Recombinant human VEGF treatment transiently increases lung edema but enhances lung structure after neonatal hyperoxia

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Abstract

Bronchopulmonary dysplasia; lung development; vascular endothelial growth factor; lung injury

 recent studies suggest that VEGF may worsen pulmonary edema during acute lung injury (ALI), but, paradoxically, impaired VEGF signaling contributes to decreased lung growth during recovery from ALI due to neonatal hyperoxia. To examine the diverse roles of VEGF in the pathogenesis of and recovery from hyperoxia-induced ALI, we hypothesized that exogenous recombinant human VEGF (rhVEGF) treatment during early neonatal hyperoxic lung injury may increase pulmonary edema but would improve late lung structure during recovery. Sprague-Dawley rat pups were placed in a hyperoxia chamber (inspired O2 fraction 0.9) for postnatal days 2–14. Pups were randomized to daily intramuscular injections of rhVEGF165 (20 μg/kg) or saline (controls). On postnatal day 14, rats were placed in room air for a 7-day recovery period. At postnatal days 3, 14, and 21, rats were killed for studies, which included body weight and wet-to-dry lung weight ratio, morphometric analysis [including radial alveolar counts (RAC), mean linear intercepts (MLI), and vessel density], and lung endothelial NO synthase (eNOS) protein content by Western blot analysis. Compared with room air controls, hyperoxia increased pulmonary edema by histology and wet-to-dry lung weight ratios at postnatal day 3, which resolved by day 14. Although treatment with rhVEGF did not increase edema in control rats, rhVEGF increased wet-to-dry weight ratios in hyperoxia-exposed rats at postnatal days 3 and 14 (P < 0.01). Compared with room air controls, hyperoxia decreased RAC and increased MLI at postnatal days 14 and 21. Treatment with VEGF resulted in increased RAC by 181% and decreased MLI by 55% on postnatal day 14 in the hyperoxia group (P < 0.01). On postnatal day 21, RAC was increased by 176% and MLI was decreased by 58% in the hyperoxia group treated with VEGF. rhVEGF treatment during hyperoxia increased eNOS protein on postnatal day 3 by threefold (P < 0.05). We conclude that rhVEGF treatment during hyperoxia-induced ALI transiently increases pulmonary edema but improves lung structure during late recovery. We speculate that VEGF has diverse roles in hyperoxia-induced neonatal lung injury, contributing to lung edema during the acute stage of ALI but promoting repair of the lung during recovery.

Brachopulmonary dysplasia; lung development; vascular endothelial growth factor; alveolarization; angiogenesis

The mechanisms that impair lung growth and cause persistent abnormalities in lung structure in premature infants with BPD remain poorly understood. Recent studies have shown that disruption of angiogenesis, in particular the VEGF pathway, plays a crucial role in the pathogenesis of BPD. Neonatal treatment with antiangiogenesis agents such as thalidomide and fumagillin as well as VEGF receptor inhibitors decreases vascular growth and decreases alveolarization (18, 25). In addition, recent studies have shown decreased VEGF levels in BPD (17). Clinical studies further support the role of impaired VEGF signaling in the pathogenesis of BPD (4, 24). These findings suggest that angiogenesis is necessary for normal alveolarization and that impaired VEGF signaling during infancy may contribute to abnormal lung growth.

Recent animal studies have shown that hyperoxia exposure in the newborn period causes lung structural changes that are similar to the lung histology of human BPD (36, 42, 43), including decreased alveolarization and vascular growth (36, 37, 42, 43). The mechanisms by which hyperoxia contributes to arrested lung growth remain unclear. However, previous studies in adult rats and newborn rabbits have demonstrated that hyperoxia downregulates lung VEGF expression (22, 30) and that lung VEGF expression remains decreased despite

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VEGF increases lung edema but improves lung structure after hyperoxia

Methods

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased (Harlan Laboratories, Indianapolis, IN) and maintained at Denver’s altitude (1,600 m; barometric pressure 630 mmHg; inspired oxygen pressure 122 mmHg) for at least 1 wk before giving birth. Pups were delivered naturally at term gestation. Litter size was standardized to 10 pups. Animals were fed ad libitum and exposed to day-night cycles alternately every 12 h throughout the study period.

Study Design

Four study groups were created, with 10 animals in each group at each time point. This resulted in five animals in each study group at each time point for each particular analysis. All pups were maintained in room air for the first 24–36 h of life to allow successful transition to postnatal life before randomization to study groups. On postnatal day 1, half of the animals were placed in 90% oxygen and half remained in room air for the next 13 days. At this point animals were also randomly assigned to treatment with rhVEGF or saline. The vehicle for rhVEGF was saline. Half the animals in each litter were treated with daily intramuscular injections of rhVEGF165 (20 μg/kg), and half were treated with normal saline daily for a total of 13 days from postnatal day 1 through day 14. The doses of rhVEGF165 used in whole animal studies vary widely throughout the literature (3, 6, 16, 38). Daily doses as small as 0.22 μg/kg intra-arterially have been used in a rabbit model of hindlimb ischemia (16). In the same model, daily intramuscular doses of 1 and 2 mg have been used (3). Becit et al. (3) showed in rabbit hindlimb ischemia that less VEGF is required to achieve the same effect when administered intra-arterially compared with intramuscularly. None of these studies addressed effects of VEGF on tissues outside of the target tissues for the respective studies. The dose of rhVEGF165 we chose to use in the present study was based on a previous study from our laboratory showing improved lung structure without evidence of lung injury (23). This dose is in the low to middle range of doses used throughout the literature. On postnatal day 14, all animals were placed in room air for 7 days to allow a period of recovery in room air. The study groups included room air + saline (R+S), room air + VEGF (R+V), hyperoxia + saline (H+S), and hyperoxia + VEGF (H+V). The animals were killed on postnatal days 3, 14, and 21, and lung tissue was harvested for analysis (Fig. 1).

Fig. 1. Study design. On postnatal day 1, rat pups were exposed to hyperoxia or room air for 13 days. On postnatal day 14, all animals were placed in room air. Daily injections of recombinant human VEGF (rhVEGF) or normal saline (NS) were administered during postnatal days 1–13. All animals were allowed to recover in room air from postnatal days 14 through 21. FlO2, inspired O2 fraction.
VEGF INCREASES LUNG EDEMA BUT IMPROVES LUNG STRUCTURE AFTER HYPEROXIA

Study Measurements

Body weight, lung weight, lung weight-to-body weight ratio. Each animal was weighed before death. At autopsy, lungs were harvested from half the animals for frozen tissue. Before freezing, wet whole lung weights were recorded. Subsequently, the postcaudal lobe of the right lung was removed and weighed immediately. This lobe was subsequently weighed daily until the weights reached a plateau. This was then considered the dry weight and was recorded. The lungs of the remaining animals were fixed with paraformaldehyde.

Fixation of lung tissue. Rat lungs were prepared and fixed in situ at the time of death on postnatal day 21. A midline sternotomy was made to expose the lungs and heart. The left atrium was incised to allow drainage of perfusate. Phosphate-buffered saline (PBS) was then infused, and the right ventricle to flush the pulmonary circulation free of blood. The trachea was cannulated, and 4% paraformaldehyde was instilled into the lungs under constant pressure (20 cmH₂O) for 30–60 min. This inflation pressure was chosen based on a previous study (2, 14). The trachea was then ligated with the lungs under continued distending pressure. Lungs were removed and submerged in fixative for 24 h at room temperature.

Lung morphometric analysis. Transverse sections were obtained from the midplane of the anterior, middle, and posterior lobes of the formalin-fixed right lung for morphometric analysis. Sections from each animal were processed and embedded in paraffin wax. Paraffin sections (5 μm thick) were cut from each block and stained with hematoxylin and eosin. Analysis of each section was carried out in a blinded fashion. Alveolarization was assessed by performing radial alveolar counts (RAC) according to the method of Emery and Mithal (see Refs. 9, 10). From the center of the respiratory bronchiole a perpendicular was drawn to the edge of the acinus (as defined by a connective tissue septum or the pleura), and the number of septa intersected by this line was counted. Six counts were performed for each animal.

For assessment of the mean linear intercept (MLI), six lung sections were selected in an unbiased fashion. Images of each section were captured with a Magnafire digital camera through an Olympus IX 81 microscope and were saved as PICT files. The images were then analyzed with the use of Stereology Toolbox software (Morphometrics, Davis, CA). The mean interalveolar distance was measured as the MLI by dividing the total length of lines drawn across the lung section by the number of intercepts encountered, as described by Cooney and Thurlbeck (10).

Immunohistochemistry and vessel volume density. Immunohistochemistry for factor VIII was performed to identify vessels for morphometric assessment. Paraffin-embedded slides from paraformaldehyde-fixed tissue were deparaffinized in CitriSolv (Fisher Scientific, Pittsburgh, PA). The sections were rehydrated by serial immersions in 100% ethanol, 95% ethanol, 70% ethanol, and water. Sections were digested with Proteinase K at a concentration of 500 μg/ml for 10 min at room temperature and then washed with PBS (mM) 2.7 KCl, 1.2 KH₂PO₄, 138 NaCl, and 8.1 Na₂HPO₄. Endogenous peroxidase activity was reduced by immersion in 3% hydrogen peroxide in methanol. After rinsing, sections were covered in 10% goat serum for 30 min and incubated with rabbit anti-factor VIII antibody (1:1,000) diluted in PBS with 1% BSA and 0.1% sodium azide for 60 min. After incubation, the sections were rinsed with PBS and incubated with biotin-labeled secondary antibody (diluted 1:200 in PBS with 2% goat serum) for 30 min. After incubation with the secondary antibody, sections were rinsed with PBS, incubated in avidin-biotin complex (Vector) for 30 min at room temperature, rinsed in PBS, and developed with diaminobenzidine and hydrogen peroxide. Slides were lightly counterstained with hematoxylin. The slides were then dehydrated by sequential immersion in 70% ethanol, 95% ethanol, 100% ethanol, and CitriSolv before coverslips were applied. Immunostaining for eNOS protein was also performed by similar methods. Four lung sections were selected and captured by digital camera for analysis. The cells positive for factor VIII were stained brown. The number of factor VIII-positive vessels per high-power field (HPF) was counted. Six lung sections were analyzed in for each animal. The magnification used was ×20. Vessels immediately adjacent to large airways were excluded.

Lung neutrophil accumulation. Unstained tissue sections were obtained from fixed lung tissue harvested at postnatal day 3 and stained for neutrophil esterase with a commercially available kit (naphthol AS-D chloroacetate esterase kit; Sigma Diagnostics, St. Louis, MO) (44).

Western blot analysis. Lung tissue was collected for Western blot analysis from H+S and H+V study groups on postnatal days 3, 7, and 14. Western blot analysis was performed according to previously published techniques (27) with a monoclonal antibody to eNOS (Transduction Laboratories, Lexington, KY) as primary antibody. Lung tissue was homogenized on ice in 25 mM Tris·HCl, pH 7.4, containing (mM) 1 EDTA, 1 EGTA, 1 phenylmethylsulfonyl fluoride, 2 leupeptin, and 1 pepstatin A with 0.1% (vol/vol) 2-mercaptoethanol. Samples were centrifuged at 1,500 g at 4°C for 10 min to remove cell debris. Lung homogenates (25 μg) were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting. Blots were blocked overnight at 4°C in 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2% (vol/vol) BSA, and 0.1% (vol/vol) Tween 20 and then incubated with the primary antibody for 1 h at room temperature. Primary antibody was diluted 1:500 in blocking buffer. Blots were then washed six times for 5 min/wash with 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, and 0.1% (vol/vol) Tween 20 at room temperature to remove unbound antibody, incubated for 1 h with anti-mouse IgG antibody coupled to horseradish peroxidase diluted in blocking buffer, and then washed again with Tris-buffered saline-Tween 20 at room temperature. Protein bands were detected by chemiluminescence after exposure to X-ray film (ECL Plus detection; Amersham, Piscataway, NJ). Densitometry was performed with a scanner and NIH Image software (National Institutes of Health, Bethesda, MD). We initially determined the accuracy and consistency of the protein loads for each gel by Ponceau S staining before applying the different antibodies. In addition, each gel was stripped and reprobed with β-actin for use as a housekeeping protein in order to compare expression between samples. The experiments were performed with at least five animals per study group.

Statistical analysis. Statistical comparison was made by analysis of variance and Fisher’s protected least significant difference test with the Statview software package (Abacus Concepts, Berkeley, CA). Differences were considered significant at P < 0.05. The results are presented as means ± SE.

RESULTS

Body Weight, Lung Weight, Lung Weight-to-Body Weight Ratio

Ten animals were represented in each study group and at each time point. There was no difference in body weights at postnatal day 3. Body weights were lower in the hyperoxia groups at postnatal days 14 and 21 compared with room air groups. There was no difference in lung weights or lung weight-to-body weight ratios at postnatal days 3, 14, and 21 between room air-raised animals and those exposed to hyperoxia. Additionally, rhVEGF treatment in the R + V group or the H + V group had no effect on body weight, lung weight, or lung weight-to-body weight ratio at any time point (Table 1).
### Table 1. Body weight, lung weight, and lung weight-to-body weight ratio in study animals

<table>
<thead>
<tr>
<th>Postnatal day 3</th>
<th>Body Wt, g</th>
<th>Lung Wt, g</th>
<th>Lung Wt:Body Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA + saline</td>
<td>9.2 ± 0.6</td>
<td>0.13 ± 0.01</td>
<td>0.019 ± 0.001</td>
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<tr>
<td>RA + VEGF</td>
<td>9.3 ± 0.7</td>
<td>0.15 ± 0.01</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>Hyperoxia + saline</td>
<td>9.5 ± 0.2</td>
<td>0.20 ± 0.01</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>Hyperoxia + VEGF</td>
<td>10.2 ± 0.2</td>
<td>0.19 ± 0.01</td>
<td>0.019 ± 0.001</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Postnatal day 14</th>
<th>Body Wt, g</th>
<th>Lung Wt, g</th>
<th>Lung Wt:Body Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA + saline</td>
<td>28.5 ± 1.9</td>
<td>0.42 ± 0.04</td>
<td>0.017 ± 0.001</td>
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<tr>
<td>RA + VEGF</td>
<td>27.8 ± 1.1</td>
<td>0.42 ± 0.01</td>
<td>0.017 ± 0.0002</td>
</tr>
<tr>
<td>Hyperoxia + saline</td>
<td>22.7 ± 0.4</td>
<td>0.38 ± 0.01</td>
<td>0.016 ± 0.0003</td>
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<td>Hyperoxia + VEGF</td>
<td>22.2 ± 0.5</td>
<td>0.37 ± 0.01</td>
<td>0.017 ± 0.001</td>
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<table>
<thead>
<tr>
<th>Postnatal day 21</th>
<th>Body Wt, g</th>
<th>Lung Wt, g</th>
<th>Lung Wt:Body Wt</th>
</tr>
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<tr>
<td>RA + saline</td>
<td>48.0 ± 1.7</td>
<td>0.46 ± 0.02</td>
<td>0.010 ± 0.0003</td>
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<tr>
<td>RA + VEGF</td>
<td>46.1 ± 1.7</td>
<td>0.46 ± 0.03</td>
<td>0.011 ± 0.001</td>
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<tr>
<td>Hyperoxia + saline</td>
<td>41.2 ± 0.8</td>
<td>0.42 ± 0.01</td>
<td>0.010 ± 0.0003</td>
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<tr>
<td>Hyperoxia + VEGF</td>
<td>43.2 ± 0.8</td>
<td>0.47 ± 0.02</td>
<td>0.011 ± 0.0004</td>
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</tbody>
</table>

Values represent means ± SE. RA, room air.

### Lung Histology and Morphometrics

In comparison with room air controls, lung histology of animals exposed to hyperoxia was characterized by decreased septation, distal air space enlargement, and reduced complexity (Fig. 3A). rhVEGF treatment during hyperoxia exposure restored normal lung structure both at postnatal day 14 (at the end of hyperoxia exposure) and at postnatal day 21, after 7 days of recovery in room air. Lung histology at these time points appeared similar to lung histology of room air-raised controls. rhVEGF treatment of animals raised in room air had no apparent effect on distal lung structure at either postnatal day 14 or 21.

To quantitate differences in lung structure, morphometric analysis was performed with RAC (Fig. 3B) and MLI. At postnatal day 14, RAC were lower in infant rats exposed to hyperoxia than in room air controls (4.6 ± 0.2 vs. 7.7 ± 0.2, P < 0.01). rhVEGF treatment during hyperoxia increased RAC compared with hyperoxia alone (8.4 ± 0.3, P < 0.01), and RAC were not different from RAC of room air-raised controls at postnatal day 14. rhVEGF treatment in room air did not change RAC compared with room air controls (7.8 ± 0.2, P = NS). On postnatal day 21, after 7 days of recovery in room air, MLI in the animals exposed to hyperoxia remained decreased compared with room air controls (4.9 ± 0.2 vs. 9.3 ± 0.2, P < 0.01). rhVEGF during hyperoxia increased RAC at postnatal day 21 (8.6 ± 0.3 vs. hyperoxia controls, P < 0.01) and were not different from room air controls at postnatal day 21. At postnatal day 21, rhVEGF treatment of room air-raised animals had no effect on RAC compared with room air controls (8.9 ± 0.2, P = NS).

MLI were also measured to quantitate histological differences in lung structure (Fig. 3C). On postnatal day 14, MLI were higher in infant rats exposed to hyperoxia than in room air controls (69.5 ± 1.9 vs. 45.0 ± 0.7, P < 0.01). rhVEGF treatment during hyperoxia decreased MLI compared with hyperoxia alone (38.5 ± 0.6, P < 0.01). Interestingly, MLI in the hyperoxia-exposed group treated with rhVEGF were lower than MLI of room air controls at postnatal day 14 (38.5 ± 0.6 vs. 45.0 ± 0.7, P < 0.01). rhVEGF-treated room air-raised animals had MLI similar to room air controls at postnatal day 14 (45.1 ± 0.7, P = NS). On postnatal day 21, after 7 days of recovery in room air, there was a persistent increase in MLI in the animals exposed to hyperoxia in the first 14 days of life compared with animals raised in room air (71.2 ± 1.9 vs. 41.9 ± 1.2, P < 0.01). Infant rats that received daily injections of rhVEGF during hyperoxia exposure had lower MLI than those receiving hyperoxia alone (41.3 ± 0.6, P < 0.01) and were not different from room air controls at postnatal day 21. At postnatal day 21 rhVEGF treatment of room air-raised animals had no effect on MLI compared with room air controls (42.2 ± 0.9, P = NS).

### Vessel Density

In comparison with room air controls, hyperoxia decreased vessel density at both postnatal days 14 and 21 (Fig. 4A). rhVEGF treatment of neonatal rats during hyperoxia increased vessel density to baseline levels at postnatal days 14 and 21.

To quantitate these differences, we counted the number of factor VIII-positive stained vessels per HPF (Fig. 4B). At
postnatal day 14, vessel count per HPF was lower in the hyperoxia-exposed animals than in animals raised in room air (7.1 ± 0.2 vs. 13.3 ± 0.3, P < 0.05). rhVEGF treatment during hyperoxia increased vessel count compared with hyperoxia alone (11.9 ± 0.2, P < 0.05), and vessel count was not different from that of room air-raised controls on postnatal day 14. rhVEGF treatment of animals raised in room air did not affect vessel count compared with room air alone (12.9 ± 0.3, P = NS). On postnatal day 21, after 7 days of recovery in room air, there was a persistent decrease in vessel number in the hyperoxia-exposed animals compared with animals raised in room air (10.9 ± 0.3 vs. 15.7 ± 0.4, P < 0.05). Vessel counts in infant rats that received daily injections of rhVEGF during hyperoxia exposure were higher than with hyperoxia alone (16.2 ± 0.3, P < 0.05) and were not different from room air controls at postnatal day 21. At postnatal day 21, rhVEGF treatment of room air-raised controls had no effect on vessel number compared with room air controls (15.7 ± 0.3, P = NS).

Inflammation

Inflammation was evaluated with esterase staining of neutrophils. Aerosolized lipopolysaccharide (LPS) exposure in adult rats was used as a positive control for inflammation.
As shown in Fig. 5, there are a considerable number of neutrophils present in the control animal exposed to LPS. At postnatal day 3, there is no apparent difference in inflammation in the hyperoxia-exposed animals compared with room air controls. Additionally, rhVEGF treatment either alone or in conjunction with hyperoxia exposure does not appear to cause inflammation at postnatal days 3, 14, and 21.

Fig. 3. A: effects of rhVEGF treatment during hyperoxia on lung structure in infant rats. a: Lung histology of 14-day-old rats raised in room air. Hyperoxia exposure through postnatal day 14 causes distal air space enlargement and decreased septation (c). These abnormalities of lung growth persist at postnatal day 21 despite a 7-day period of recovery in room air (d). rhVEGF treatment during hyperoxia through postnatal day 14 improves alveolarization both at day 14 (e) and after room air recovery at day 21 (f). Bars, 100 μm. B: effects of rhVEGF treatment during hyperoxia on radial alveolar counts (RAC) in infant rats. At postnatal day 14, hyperoxia decreases RAC. rhVEGF treatment during hyperoxia increases RAC to levels similar to those seen in animals raised in room air. At postnatal day 21, after 7 days of recovery in room air, RAC remain decreased in the hyperoxia-exposed animals. At day 21 RAC are increased to control values in animals exposed to hyperoxia and treated with rhVEGF during the first 14 days of life. C: effects of rhVEGF treatment during hyperoxia on mean linear intercepts (MLI) in infant rats. At postnatal day 14, hyperoxia increases MLI, but rhVEGF treatment during hyperoxia decreases MLI to levels similar to room air controls. At postnatal day 21, after 7 days of recovery in room air, MLI remain increased in the hyperoxia-exposed animals. At day 21, MLI are decreased to control values in animals exposed to hyperoxia and treated with rhVEGF during the first 14 days of life.
Lung eNOS Expression

Western blot analysis of eNOS protein was performed to determine the effects of hyperoxia and rhVEGF treatment on eNOS expression. As shown in Fig. 6A, rhVEGF treatment during hyperoxia significantly increases the eNOS-to-β-actin ratio on postnatal day 3 (2.49 ± 0.68 vs. 0.77 ± 0.20, P < 0.05). At postnatal days 14 and 21, lung eNOS expression in hyperoxia-exposed animals treated with rhVEGF was not different from animals exposed to hyperoxia alone (1.0 ± 0.1 vs. 0.8 ± 0.1, P = NS) and (0.6 ± 0.1 vs. 1.0 ± 0.1, P = NS).

Immunostaining with eNOS was also performed to determine effects of hyperoxia and rhVEGF treatment on localization. As shown in Fig. 4B, eNOS is localized to endothelial cells and airway epithelial cells. There appears to be no difference in localization in 3-day-old and 14-day-old rat pups exposed to hyperoxia and treated with rhVEGF compared with rat pups of the same age exposed to hyperoxia alone.

DISCUSSION

We found that exposure of infant rats to hyperoxia increases lung edema and impairs distal lung growth. These changes persist despite prolonged recovery in room air. We further report that treatment with rhVEGF protein during hyperoxia exposure worsens lung edema early, as demonstrated by increased lung wet-to-dry ratios at postnatal day 3. However, rhVEGF treatment improves late lung architecture, as shown by increased septation and increased vessel density. We also show that rhVEGF treatment of infant rats during hyperoxia exposure preserves eNOS protein levels in the lung. Overall, these findings demonstrate that rhVEGF treatment transiently worsens lung edema during neonatal hyperoxia but preserves lung growth and structure.

These findings further support the hypothesis that VEGF regulates angiogenesis and promotes lung growth and that arrested alveolarization after neonatal hyperoxia may be due to...
sustained impairment in VEGF signaling. Because VEGF treatment during hyperoxic lung injury worsens pulmonary edema, we further speculate that VEGF also has an important role in ALI. In particular, in the early stages of lung injury VEGF may increase vascular permeability, as suggested in other models of ALI (20, 29). VEGF may have diverse roles depending on the timing of upregulation or treatment relative to the timing of lung injury.

These findings are especially interesting in light of previous animal studies that have shown enhanced alveolarization and vascular growth in animals treated with VEGF during recovery after hyperoxia (23). In this study, we report that rhVEGF treatment after hyperoxia improved late lung structure in neonatal rat pups. Additionally, Thebaud et al. (39) demonstrated preservation of alveolarization and promotion of angiogenesis during hyperoxia-induced lung injury by intratracheal VEGF gene treatment with adenovirus-mediated vector. In contrast to the studies showing improved lung structure after VEGF treatment, VEGF has also been implicated in various models of ALI. VEGF has been shown to cause pulmonary hemorrhage and air space enlargement with genetic overexpression in transgenic mouse models (26). Patients with high-altitude pulmonary edema have increased VEGF levels (20). This is the first report to show that although VEGF may worsen initial lung injury it may also improve lung structure during progression and recovery from neonatal lung injury. Thus VEGF may have biphasic effects at different stages of lung injury. Our results suggest that VEGF treatment during hyperoxic lung injury worsens pulmonary edema early in lung injury but hastens normalization of lung structure late in lung injury and after recovery from lung injury.

Potential mechanisms by which VEGF treatment improves late lung structure after hyperoxic lung injury are uncertain but may be due to effects on endothelial cells, epithelial cells, or both. Although VEGF is well established as a permeability factor, it is also an endothelial cell survival factor enhancing endothelial cell growth and function and may therefore increase vascular growth that is necessary to sustain normal alveolarization (18). VEGF may also have a direct effect on angiogenesis and alveolarization through liberation of NO (11, 13, 15, 35), and we found that rhVEGF increased lung eNOS protein content after hyperoxia. Recent studies in our lab have shown VEGF and eNOS protein levels in the lung to be downregulated during and after hyperoxia exposure despite recovery in room air. Treatment with inhaled NO (iNO) after hyperoxia exposure preserved normal lung growth (28). Additionally, several animal studies have shown that iNO during hyperoxia exposure may reduce ALI (12, 17, 32, 33, 41).

Fig. 5. Effects of hyperoxia and rhVEGF treatment on inflammation in infant rats. A: esterase staining of an adult rat lung exposed to lipopolysaccharide (LPS). Arrows indicate esterase positive cells. Bar, 100 μm. B: lung histology of a 3-day-old animal raised in room air. As expected, there is less inflammation in the LPS control. There is no increase in inflammation in hyperoxia-exposed animals at postnatal day 3 (C), day 14 (D), and day 21 (E). Additionally, rhVEGF treatment did not change inflammation in both room air (F) and hyperoxia (G)-exposed animals at postnatal day 3. There is no increase in inflammation in hyperoxia-exposed animals treated with rhVEGF at postnatal day 14 (H) and day 21 (I).
Collectively, these studies suggest a critical role for the VEGF-NO signaling pathway during normal early postnatal lung development. Alternatively, VEGF may have a direct effect on the pulmonary epithelium in promoting alveolarization. A recent study demonstrated that VEGF receptor-2 transcripts are expressed by alveolar type II cells and that type II cells respond to VEGF treatment with increased surfactant protein-B and -C production (8). Additionally, it has been shown that VEGF may be involved in epithelial growth in fetal human lung explants in vitro and that exogenous VEGF treatment can increase epithelial proliferation (5). Whether the effects of rhVEGF treatment in our study are primarily due to effects on the vasculature or on type II cells is unknown.

There are several potential limitations to this study. Although we show improvement in distal lung structure, we also observed worsening of ALI early with VEGF treatment. The mechanisms by which VEGF treatment enhances lung growth still need further study before we can understand the diverse roles of VEGF in lung injury and repair. In addition, VEGF may have systemic effects on other organs. We only collected lung tissue and thus are unable to evaluate the effects of VEGF treatment on other organs and tissues. In particular, VEGF has been associated with abnormal vascular proliferation, which is responsible for retinopathy of prematurity (ROP). This is a condition very common in extremely low-birth-weight infants who are at high risk for developing BPD, and whether systemic VEGF treatment would worsen ROP is unknown. It is important to address the effects of VEGF on other organs in future studies. In addition, we saw no changes in inflammation with either hyperoxia or VEGF treatment. It is possible that inflammation occurred after postnatal day 3 and resolved by day 14 of exposure or perhaps that it occurred beyond day 21. Finally, the body weights on postnatal day 3 were the same in the hyperoxia and room air groups, but on postnatal days 14 and 21...
the body weights of the hyperoxia group were lower than the room air group. However, VEGF did not alter body weight in either hyperoxia or room air. Hyperoxia may have impaired metabolism, contributing to lower body weight in this group.

In summary, we found that hyperoxia increases pulmonary edema early during exposure but the edema resolves during recovery. Treatment with rhVEGF during hyperoxia worsens lung edema both early and late in the development of ALI; however, the edema resolves after hyperoxia. Inflammation does not appear to be associated with hyperoxia exposure, rhVEGF treatment, or hyperoxia in conjunction with rhVEGF in early lung injury. We also report that hyperoxia exposure inhibits alveolarization and vascular growth, which persists despite a recovery period in room air. rhVEGF treatment during neonatal hyperoxia exposure restores normal lung architecture and vessel density. Further studies are needed to determine the exact mechanism by which rhVEGF treatment improves alveolarization, and we speculate that this may be partly due to upregulation of eNOS. Further studies are warranted to better understand the different mechanisms through which VEGF acts on the developing lung, especially in the setting of neonatal lung injury.

REFERENCES