intensive care, with an estimated 10,000 affected infants in the United States each year. The medical and socio-economic impact of BPD is substantial, with many infants requiring frequent physician visits and hospitalizations due to recurrent respiratory infections, reactive airways disease, upper airway obstruction, cor pulmonale, and exercise intolerance. In many cases, signs and symptoms of chronic lung disease continue into adolescence and adulthood.

With the introduction of surfactant therapy, maternal steroids, new ventilator strategies, aggressive management of the patent ductus arteriosus, improved nutrition, and other treatments, the clinical course and outcomes of premature newborns with RDS have dramatically changed over the past 30 years. During the “surfactant era,” BPD was directly related to the severity of acute respiratory distress syndrome (RDS) and was often present in premature infants who were born at relatively large birth weights and gestational ages. For example, in the original report of Northway and coworkers, the premature infants with BPD were born at 34-weeks gestation, weighed 2200 g, and mortality was 67% (135). Since that time, mortality of preterm infants has markedly decreased, with survival increasing from less than 10% to presently over 50% in even the most extremely preterm newborn (24–26-weeks gestation). The risk of BPD rises with decreasing birth weight, with an incidence up to 85% for newborns between 500–699 g (135, 136). In extremely immature infants, even minimal exposure to oxygen and mechanical ventilation may be sufficient to contribute to BPD (137–139). A recent report demonstrated that about two thirds of infants who develop BPD have only mild respiratory distress at birth (137). These findings suggest that developmental timing of lung injury is a critical factor in the etiology of BPD.

In parallel with this changing epidemiologic and clinical pattern, key features of lung histology in BPD have also changed. Original studies of BPD described a
continuous process through distinct stages of disease, progressing from acute lung injury, or an exudative phase with diffuse pulmonary edema, proteinaceous debris, and inflammation, to a proliferative phase with structural features of chronic lung disease. Older reports described the gross cobblestone appearance of the lungs, representing alternating areas of atelectasis, marked scarring, and regional hyperinflation (or emphysema). Typical histologic features of BPD included marked airway changes, such as squamous metaplasia of large and small airways, increased peribronchial smooth muscle and fibrosis, chronic inflammation and airway edema, and hyperplasia of submucosal glands. Distal parenchymal lung disease was characterized by heterogeneous changes, including regions of volume loss from atelectasis and septal fibrosis alternating with areas of overdistension or emphysematous regions. Mesenchymal thickening with increased cellularity and destruction of septae with alveolar hypoplasia were also noted in early autopsy studies, along with hypertensive structural remodeling of small pulmonary arteries, including smooth muscle hyperplasia and distal extension of smooth muscle growth into vessels that are normally nonmuscular.

There is now growing recognition that infants with persistent lung disease after premature birth have a different clinical course and pathology than was traditionally observed in infants dying with BPD during this presurfactant era (140, 141). The classic progressive stages that first characterized BPD are often absent owing to changes in clinical management, and BPD has clearly changed from being predominantly defined by the severity of acute lung injury to its current characterization, which is primarily defined by a disruption of distal lung growth. In contrast to classic BPD, the new BPD develops in preterm newborns who generally require minimal ventilator support and relatively low FiO₂ (fraction of inspired oxygen) during the early postnatal days. Pathologic signs of severe lung injury with striking fibroproliferative changes have become rare. At autopsy, lung histology now displays more uniform and milder regions of injury, and signs of impaired alveolar and vascular growth are more prominent. These features include a pattern of alveolar simplification with enlarged distal airspaces and reduced growth of the capillary bed, with vessels that are often described as dysmorphic because of their centralized location in the thickened mesenchyme (Figure 2).

Thus, the so-called new BPD of the postsurfactant period represents inhibition of lung development with altered lung structure, growth, and function of the distal airspaces and vasculature (see below). Physiologically, these findings suggest a marked reduction in alveolo-capillary surface area, potentially contributing to impaired gas exchange with increased risk for exercise intolerance, pulmonary hypertension, and poor tolerance of acute respiratory infections (133, 142–145, see below).

Pathogenesis of BPD

BPD represents the response of the lung to injury during a critical period of lung growth, that is, during the canalicular period (17 to 26 weeks in the human), a
Figure 3 Abnormal pulmonary circulation in chronic lung disease of infancy (BPD) Schematic illustrating pathogenetic abnormalities of the pulmonary circulation in BPD.

Hyperoxia and oxidant stress are critical factors in the development of BPD (149, 150). The transition of the premature newborn from the low-oxygen tension environment of the normal fetus to the relative hyperoxia of extrauterine life increases the risk for BPD with decreased alveolarization and a dysmorphic vasculature. This premature change in the oxygen environment is likely to impede normal epithelial-mesenchymal interactions leading to alterations in endothelial cell survival, differentiation, and organization in the microvasculature, the mechanisms of which are partly described above. The premature infant is especially susceptible to reactive oxidant species (ROS)-induced damage owing to the lack of adequate antioxidants after premature birth. Antioxidant enzymes [e.g., superoxide dismutase (SOD), catalase, and glutathione peroxidase] markedly increase during late gestation (151). In addition, the ability to increase synthesis of antioxidant enzymes in response to hyperoxia is decreased in preterm animals, suggesting that premature birth may precede the normal up-regulation of antioxidants, which
persists during early postnatal life. Experimental studies have shown that hyperoxia, even in the absence of ventilation, can induce lung injury that mimics RDS and late sequelae that are similar to BPD (152, 153; see below). Endothelial and alveolar type II cells are both extremely susceptible to hyperoxia and ROS-induced injury, leading to increased edema, cellular dysfunction, and impaired cell survival and growth (152–154). The critical role of host antioxidant defenses in protecting the developing lung from hyperoxia-induced injury is further shown by studies of transgenic SOD mice and treatment with exogenous SOD (155–157).

Even in the absence of overt signs of baro- or volutrauma, treatment of premature neonates with mechanical ventilation initiates and promotes lung injury with inflammation and permeability edema, and contributes to BPD. Ventilator-associated lung injury (VALI) results from stretching distal airway epithelium and capillary endothelium, which increases permeability edema, inhibits surfactant function, and provokes a complex inflammatory cascade (158). Experimental studies have clearly shown that overdistension, and not pressure per se, is responsible for lung injury in the surfactant-deficient lung (159, 160). Even brief periods of positive-pressure ventilation, such as during resuscitation in the delivery room, can cause bronchiolar epithelial and endothelial damage in the lung, setting the stage for progressive lung inflammation and injury (161, 162).

As described above, lung inflammation, whether induced prior to birth (from chorioamnionitis) or during the early postnatal period (due to hyperoxia or VALI) plays a prominent role in the development of BPD (163–165). Numerous clinical and experimental studies have shown that the risk for BPD is associated with sustained increases in tracheal fluid neutrophil counts, activated macrophages, high concentrations of lipid products, oxidant-inactivated α-1-antitrypsin activity, and proinflammatory cytokines, including IL-6 and IL-8, and decreased IL-10 levels (166–168). Release of early response cytokines, such as TNF-α, IL-1β, IL-8, and TGF-β, by macrophages and the presence of soluble adhesion molecules (i.e., selectins) may impact other cells to release chemoattractants that recruit neutrophils and amplify the inflammatory response (169, 170). Elevated concentrations of proinflammatory cytokines in conjunction with reduced anti-inflammatory products (i.e., IL-10) usually appear in tracheal aspirates within a few hours of life in infants subsequently developing BPD. Increased elastase and collagenase release from activated neutrophils may directly destroy the elastin and collagen framework of the lung, and markers of collagen and elastin degradation have been recovered in the urine of infants with BPD (171). Infection from relatively low virulence organisms, such as airway colonization with Ureaplasma urealyticum, may augment the inflammatory response, further increasing to the risk for BPD (172). Finally, other factors, such as nutritional deficits and genetic factors, such as vitamin A and E deficiency or single nucleotide polymorphism variants of the surfactant proteins, respectively, are likely to increase risk for BPD in some premature newborns (173, 174). Thus multiple stimuli act on the susceptible lung at a critical stage of development after premature birth, leading to disruption of normal vascular growth and impaired alveolarization.
Pulmonary Circulation in Human BPD

In addition to adverse effects on the airway and distal airspace, acute lung injury also impairs growth, structure, and function of the developing pulmonary circulation after premature birth (13, 133, 153). Endothelial cells have been shown to be particularly susceptible to oxidant injury through hyperoxia or inflammation (152, 153, 157). The media of small pulmonary arteries may also undergo striking changes, including smooth muscle cell proliferation, precocious maturation of immature mesenchymal cells into mature smooth muscle cells, and incorporation of fibroblasts/myofibroblasts into the vessel wall (176, 177). Structural changes in the lung vasculature contribute to high pulmonary vascular resistance (PVR) through narrowing of the vessel diameter and decreased vascular compliance. In addition to these structural changes, the pulmonary circulation is further characterized by abnormal vasoreactivity, which also increases PVR (127, 130). Finally, decreased angiogenesis may limit vascular surface area, causing further elevations of PVR, especially in response to high cardiac output with exercise or stress.

Overall, early injury to the lung circulation leads to the rapid development of pulmonary hypertension, which contributes significantly to the morbidity and mortality of severe BPD. Even from the earliest reports of BPD, pulmonary hypertension and cor pulmonale were recognized as being associated with high mortality (128, 131). Walther et al. showed that elevated pulmonary artery pressure in premature newborns with acute RDS (determined from serial echocardiograms) was associated with severe disease and high mortality (131). Past studies have also shown that persistent echocardiographic evidence of pulmonary hypertension beyond the first few months of life is associated with up to 40% mortality in infants with BPD (128). High mortality rates have also been reported in infants with BPD and pulmonary hypertension who require prolonged ventilator support (132). Although pulmonary hypertension is a marker of more advanced BPD, elevated PVR also causes poor right ventricular function, impaired cardiac output, limited oxygen delivery, increased pulmonary edema and, perhaps, a higher risk for sudden death.

Physiologic abnormalities of the pulmonary circulation in BPD include elevated PVR and abnormal vasoreactivity, as evidenced by the marked vasoconstrictor response to acute hypoxia (127, 130, 132, 178). Cardiac catheterization studies have shown that even mild hypoxia causes marked elevations in pulmonary artery pressure in infants with modest basal levels of pulmonary hypertension. Treatment levels of oxygen saturations above 92–94% effectively lower pulmonary artery pressure (130). Strategies to lower pulmonary artery pressure or limit injury to the pulmonary vasculature may limit the subsequent development of pulmonary hypertension in BPD. Recent data suggest that high pulmonary vascular tone continues to elevate PVR in older patients with BPD, as demonstrated by responsiveness to altered oxygen tension and inhaled nitric oxide (178).

In addition to pulmonary hypertension, clinical studies have also shown that metabolic function of the pulmonary circulation is impaired, as reflected by the impaired clearance of circulating norepinephrine (NE) across the lung (179).
Normally, 20–40% of circulating NE is cleared during a single passage through the lung, but infants with severe BPD have a net production of NE across the pulmonary circulation. It is unknown whether impaired metabolic function of the lung contributes to the pathophysiology of BPD by increasing circulating catecholamine levels, or if it is simply a marker of severe pulmonary vascular disease. It has been speculated that high catecholamine levels may lead to left ventricular hypertrophy or systemic hypertension, which are known complications of BPD (179–181).

Prominent bronchial or other systemic-to-pulmonary collateral vessels were noted in early morphometric studies of infants with BPD and can be readily identified in many infants during cardiac catheterization (182, 183). Although these collateral vessels are generally small, large collaterals may contribute to significant shunting of blood flow to the lung, causing edema and the need for higher FiO2. Interestingly, this enlargement of the bronchial circulation is similar to that described in adults with emphysema, chronic atelectasis, and/or high PVR, again supporting the notion that obstruction to blood flow in the pulmonary circulation is a significant stimulus for growth of the bronchial circulation (see above). Collateral vessels have been associated with high mortality in some patients with severe BPD who also had severe pulmonary hypertension. Some infants have improved after embolization of large collateral vessels, as reflected by a reduced need for supplemental oxygen, ventilator support, or diuretics. However, neither the actual contribution of systemic collateral vessels to the pathophysiology of BPD nor the cellular mechanisms driving their enlargement is known.

Finally, pulmonary hypertension and right heart function remain major clinical concerns in infants with BPD. However, it is now clear that pulmonary vascular disease in BPD also includes reduced pulmonary artery density owing to impaired growth, which contributes to physiologic abnormalities of impaired gas exchange, as well as to the actual pathogenesis of BPD (184, 185). As discussed below, experimental data support the hypothesis that impaired angiogenesis can impede alveolarization (186, 187) and that strategies preserving and enhancing endothelial cell survival, growth, and function may provide new therapeutic approaches for the prevention of BPD.

**Altered Signaling of Angiogenic Factors in Human BPD**

As described above, multiple growth factors and signaling systems have been shown to play important roles in normal lung vascular growth (7, 13) (Table 1). Several studies have examined how premature delivery and changes in oxygen tension, inflammatory cytokines, and other signals alter normal growth factor expression and signaling and thus lung/lung vascular development. The majority of studies have focused on VEGF. Impaired VEGF signaling has been associated with the pathogenesis of BPD in the clinical setting (184, 185). Bhatt and coworkers first demonstrated decreased lung expression VEGF and VEGFR-1 in the lungs of premature infants who died with BPD (184). In another study, VEGF was found
to be lower in tracheal fluid samples from premature neonates who subsequently develop BPD than those who do not develop chronic lung disease (185). Experimentally, hyperoxia down-regulates lung VEGF expression, and pharmacologic inhibition of VEGF signaling in newborn rats impairs lung vascular growth and inhibits alveolarization (188–191; see below). Thus the biologic basis for impaired VEGF signaling leading to decreased vascular growth and impaired alveolarization is well established. Additionally, lung VEGF expression is impaired in primate and ovine models of BPD induced by mechanical ventilation after premature birth, further supporting the hypothesis that impaired VEGF signaling contributes to the pathogenesis of BPD.

In addition, a role for TGF-β in the pathogenesis of BPD has been raised. For example, high levels of TGF-β inhibit lung morphogenesis, and increased levels of TGF-β-1 are found in tracheal fluid samples early in the course of infants developing BPD, which may predict high risk for BPD (192). Thus while TGF-β may be necessary for certain stages in normal lung development, excessive expression, particularly at the wrong time, may induce an inhibition of lung morphogenesis and cause progressive pulmonary fibrosis (193). In fact, overexpression of TGF-β-1 inhibits alveolar growth and remodeling during the neonatal period (194). These observations make TGF-β a particularly intriguing target when considering strategies to ameliorate the structural abnormalities that characterize BPD.

Ongoing studies of the regulation and activities of diverse growth factors are likely to lead to greater understanding of the pathobiology and treatment of BPD.

Relationship of Vascular Growth and Alveolarization

As described above, close coordination of growth between airways and vessels is essential for normal lung development. Thus we and others have hypothesized that failure of pulmonary vascular growth during a critical period of lung growth (saccular or alveolar stages of development) could decrease septation and ultimately contribute to the lung hypoplasia that characterizes BPD.

To determine whether angiogenesis is necessary for alveolarization, we studied the effects of the antiangiogenesis agents, thalidomide and fumagillin, on lung growth in the newborn and infant rat (187). In comparison with vehicle-treated controls, postnatal treatment with these inhibitors of angiogenesis reduced lung vascular density, alveolarization, and lung weight. These findings suggest that angiogenesis is necessary for alveolarization during lung development and that mechanisms that injure and inhibit lung vascular growth may impede alveolar growth after premature birth. Because VEGF has potent angiogenic effects during lung development and may synchronize vascular growth with neighboring epithelium, we also studied the effects of SU5416, a selective VEGF receptor inhibitor, on alveolarization during early postnatal life (191). Treatment of neonatal rats with a single injection of SU5416 caused pulmonary hypertension, reduced lung vascular density, and reduced alveolarization in infant rats (191) (Figure 4). Thus inhibition of lung vascular growth during a critical period of postnatal lung growth
Figure 4  Inhaled NO increases lung vascular and alveolar growth in rats treated with the VEGF receptor inhibitor, SU5416. The upper panels illustrate improved vascular growth after inhaled NO therapy of SU5416-treated rats. Inhaled NO improved pulmonary artery wall thickness, vascular growth, and alveolarization (radial alveolar counts, RAC) (88).

impairs alveolarization, suggesting that endothelial-epithelial cross talk, especially via VEGF signaling, is critical for normal lung growth following birth.

The mechanisms through which impaired VEGF signaling inhibits vascular growth and alveolarization are uncertain, but, in part, may be mediated by altered NO production. Past in vitro and in vivo studies have shown that VEGF stimulates endothelial NO synthase (eNOS) expression in isolated endothelial cells from the systemic circulation (195–201), but whether VEGF is an important regulator of lung eNOS expression, especially during development, and what role NO plays on lung growth are incompletely understood. In addition to its effects on vascular tone, NO can alter angiogenesis, but data are conflicting on its effects. Although NO inhibits endothelial cell proliferation in some models, most studies have shown that NO mediates the angiogenic effects of VEGF via activation of VEGFR-2 and stimulation of the Akt-PI3K pathway (196, 198–201). Proliferating bovine aortic endothelial cells express greater eNOS mRNA and protein than confluent cells, but NOS inhibition does not apparently affect their rate of proliferation. Studies with the eNOS−/− fetal mouse model suggest that NO plays a critical role in
vascular and alveolar growth in utero and that eNOS−/− newborns are more susceptible to hypoxia-induced inhibition of alveolarization (202–205). Interestingly, lung eNOS expression is down-regulated in primate and ovine models of BPD (206, 207). More recently, treatment of newborn rats with SU5416, the VEGF receptor antagonist, was shown to decrease eNOS expression and NO production during infancy, and that prolonged treatment with inhaled NO prevented the development of pulmonary hypertension, improved vascular growth, and enhanced alveolarization (208) (Figure 4). Previous studies have shown that inhaled NO attenuates hyperoxia-induced acute lung injury (209–211), which may enhance subsequent vascular and lung growth. These studies suggest that decreased VEGF signaling down-regulates lung eNOS expression, and that impaired NO production may contribute to abnormal lung growth during development. Importantly, a recent randomized single-center study has shown that inhaled NO treatment reduced the combined endpoint of BPD and death in human premature newborns with moderate RDS (212). Ongoing multicenter clinical trials are evaluating the potential efficacy of inhaled NO in reducing BPD in larger population studies.

MODELS OF BPD

For more than 20 years, exposure of newborn rats to hyperoxia has been used as a model to study mechanisms of BPD due to the effects of enriched oxygen on alveolarization and vascular growth. During the alveolar phase of lung development, the first 2 weeks of postnatal life in the rat, the lung also undergoes a striking proliferation of vascular growth. Exposure of newborn rats to either 100% or 60% FiO2 markedly decreases capillary density when compared with that of air-breathing controls (153, 175). During the room air recovery period after relatively short exposures to hyperoxia, the number of capillaries and alveoli tend to increase toward normal values in infant rats. However, if the period of oxygen exposure is more prolonged, the decrease in pulmonary vascular density persists despite room air recovery.

Although multiple mechanisms are likely involved in the arrest of lung growth after hyperoxia, several studies have demonstrated marked and sustained down-regulation of lung VEGF and VEGFR-2 expression, suggesting that impaired VEGF signaling contributes to these long-lived abnormalities in lung structure. Thus hyperoxic exposure provides a useful model for the study of basic mechanisms that disrupt normal pulmonary vascular growth and development.

Recently, the gene expression profile induced by prolonged oxidative stress has been studied. Using DNA-microarray analysis, which was then largely confirmed by real-time RT-PCR, Wagenaar et al. demonstrated that prolonged oxidative stress induces changes in a complex orchestra of genes involved in inflammation, coagulation, fibrinolysis, extracellular matrix turnover, cell cycle, signal transduction, and alveolar development (213). The changes in gene expression, both up-regulated as well as down-regulated, appear consistent with the pathologic...
changes in immature lungs developing a BPD-like picture. One of the major effects of hyperoxia on postnatal lung development in the rat is the reduction of secondary septation and the enlargement of alveoli. This phenomenon was accompanied by a down-regulation of FGFR-4 from day 3 onward and of the Flk-1 receptor later in the course (day 10). These observations are consistent with previous findings demonstrating that the lungs of FGFR-3\(^{-/-}\)/FGFR-4\(^{-/-}\) mice are normal at birth but then develop a complete block in alveologenesis and do not form secondary septa (214). Down-regulation of VEGFR-2 (or Flk-1) confirmed in this array analysis on day 10 coincided with the presence of enlarging alveoli and is consistent with the role of VEGF/VEGFR-2 signaling in the maintenance of alveolar structures (215). These findings provide further support for a critical role of VEGF/VEGFR-2 signaling in the pathogenesis of BPD.

Another set of genes up-regulated by hyperoxia is linked to the inflammatory response and is consistent with observations in the human as described above. The influx of leukocytes appears to be mediated by chemokines such as IL-8, CINC-1, and MIP-2 via the activation of the CXCR2 receptor. The Wagenaar study confirms the up-regulation of these molecules observed in other hyperoxic models and implicates them in the disease process. The importance of these chemokines in hyperoxia-induced lung injury is demonstrated by the fact that antichemokine treatment, which reduces neutrophil influx into the lung, preserved alveolar development in newborn rats exposed to hyperoxia (216, 217).

Several studies have demonstrated that extravascular fibrin deposits are frequently observed in the septa and alveoli of infants with BPD. Again, in the hyperoxic model described, gene array analysis demonstrates the up-regulation of the procoagulant tissue factor (TF), down-regulation of the anticoagulant thrombomodulin (TM), and up-regulation of the fibrinolytic inhibitor, PAI-1. These changes would lead to a procoagulant and antifibrolytic environment resulting in fibrin deposition. The significance of PAI-1 in hyperoxia-induced fibrin deposition has been demonstrated in PAI-1-deficient mice, which fail to develop intra-alveolar fibrin deposits and show a less severe phenotype in response to hyperoxia-induced injury (218). The accumulation of fibrin can contribute to injury in several ways. It can function as a ligand for receptors on circulating leukocytes, including the integrin Mac-1, and can promote both their migration and activation (219). Fibrin can also activate and induce proliferation and migration of fibroblasts, probably via activation of NF-κB and AP-1 (220). Therefore, unwanted activation of leukocytes and fibroblasts can contribute to the abnormalities of lung vascular growth that characterize BPD.

The combination of rat models and gene array analysis thus provides important new insights into the mechanisms through which interrupted fetal lung development can lead to significant abnormalities in postnatal pulmonary vascular and lung development.

Extensive work from the Bland laboratory has shown that prolonged mechanical ventilation of premature lambs after cesarean-section delivery at 120–130 days...
gestation (term 147 days) for 2–3 weeks causes a chronic lung disease (CLD) that shares many features of human BPD (221). In addition to airway abnormalities, pulmonary vascular growth, structure, and function are impaired after chronic ventilation. In comparison with controls, CLD lambs have reduced pulmonary artery density with reduced alveolarization and hypertensive pulmonary artery remodeling. Although the mechanisms of altered lung growth are unclear, lung eNOS and soluble guanylate cyclase expression are reduced in CLD lambs, suggesting that impaired NO-cGMP signaling may contribute to pulmonary vascular disease in this model.

Primate Model of BPD

An extremely important model of BPD in primates has been developed under the guidance of Coalson (141). In this model, premature baboons are delivered by cesarean section at 140 days (or 0.75 term) and treated with mechanical ventilation with high FiO₂ (222). These animals develop severe chronic lung disease, with histologic lesions that mimic human BPD (222). A newer and perhaps more relevant model for the new BPD is one in which extremely premature baboons are delivered at 125 days (or 0.67 term; which is roughly equivalent to human gestation of 26–27 weeks) and are treated with exogenous surfactant and mechanical ventilation, but are managed with lower (as needed) levels of supplemental oxygen. These animals develop structural lesions characterized by severe alveolar hypoplasia with decreased and dysmorphic vascular growth. As in the premature lamb model of BPD, lung eNOS expression is impaired (206). In addition, lung VEGF mRNA content is markedly decreased, and the localized epithelial pattern of VEGF expression is absent (223). Expression of mRNA for VEGFR-1 also decreases by 30–40% in treated animals but the VEGFR-2 mRNA expression remained unchanged. Whether interventions such as inhaled NO or VEGF treatment can reduce the abnormalities of lung vascular growth and impaired alveolarization in this model is uncertain.

CONCLUSIONS

Past studies have primarily focused on how altered lung vascular growth and development contributes to pulmonary hypertension. Recently, basic studies of vascular growth have led to novel insights into mechanisms underlying development of the normal pulmonary circulation and the essential relationship of vascular growth to lung alveolar development. These observations have led to new concepts underlying the pathobiology of developmental lung disease, especially the inhibition of lung growth that characterizes BPD. We speculate that understanding basic mechanisms that regulate and determine vascular growth will lead to new clinical strategies to improve the long-term outcome of premature babies with BPD.
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Figure 2  Radiographic and histologic features of bronchopulmonary dysplasia (BPD). This chest radiograph demonstrates late features of BPD, including marked hyperinflation (upper left panel). Pulmonary angiography illustrates striking pruning of the pulmonary circulation from this patient, who also had severe pulmonary hypertension (lower left panel). Marked fibroproliferative changes with interstitial thickening and decreased septation are shown (right panels). Abnormal vascular growth, as reflected by factor VIII staining of endothelium, is shown in the lower right panel.
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