VEGF causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice

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Le Cras, T. D., R. E. Spitzmiller, K. H. Albertine, J. M. Greenberg, J. A. Whitsett, and A. L. Akeson. VEGF causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. Am J Physiol Lung Cell Mol Physiol 287:L134–L142, 2004.—To determine whether increased levels of VEGF disrupt postnatal lung formation or function, conditional transgenic mice in which VEGF 164 expression was enhanced in respiratory epithelial cells were produced. VEGF expression was induced in the lungs of VEGF transgenic pups with doxycycline from postnatal day 1 through 2 and 6 wk of age. VEGF levels were higher in bronchoalveolar lavage fluid (BALF) and lung homogenates of VEGF transgenic mice compared with endogenous VEGF levels in controls. Neonatal mortality was increased by 50% in VEGF transgenic mice. Total protein content in BALF was elevated in VEGF transgenic mice. Surfactant protein B protein expression was unaltered in VEGF transgenic mice. Although postnatal alveolar and vascular development were not disrupted by VEGF expression, VEGF transgenic mice developed pulmonary hemorrhage, alveolar remodeling, and macrophage accumulation as early as 2 wk of age. Electron microscopy demonstrated abnormal alveolar capillary endothelium in the VEGF transgenic mice. In many locations, the endothelium was discontinuous with segments of attenuated endothelial cells. Large numbers of hemosiderin-laden macrophages and varying degrees of emphysema were observed in adult VEGF transgenic mice. Overexpression of VEGF in the neonatal lung increased infant mortality and caused pulmonary hemorrhage, hemosiderosis, alveolar remodeling, and inflammation in chronic lung disease of infancy; bronchopulmonary dysplasia; respiratory distress syndrome; sudden infant death syndrome; emphysema; surfactant protein B.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF-A) plays a vital role in vascular development, stimulating both angiogenesis and vasculogenesis (20). VEGF is both a proangiogenic and survival factor for endothelial cells (20, 22, 46). Removal of VEGF causes endothelial cell apoptosis in vitro and in vivo (4, 32, 46). Chemotaxis and vascular permeability effects of VEGF on endothelial cells may be mediated through several intracellular signaling pathways (7, 46). VEGF can have a direct vascular effect, stimulating vasodilation in the fetal lung through activation of phosphatidylinositol-3-kinase and NO release (27). In addition to its effects on endothelial cells, VEGF can also stimulate activation and migration of monocytes (14).

Several VEGF-A isoforms are generated by alternative splicing from a single gene (44). Differential expression of these isoforms reflects distinct functions during vascular development (39). The larger isoforms, including VEGF 206, 188, and 164, bind to heparin proteoglycans and extracellular matrix, and are not as freely diffusible as the smaller VEGF 120 isoform. VEGF 164 has intermediate properties and can diffuse, as well as bind to, heparin proteoglycans (22). The differential roles of the VEGF isoforms have been investigated in mice in which the larger heparin-binding VEGF 188 and 164 isoforms were deleted, leaving only VEGF 120 expression (24). The VEGF 120 mice developed to term but died at birth with abnormalities in pulmonary vascular development (24), indicating the developmental importance of the larger VEGF isoforms.

A number of clinical studies in humans have associated reductions in VEGF with bronchopulmonary dysplasia (BPD) (8, 34, 35). VEGF was reduced in lung aspirates of newborns with respiratory distress syndrome (RDS) (8) and in lung tissue from infants who died with BPD (34, 35). The role of VEGF after birth was explored in a study by Gerber et al. (25), in which the VEGF gene was conditionally deleted or newborn mice were treated with a soluble VEGF receptor. In both cases, the mice failed to thrive, and morphogenesis in many organs, including the lung, was altered. In a recent study, treatment of newborn rats with a VEGF receptor inhibitor disrupted postnatal lung development, causing alveolar simplification, reduced vascular morphogenesis, and pulmonary hypertension (37). When adult rats were treated with the same VEGF receptor inhibitor, apoptosis of endothelial and epithelial cells resulted in loss of small pulmonary arteries, emphysema, and pulmonary hypertension (32, 42). These experimental studies suggest an important role for VEGF in postnatal lung development and for normal endothelial cell function and vascular maintenance after morphogenesis is complete.

VEGF gene expression is controlled by oxygen tension through the binding of hypoxia-inducible factors (HIF) to hypoxia response elements in the VEGF gene promoter (19, 23). Deletion of the HIF-2α gene was recently shown to cause RDS and death at birth in mice (15). HIF-2α null mice had reduced VEGF levels, and deletion of the hypoxia response element in the VEGF gene also caused RDS (15). In this study, premature mouse pups with RDS were treated with VEGF. Intratracheal VEGF treatment improved short-term survival, which led the authors to suggest that VEGF treatment might be a potential therapy of RDS in premature infants (15). However, the effects of more prolonged increases in VEGF in the newborn lung are unclear.

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Chronic and conditional overexpression of VEGF in the prenatal lung disrupted distal air space and pulmonary vascular development, causing death at birth (2, 47). In the adult lung, transfection of VEGF using an adenoviral vector caused pulmonary edema (31). The goal of the present study was to determine whether chronic in term newborn pups. In addition, surfactant protein B (SP-B) expression was examined to determine whether chronic increases in VEGF increase SP-B expression in the neonatal lung.

MATERIALS AND METHODS

Animals. All animal procedures and protocols were approved by the Animal Care and Use Committee at the Cincinnati Children’s Hospital Research Foundation (Cincinnati, OH). All mice were FVB/N strain. An inducible or conditional transgenic system was used to induce VEGF 164 expression in the lungs of newborn mice (2). Generation and characterization of the (tetO)7 -VEGF transgenic mice were previously described (2). To generate mice for this study that have the conditional lung-specific transgenic system, we used two different transgenic mouse lines: 1) “activator” mice (CCSP-rtTA) expressing the reverse tetracycline transactivator transcription factor (rtTA) under the control of a lung-specific Clara cell secretory protein (CCSP) gene promoter (43) and 2) “responder” transgenic mice [(tetO)7-VEGF], in which a mouse transgene coding for the 164 amino acid isoform of VEGF-A is under the control of tetracycline response elements [(tetO)7]. To generate mouse pups for this study, homozygous CCSP-rtTA+/+ mice were mated to heterozygous (tetO)7-VEGF+/− mice. The resulting litters contained bitransgenic [CCSP-rtTA+/−, (tetO)7-VEGF+/−] and single transgene control (CCSP-rtTA+/−) mice. We induced VEGF in the lungs of bitransgenic pups [CCSP-rtTA+/−, (tetO)7-VEGF+/−] by giving the nursing dams chow and drinking water containing doxycycline (Dox; 0.625 mg/kg and 1 mg/ml, respectively) from postnatal day 1 and thereafter (40). Bitransgenic mice [CCSP-rtTA+/−, (tetO)7-VEGF+/−] exposed to Dox are hereafter referred to as VEGF transgenic mice. Single transgene (CCSP-rtTA+/−) littersmates exposed to Dox and bitransgenic mice [CCSP-rtTA+/−, (tetO)7-VEGF+/−] not exposed to Dox were used as controls. All mice were genotyped by PCR analysis of tail DNA for CCSP-rtTA (40) and (tetO)7-VEGF transgenes (2). Mice

Fig. 1. Conditional expression of VEGF in the lungs of postnatal mice. A: VEGF was measured in lung homogenates from 2-, 3-, 8-, and 14-day-old pups. Dams were placed on doxycycline (Dox) from postnatal day 1 to 14. VEGF transgenic mice [(CCSP-rtTA)+/− (tetO)7-VEGF+/−, n = 4–8] and single transgene control (CCSP-rtTA+/−, n = 4–7) littersmates were all exposed to Dox. VEGF-A levels in lung homogenates were normalized to total lung protein and then expressed as a percentage of control values. *P < 0.05 vs. control littermates. Levels of VEGF in lung homogenates were increased after 2 days of Dox and then remained elevated after 7 and 13 days of Dox exposure. B: VEGF was measured by an ELISA in bronchoalveolar lavage fluid (BALF) from 2-wk-old mice. Dams were exposed to Dox from postnatal day 1 to 14. VEGF transgenic mice (n = 8) and control (n = 6) littermates. Levels of VEGF in BALF were increased 14.6-fold in VEGF transgenic mice. *P < 0.05 vs. control littermates. C: VEGF was measured by an ELISA in lung homogenates from 2-wk-old mice. Dams were exposed to Dox from postnatal day 1 to 14. VEGF-A levels (pg) in lung homogenates were normalized to total lung protein (mg). Results are from VEGF transgenic mice [(CCSP-rtTA)+/− (tetO)7-VEGF+/−, n = 8] and single transgene control (CCSP-rtTA+/−, n = 6) littersmates. Levels of VEGF in lung homogenates were increased by 30% in VEGF transgenic mice. *P < 0.05 vs. control littermates.
were killed by a pentobarbital sodium (26%) euthanasia solution (Fort Dodge Animal Health, Fort Dodge, IA).

**VEGF and total protein measurements in bronchoalveolar lavage fluid and lung homogenates.** To determine concentrations of VEGF induced in the VEGF transgenic mice, we treated litters of mice with Dox from postnatal day 1 to 14. Mice were genotyped on postnatal day 1, and the surviving pups were genotyped again on day 14. When dead mice were recovered, tail DNA was also removed for genotyping. Nursing dams were given Dox from postnatal day 1 to 14 so that both single transgene controls and VEGF transgenic littermates were exposed to Dox. Cumulative survival from a Kaplan Meier analysis of the mortality data is shown. By 2 wk of age, there was a 50% increase in mortality in VEGF transgenic pups compared with controls [Logrank (Mantel-Cox) test, \( P < 0.001 \)].

**Lung histology and histochemical analysis.** Lungs were inflation fixed by tracheal installation of 4% paraformaldehyde in PBS (pH 7.4) at constant pressure (25 cmH\(_2\)O). After 24 h, lungs were transferred to 70% ethanol. After another 24 h, the lungs were cut into 1-mm sections starting at the point where the bronchus enters the left lung. Three 1-mm sections per animal were embedded in paraffin. Five-micrometer-thick paraffin sections were cut and stained with hematoxylin and eosin. Gomori's modified iron stain (Prussian blue) (26, 41) was performed on sections to detect cells that had accumulated free iron, which is indicative of hemosiderosis. For Gomori's modified iron stain, sections were hydrated and immersed in 10% potassium ferrocyanide followed by equal volumes of potassium ferrocyanide and 20% hydrochloric acid. After being washed in distilled water, sections were counterstained with nuclear fast red, dehydrated, and mounted. At least three lung sections were examined from each animal.

**Western blot analysis.** To quantitate levels of the endothelial marker proteins platelet endothelial cell adhesion molecule (PECAM) and Tie2, we performed protein blot analysis by standard methods previously described (36). Polyclonal antibodies to PECAM (CD31; Santa Cruz Biotechnology, Santa Cruz, CA) and Tie2 (Santa Cruz Biotechnology) were diluted 1:500 and 1:2,000, respectively. After chemiluminescent detection, the blots were stripped and reprobed with an antibody to actin to control for protein loading and transfer.
levels in BALF and lung homogenates were similar in control and VEGF transgenic mice. Levels of SP-B in lung homogenates were normalized to actin levels. SP-B for actin. Levels of SP-B protein were determined after scanning densitometry. The levels of SP-B in lung homogenates were normalized to actin levels. SP-B levels in BALF and lung homogenates were similar in control and VEGF transgenic mice ($P > 0.05$).

Densitometry was performed with a scanner and ImageQuant (Molecular Dynamics, Sunnyvale, CA). Densitometry values for PECAM and Tie2 were normalized to the values for actin. For detection of dimeric SP-B, Western blots were generated using 10 μg of lung protein were separated under nonreducing conditions (6). SP-B was detected with a rabbit polyclonal antibody diluted 1:5,000 (Chemicon, Temecula, CA).

**Immunohistochemical staining for PECAM and Mac3.** Immunostaining for PECAM (CD31) was performed to assess pulmonary vascular structure. PECAM was detected with an anti-CD31 monoclonal antibody (clone MEC13.3; Pharmingen, San Diego, CA) as previously described (36). Immunostaining of macrophages was performed with a rat monoclonal antibody to mouse Mac3 (Pharmingen) by standard techniques. Sections were lightly counterstained with nuclear fast red before dehydration and mounting.

**Electron microscopy.** Lungs of 2-wk-old control and VEGF transgenic mice were analyzed by transmission electron microscopy. Briefly, after the trachea was cannulated, lungs were inflation-fixed with 2.5% glutaraldehyde-1% paraformaldehyde in buffer (pH 7.4, 310 mosM/kgH$_2$O, 4°C for 24 h) in situ at 25-cmH$_2$O pressure (3). Principles of systematic, uniform, random sampling were used to collect tissue blocks from all lobes (1 mm$^3$, 8–10 per lobe) (10). The tissue blocks were postfixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and infiltrated and embedded in epoxy resin. Thin sections (80-nm thickness) were cut with the aid of a diamond knife and counterstained with uranyl acetate and lead citrate. A Philips Tecnai 12 transmission electron microscope was used to observe and photograph the thin sections. Cross sections of capillary profiles were photographed at the same magnification in the upper left corner of each grid square for an entire thin section per tissue block. Thin sections from four tissue blocks per lobe were photographed.

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was performed with the Statview software package (Abacus Concepts, Berkeley, CA). Statistical comparisons were made using analysis of variance, Fisher’s protected least significant differences test, and regression analysis. Kaplan Meier survival analysis and the Logrank (Mantel-Cox) test were performed on the neonatal mortality data. $P < 0.05$ was considered significant.

## RESULTS

**Conditional expression of VEGF.** To characterize the time course of Dox-induced VEGF expression in VEGF transgenic mice, we measured VEGF protein levels in lung homogenates and BALF by ELISA. Litters of mice containing control and VEGF transgenic mice were treated with Dox from postnatal days 1 to 2, 1 to 3, 1 to 8, and 1 to 14. Levels of VEGF in lung homogenates from VEGF transgenic mice were increased 60% after 2 days of Dox treatment and then remained elevated with 7 and 13 days of Dox treatment (Fig. 1A). VEGF levels in BALF from 2-wk-old VEGF transgenic mice were 14.6-fold higher than in BALF from control littermates (Fig. 1B). Total VEGF levels were 30% higher in the lung tissue of 2-wk-old VEGF transgenic mice compared with endogenous VEGF levels in the lung tissue of control mice (Fig. 1C).

**Increased mortality in infant VEGF mice.** To determine whether induction of VEGF in the lungs of infant mice increased neonatal mortality, we placed 10 litters of mice on Dox from postnatal day 1 to 14. Litters contained control and VEGF transgenic mice. Mice were genotyped on postnatal day 1 and 14, and tails were also removed from dead mice for genotyping. By 2 wk of age, 22 of 39 VEGF transgenic pups (56%) had died vs. 3 of 46 single transgenic controls (6%). The three control pups that died were from large litters and showed a failure to thrive consistent with runting. This mortality rate is common when litters contain eight or more pups and is seen in litters from wild-type, as well as transgenic, mice. Kaplan Meier analysis was performed on the mortality data from the 10 Dox-treated litters and a Logrank (Mantel-Cox) test showed that there was a 50% increase in mortality in the VEGF transgenic mouse pups compared with controls ($P < 0.001$) (Fig. 2).

**VEGF increased BALF protein.** Total protein levels were measured in BALF from 2-wk-old control ($n = 7$) and VEGF transgenic ($n = 8$) mice. Dams were treated with Dox from day 1 to 14. A: total protein levels were 6.2-fold higher in BALF from 2-wk-old VEGF transgenic mice vs. controls ($P < 0.05$). B: regression analysis showed that protein concentrations in BALF were positively correlated with VEGF levels.
Pulmonary vascular development in VEGF transgenic mice. Because VEGF is a proangiogenic factor, pulmonary vascular development was assessed in VEGF transgenic mice. PECAM immunostaining, which detects the vascular endothelium, and Western blot analysis on endothelial marker proteins were performed to determine whether conditional overexpression of VEGF in the infant lung altered pulmonary vascular development. PECAM immunostaining was performed on lung sections from mice exposed to Dox from postnatal day 1 to 14. A normal pattern of PECAM staining was seen in VEGF transgenic [CCSP-rtTA/+/(tetO)-VEGF/+] mice compared with control pups, indicating that pulmonary vascular development was normal (Fig. 3A). Western blot analysis of endothelial marker proteins PECAM and Tie2 did not detect any differences in their concentrations in lung homogenates from VEGF transgenic and control mice (Fig. 3B, P > 0.05).

SP-B protein expression was not altered in VEGF transgenic mice. Recently, Compernolle et al. (15) reported that exogenous VEGF treatment induced surfactant protein expression in the lungs of preterm mouse pups and in type II cells in culture. To determine whether VEGF increases surfactant protein expression in the lungs of term mouse pups and in type II cells in culture. To determine whether VEGF increases surfactant protein expression in the lungs of term mouse pups, SP-B protein levels were measured in BALF and lung homogenates of VEGF transgenic mice. SP-B protein levels were measured in BALF and lung tissue homogenates from VEGF transgenic and control mice treated with Dox from postnatal days 1–14. No differences in SP-B concentrations were detected in BALF or lung homogenates from VEGF transgenic mice compared with controls (Fig. 4, P > 0.05).

Vascular leak, pulmonary hemorrhage, inflammation, hemosiderosis, and alveolar remodeling in VEGF transgenic mice. Because VEGF can increase vascular leak, total protein was measured in BALF and lung histology was performed on VEGF transgenic and control mice following Dox activation. Control and VEGF transgenic mice were treated with Dox from postnatal day 1 to 14. Consistent with the increase in VEGF levels, total protein levels were 6.2-fold higher in BALF from 2-wk-old VEGF transgenic mice vs. controls (P < 0.05), indicating vascular leak (Fig. 5A). Protein concentrations in BALF were positively correlated with VEGF concentrations (P < 0.05) (Fig. 5B). At 2 wk of age, histology showed that there was evidence of pulmonary hemorrhage in the VEGF transgenic mice (Figs. 6 and 7). Hemosiderin-laden macrophages were observed in mice with severe pulmonary hemorrhage (Fig. 6). Accumulation of macrophages and air space remodeling were also observed in the VEGF transgenic mice (Fig. 7). VEGF transgenic mice treated with Dox had histological evidence of pulmonary hemosiderosis and emphysema.

Fig. 6. VEGF caused pulmonary hemorrhage and hemosiderosis. Lung histology from control (left) and VEGF transgenic (center and right) mice was assessed at 2 wk of age. Dams were placed on Dox from postnatal day 1 to 14. Hematoxylin and eosin (H&E) stains of lung sections are shown at left and center. Areas of extensive pulmonary hemorrhage and accumulation of serum proteins in the distal air spaces were seen in VEGF transgenic mice. Panel on the right is a high-power view of Gomori’s modified iron stain of the same lung tissue showing hemosiderin-containing macrophages (blue stain, arrows) and intracellular erythrocytes (yellow, arrowheads). Scale bar = 100 μm (left and center) and 50 μm (right).

Fig. 7. VEGF caused inflammation and air space remodeling. Lung histology of 2-wk-old mice, control (left) and VEGF transgenic (center, right), on Dox from postnatal day 1 to 14. H&E stains of sections are shown at low power (left, center) and high power (right). Cell infiltrates and remodeling were observed in VEGF-expressing mice. *Areas of hemorrhage, inflammatory cell infiltrates, and remodeling (center). Arrows, inflammatory cells; arrowheads, areas of early remodeling (right). Scale bar = 100 μm.
ranging from mild to severe (40%) when killed at 6 wk of age (Figs. 8 and 9), suggesting that surviving VEGF transgenic mice had experienced sublethal but chronic vascular leak. Immunostaining for Mac3 confirmed that hemosiderin-laden cells were macrophages (Fig. 10), many of which were clustered around vessels and along alveolar septae (Figs. 8–10). PECAM immunostaining showed endothelial cells in the alveolar septae (Fig. 10), although there appeared to be a paucity of staining in alveolar septae that were immediately adjacent to large accumulations of hemosiderin-laden macrophages.

**Gaps in the capillary endothelium of VEGF transgenic mice.** Electron microscopy of lungs from 2-wk-old mice showed normal ultrastructural appearance of alveolar capillaries in control mice. The capillaries were lined by a continuous layer of endothelial cells (Fig. 11). By contrast, alveolar capillaries in the VEGF transgenic mice were not normal (Fig. 11). Segments of endothelial cells in the lungs of the VEGF transgenic mice were attenuated, and in many locations, the endothelium was discontinuous. The discontinuities were visible as beads of cytoplasm interrupted by small or large gaps.

**DISCUSSION**

A conditional lung-specific transgenic system was used to increase VEGF expression in the lungs of neonatal mice. Although chronic increases in VEGF did not alter postnatal lung morphogenesis or SP-B expression, vascular leak and pulmonary hemorrhage were observed in VEGF transgenic mice, resulting in a 50% increase in neonatal mortality. In addition to alveolar hemorrhage, evidence of inflammation, air space remodeling, and pulmonary hemosiderosis were observed as early as 2 wk of age. In VEGF transgenic mice that survived to adulthood, pulmonary hemosiderosis and emphysema were observed. Electron microscopy showed that the endothelium of alveolar capillaries in VEGF transgenic mice was abnormal and discontinuous.

In the present study, increased VEGF levels caused a sixfold increase in BALF protein levels and pulmonary hemorrhage in neonates. Half of the VEGF transgenic mice died by 2 wk of age, most likely due to pulmonary hemorrhage, since the lungs of VEGF transgenic mice euthanized because of respiratory distress had gross blood (data not shown). In VEGF mice that survived to 2 wk of age, pulmonary hemorrhage, as well as the early signs of pulmonary hemosiderosis, was observed. Pulmonary hemosiderosis and emphysema were seen in varying degrees in the VEGF transgenic mice that survived beyond 2 wk into adulthood. This is likely due to variability in the severity of vascular leak and pulmonary hemorrhage induced by VEGF or to the host response to the leak/hemorrhage. Modifier genes are now recognized as playing an important role in the pathological responses to injurious stimuli and also single gene defects. For example, patients with cystic fibrosis show a large spectrum of disease severity and clinical course,
even though a defect in a single gene, the CFTR, is considered to be the prime initiating factor (1).

A number of potential mechanisms may have contributed to VEGF-induced increases in vascular permeability in this study, including formation of caveolae, vesicular-vacuolar organelles, and fenestrae (16, 33, 46). Electron microscopy showed that there were gaps in the endothelium of capillaries in the alveolar septae of VEGF transgenic mice. Molecular mechanisms accounting for the effects of VEGF in the present study may include Src-mediated signaling and increases in nitric oxide and prostacyclin that have been suggested to mediate the effects of VEGF on vascular permeability (46).

Fig. 10. Immunohistochemical detection of Mac3 and PECAM in lungs of adult VEGF-expressing mice. Lung histology of adult VEGF transgenic mice exposed to Dox from postnatal day 1 through 6 wk of age. Iron in hemosiderin-laden macrophages around a vessel (left), Mac3 macrophage-specific immunostaining shows that these are macrophages (center), and PECAM immunostaining shows endothelial cells in alveolar septae and a paucity of PECAM staining around an area where there is a large aggregate of hemosiderin-laden macrophages (right). Scale bar = 50 μm.

Fig. 11. Electron microscopy of VEGF transgenic mice shows gaps in capillary endothelium. Transmission electron micrographs of alveolar capillaries (Cap) in 2-wk-old mouse lungs. A: wild-type mouse lung. The capillary profile is normal, meaning that it is lined by continuous endothelium. B–D: VEGF transgenic mice lungs. Capillary profiles are abnormal. Portions of the endothelial lining are attenuated (B); have multiple, short discontinuities that give a beaded appearance (C); or have large gaps (D, arrows). All micrographs are at the same magnification. Scale bar = 1 μm. Alv, alveolus.
Pulmonary hemosiderosis is defined as an abnormal accumulation of hemosiderin in the lungs, which results from diffuse alveolar hemorrhage (9, 38). Occasional hemosiderin-positive macrophages were seen in the lungs of VEGF transgenic mice as early as 2 wk of age. However, by 6 wk of age, severe pulmonary hemosiderosis was seen in a number of VEGF transgenic mice that had survived. Emphysema was also observed in VEGF-expressing mice that survived and reached adulthood. This was usually associated with pulmonary hemosiderosis. The pathogenesis of emphysema in this model is unclear. Pediatric cases of pulmonary hemosiderosis are uncommon but have been described in scimitar syndrome associated with severe pulmonary hypertension (11), with hyperreactivity to cow’s milk protein (Heiner’s syndrome) (28), in association with fungal exposure (18), following exposure to toxic chemical agents (13), and also in some patients who died from sudden infant death syndrome (17). Pulmonary hemosiderosis can also be secondary to cardiac diseases, bleeding disorders, collagen vascular diseases, or systemic vasculitis (9, 38). Primary or idiopathic pulmonary hemosiderosis accounts for the majority of pediatric cases (38), although the pathogenesis and causes of the disease remain unclear. Whether VEGF is involved in the pathogenesis of pulmonary hemosiderosis in these clinical conditions is at present unknown.

Increased expression of VEGF has been associated with several respiratory diseases. VEGF levels were increased in the induced sputum of patients with bronchitis and airflow limitation (30). Increased vascularity in the airway mucosa of patients with asthma correlated with increased VEGF and VEGF receptor mRNA (29), and VEGF was increased in induced sputum from patients with asthma (5). In the Hoshino et al. (29) study, macrophages, eosinophils, and CD34+ cells were the major sources of increased VEGF mRNA. Increased VEGF mRNA and protein were detected in activated alveolar macrophages, epithelial cells, and in the multinuclear giant cells of granulomas in patients with pulmonary sarcoidosis (45). Hence, several studies have associated airway remodeling with increases in VEGF. In the present study, clusters of inflammatory cells and air space remodeling were observed as early as 2 wk of age. Whether the associated inflammation is due to the effects of VEGF on monocyte activation and chemotaxis (14, 46) or is related to the presence of red blood cells in the alveolar space is unclear.

VEGF treatment was recently shown to improve short-term survival in a subgroup of prematurely delivered mice with RDS, which was attributed to increases in surfactant production and accelerated septal thinning (15). Compernelle et al. (15) also reported that VEGF increased SP-B mRNA levels in cultured rat type II epithelial cells and surfactant production in premature mouse pups. In the present study, lung SP-B protein levels were unchanged in VEGF-expressing mice, despite a 14-fold increase in VEGF in BALF. However, our study did not evaluate the effects of VEGF in the premature lung, and SP-B levels were evaluated after chronic, not acute, increases in VEGF expression.

Local concentrations of VEGF in the lung and other organs are under stringent control. Deletion of even one allele of the VEGF gene is fetal lethal (12, 21), and even relatively modest increases in VEGF may lead to undesirable increases in vascular permeability. Conditional expression of VEGF in the neonatal lung increased neonatal mortality and led to vascular leak and pulmonary hemorrhage. Pulmonary hemosiderosis and emphysema were observed in VEGF-expressing mice that survived into adulthood. Therapeutic delivery of VEGF to the air spaces of newborns should be approached cautiously, since VEGF can increase vascular leak and cause pulmonary hemorrhage and hemosiderosis.

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