Early inhaled nitric oxide treatment decreases apoptosis of endothelial cells in neonatal rat lungs after vascular endothelial growth factor inhibition

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Am J Physiol Lung Cell Mol Physiol 293: L1271–L1280, 2007. First published September 7, 2007; doi:10.1152/ajplung.00224.2007.—Vascular endothelial growth factor (VEGF) receptor blockade impairs lung growth and decreases nitric oxide (NO) production in neonatal rat lungs. Inhaled NO (iNO) treatment after VEGF inhibition preserves lung growth in infant rats by unknown mechanisms. We hypothesized that neonatal VEGF inhibition disrupts lung growth by causing apoptosis in endothelial cells, which is attenuated by early iNO treatment. Three-day-old rats received SU-5416, an inhibitor of VEGF receptor, or its vehicle and were raised in room air with or without iNO (10 ppm). SU-5416 reduced alveolar counts and lung vessel density by 28% (P < 0.005) and 21% (P < 0.05), respectively, as early as at 7 days of age. SU-5416 increased lung active caspase-3 protein by 60% at 5 days of age (P < 0.05), which subsided by 7 days of age, suggesting a transient increase in lung apoptosis after VEGF blockade. Apoptosis primarily colocalized to lung vascular endothelial cells, and SU-5416 increased endothelial cell apoptotic index by eightfold at 5 days of age (P < 0.0001). iNO treatment after SU-5416 prevented the increases in lung active caspase-3 and in endothelial cell apoptotic index. There was no difference in alveolar type 2 cell number between control and SU-5416-treated rats. We conclude that neonatal VEGF receptor inhibition causes transient apoptosis in pulmonary endothelium, which is followed by persistently impaired lung growth. Early iNO treatment after VEGF inhibition reduces endothelial cell apoptosis in neonatal lungs. We speculate that enhancing endothelial cell survival after lung injury may preserve neonatal lung growth in bronchopulmonary dysplasia.

vascular growth; alveolar growth; developing lung; bronchopulmonary dysplasia

IMPAIRED ALVEOLAR AND VASCULAR growth in the developing lung is characteristic of bronchopulmonary dysplasia (BPD), a chronic lung disease that follows premature birth and subsequent intervention. The development of new therapeutic modalities to prevent BPD requires a better understanding of its pathogenesis. Mechanisms that disrupt lung growth and cause persistent abnormalities of lung structure are uncertain. Autopsy findings show increased apoptosis in alveolar epithelial cells in preterm infants with BPD and severe respiratory distress syndrome (RDS) (16, 28, 32). However, mechanisms that increase apoptosis in the developing lung are unclear, and whether increased apoptosis contributes to abnormal lung structure in BPD is uncertain.

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen and survival factor, stimulates angiogenesis and protects against endothelial injury (13). Clinical studies show reduced lung VEGF gene and protein expression in infants with BPD (6) and reduced VEGF levels in tracheal fluid samples from premature infants with RDS who develop BPD (24). In addition, neonatal VEGF receptor blockade decreases vessel density and alveolarization in infant rat lungs, suggesting that disruption of VEGF signaling contributes to structural features of BPD (18, 25). Moreover, experimental models of BPD utilizing hyperoxia in rodents, chronic ventilation in premature rabbits and baboons, and intra-amniotic endotoxin in preterm sheep have strongly implicated the role of impaired VEGF signaling in the pathogenesis of BPD (4, 8, 19, 21, 23, 27, 30, 31, 41, 42). However, mechanisms by which VEGF regulates vascular and alveolar growth in the developing lung are unknown, and mechanisms by which impaired VEGF signaling in the newborn leads to persistent lung structural abnormalities are unclear.

Prolonged treatment of adult rats with SU-5416, a VEGF receptor inhibitor, causes emphysema, which may be due to increased apoptosis of alveolar septal cells (20). Although neonatal SU-5416 treatment decreases lung vascular and alveolar growth in infant rats (18, 25), it remains unclear whether these abnormalities of infant lung structure are due to apoptosis during the early neonatal period. In addition, past studies suggest that disruption of angiogenesis impairs neonatal lung growth (18, 25), but whether apoptosis of lung endothelial cells contributes to abnormal lung growth is unknown. Alternatively, whether VEGF receptor inhibition causes apoptosis of alveolar type 2 (AT2) cells, which may also express VEGF receptors (9), and subsequently disrupts lung growth, is unknown.

We have previously shown that inhibition of VEGF signaling downregulates endothelial nitric oxide synthase (eNOS) and decreases nitric oxide (NO) bioavailability in newborn rat lungs (38). Furthermore, inhaled NO (iNO) after neonatal VEGF receptor inhibition preserves normal alveolar and vascular growth in the infant rat lungs (38). However, mechanisms by which iNO protects and enhances lung growth after disruption of VEGF signaling in the newborn are unclear.

Therefore we hypothesized that inhibition of VEGF signaling disrupts neonatal lung growth by inducing early apoptosis.
of endothelial cells, which is attenuated by early iNO treatment. The present experiments were designed to determine whether 1) VEGF inhibition in newborn rats induced apoptosis in lung endothelial or AT2 cells that precedes the disruption of alveolar and vascular growth, and 2) early treatment with iNO preserves lung growth by preventing apoptosis following neonatal VEGF inhibition. We report that neonatal VEGF inhibition immediately and transiently induces apoptosis in pulmonary vascular endothelial cells, which is followed by an early and persistent reduction of vascular and alveolar growth in infant rats. Furthermore, early treatment with iNO decreases apoptosis in pulmonary vascular endothelial cells in this experimental model of BPD.

MATERIALS AND 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 from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver’s altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 wk before giving birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Study Design

Three-day-old rats received a single dose of SU-5416 (20 mg/kg) or its vehicle (carboxymethyl cellulose) by subcutaneous injection. The selection of SU-5416 and its dose were based on the results of our previous studies, in which a single dose of SU-5416 (20 mg/kg) in newborn rats caused pulmonary hypertension and impaired lung growth throughout infancy (25, 38). Each litter of infant rats was randomly divided between SU-5416 treatment and vehicle controls. After injection, the whole litters were maintained in room air or exposed to inhaled nitric oxide (iNO) (10 ppm) until the animals were killed at 5, 7, and 10 days of age for lung harvest, as illustrated in Fig. 1. We studied three to six rats from each group for each measurement at each time point, as described below.

Study Measurements

Tissue for histological analysis. Animals were killed with intraperitoneal injections of pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated and maintained at 20 cmH2O pressure for at least 20 min. For morphometric analysis and immunohistochemistry, lungs were inflated with 4% paraformaldehyde in PBS. For immunofluorescence staining, lungs were inflated with 30% Optimal Cutting Temperature compound (OCT; Sakura Finetek, Torrance, CA) in PBS. A ligature was tightened around the trachea to maintain pressure, and then the tracheal cannula was removed. For morphometric analysis and immunohistochemistry, lungs were immersed in 4% paraformaldehyde at room temperature overnight for fixation. One transverse section was taken from the midplane of the right upper, middle, and lower lobes and the left upper lobe of the fixed lungs per animal, respectively. The four sections from each animal were processed and embedded in paraffin wax. For immunofluorescence staining, inflated lungs were immersed in 100% OCT and preserved at −80°C before cryosectioning. Sections were cut from the paraffin- or OCT-embedded blocks with a microtome at 5 μm and mounted on RNase-free slides for histochemical analysis.

Immunohistochemistry

The slides with 5-μm paraffin sections were deparaffinized in xylene, rehydrated by serial immersions in 100% ethanol, 95% ethanol, 70% ethanol, and deionized water, treated with proteinase K (50 μg/ml) for 5 min, and then washed with PBS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in methanol and rinsed with PBS. For von Willebrand factor (vWF) immunohistochemistry, sections were incubated with rabbit anti-human polyclonal vWF antibody (1:1,000 dilution; A0082, Dako, Carpinetia, CA) for 1 h at room temperature. Sections were incubated with biotin-labeled goat anti-mouse secondary antibody diluted 1:200 for 15 min at room temperature. For prosurfactant protein C (proSP-C) immunohistochemistry, sections were incubated with anti-proSP-C goat anti-mouse polyclonal antibody (1:200 dilution; cat. no. SC7706, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by biotin-labeled anti-goat secondary antibody diluted 1:500 for 2 h at room temperature. For Ki67 immunohistochemistry, sections were incubated with anti-Ki67 rabbit monoclonal antibody (1:200 dilution; cat. no. RM-9106, Lab Vision NeoMarkers, Fremont, CA) for 1 h at room temperature and then were incubated with rabbit-labeled polymer horseradish (HRP) peroxidase for 30 min at room temperature. Following the secondary antibody, sections were incubated with ABC complex (Vector) for 30 min at room temperature, rinsed in PBS, and developed with diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) and hydrogen peroxide. Washing with water stopped the DAB reaction. A light hematoxylin counterstain was applied. Sections were dehydrated by sequential immersion in 70% ethanol, 95% ethanol, 100% ethanol, and then xylene before placing a coverslip on the section.

Morphometric Analysis

A transverse section was taken from the midplane of the right upper, middle, and lower lobes and the left upper lobes of the fixed lungs per animal, respectively. The four sections from each animal were processed and embedded in paraffin wax. Thus, each slide carries four sections cut from the paraffin-embedded block from each animal. At least 10 counts from 10 fields from these 4 sections were performed per animal for morphometric analyses, including radial alveolar counts, pulmonary vessel density, and AT2 cell density, by light microscopy in a blinded fashion, as described below.

Radial alveolar counts. Paraffin sections were stained with hematoxylin and eosin. Alveolarization was assessed by the radial alveolar count (RAC) method of Emery and Mithal as described (10, 11). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall (35). From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective tissues or septum or pleura, and the number of septae intersected by this line was counted. At least 10 counts from 10 fields...
were performed for each animal. The fields containing respiratory bronchiole(s) were chosen, but no count was made if the respiratory bronchiole was closer to the edge of the slide than the nearest connective septum.

**Pulmonary vessel density.** Pulmonary vessel density was determined by counting vessels per high-power field (×200 magnification) based on vWF staining. At least 10 counts from 10 fields were performed for each animal for analysis. The fields were chosen randomly in areas not containing large airways or vessels.

**AT2 cell density.** The numbers of AT2 cells per ×400 field were counted. AT2 cells were identified by the location in the distal lung, the morphology, and positive immunostaining of proSP-C. Five to ten counts were performed in five to ten fields for each animal.

**Western Blot Analysis for Active Caspase-3**

Lung tissue was homogenized on ice in 25 mM Tris·HCl buffer containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercapto-

![Fig. 2. Effects of SU-5416 treatment on radial alveolar counts (RACs) in infant rats raised in room air.](image)

A: lung histology from controls and SU-5416-treated rats at 5, 7, and 10 days of age stained with hematoxylin and eosin. Micrographs are representative and were obtained at the same magnification. Internal scale bar = 100 μm. B: compared with controls, RACs decreased in SU-5416-treated rats at 7 and 10 days of age, respectively. *P < 0.005 compared with age-matched controls. From 5 to 7 days of age, RACs significantly increased in controls (P < 0.001) but remained steady in SU-5416-treated rats. RACs remained steady from 7 to 10 days of age in both controls and SU-5416-treated rats.
ethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM pepstatin A. Homogenates were centrifuged at 1,500 g at 4°C for 10 min to remove cell debris, and supernatants were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting. Blots were blocked overnight at 4°C in 5% of nonfat dried milk. Immunodetection was performed using a rabbit polyclonal antibody to active caspase-3 (cat. no. AB3623; Chemicon International, Temecula, CA) diluted 1:1,000 for 1 h at room temperature. After the blots were washed to remove unbound antibody, a secondary antibody, goat anti-rabbit HRP (cat. no. SC2054, Santa Cruz Biotechnology), was diluted in blocking buffer (1:2,000) and applied for 30–60 min. After three washes, ECL Plus detection (Amersham, Piscataway, NJ) was performed. 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 as a housekeeping protein to compare expression between samples. The experiments were performed with at least four animals per study group.

Fig. 3. Effects of SU-5416 treatment on pulmonary vessel density in infant rats raised in room air. A: lung histology from controls and SU-5416-treated rats at 5, 7, and 10 days of age stained with von Willebrand Factor (vWF; a marker of endothelial cells). Micrographs are representative and were obtained at the same magnification. Internal scale bar = 100 μm. B: compared with controls, the number of vWF-stained pulmonary vessels per high-power field decreased in SU-5416-treated rats at 7 and 10 days of age, respectively. *P < 0.05 compared with age-matched controls. The number of pulmonary vessel per high-power field significantly increased from 5 to 7 days of age (P < 0.01) and then remained steady from 7 to 10 days of age, within both controls and SU-5416 treated rats.
Colocalization Studies

To identify cells undergoing apoptosis, immunofluorescence double staining was performed on 5-μm cryosections cut from the OCT-embedded lung blocks. The sections were blocked with BSA in PBS for 1 h and washed with PBS. Anti-active caspase-3 antibody (1:50 dilution; cat. no. AB3623, Chemicon International), along with either anti-vWF antibody (1:100 dilution; cat. no. SC8068, Santa Cruz Biotechnology) or anti-proSP-C antibody (1:200 dilution; cat. no. SC7706, Santa Cruz Biotechnology), was applied overnight at 4°C. Sections were washed, and secondary antibodies (donkey-anti-rabbit Alexa Fluor 488, #A-21206; Green, 1:500 dilution, and donkey-antigoat 594, #A-11058; Red, 1:500 dilution; Molecular Probes, Eugene, OR) were applied for 2 h at room temperature and washed. The sections were then mounted with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). After immunostaining tissues, sections were visualized with an Olympus IX71 fluorescence microscope (Olympus America, Center Valley, PA). Different fluorescent filters were used to acquire images of each field displaying nuclei (DAPI, blue), vWF or proSP-C (red), and active caspase-3 (green).

Quantification of Apoptosis in Endothelial Cells

Five fields per animal were obtained from four animals for each treatment group. Fields that contained a large airway or blood vessel were rejected. For each field, the cells stained with DAPI and vWF were counted. Next, active caspase-3(+) (fluorescein-labeled) that merged with the DAPI(+)vWF(+) cells were counted. The apoptotic index of endothelial cells was calculated by dividing the number of active caspase-3(+)vWF(+)DAPI(+) cells and then multiplying by 100 for each field. The ratio was summed and averaged in each animal.

Statistical Analysis

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

RESULTS

Serial Changes in Lung Histology, RACs, and Pulmonary Vessel Density

To establish the time course and subsequence of events leading to changes in lung structure, we initially studied lung tissues from infant rats at different time points by morphometric analysis. Figure 2A shows lung histology from controls and SU-5416-treated rats raised in room air at 5, 7, and 10 days of age. Alveolar numbers, as determined by RACs, were similar between controls and SU-5416-treated rats at 5 days of age, 2 days after SU-5416 treatment (Fig. 2B). At 7 and 10 days of age, SU-5416-treated rats demonstrated fewer and larger alveoli, respectively, compared with controls (Fig. 2A). SU-5416 treatment decreased RACs by 28% (P < 0.05) and 42% (P < 0.005) at 7 and 10 days of age, respectively, compared with controls (Fig. 2B). From 5 to 7 days of age, RACs increased significantly in controls (P < 0.001) but did not change in SU-5416-treated rats. Moreover, the number of pulmonary vessels per high-power field in the distal lungs of SU-5416-treated rats decreased from control values at 7 and 10 days of age by 21% (P < 0.05) and 23% (P < 0.01), respectively (Fig. 3, A and B). As early as at 5 days of age, the number of pulmonary vessels per high-power field in SU-5416-treated rats decreased by 29% from control values, but this did not achieve statistical significance (P = 0.099).

Lung Active Caspase-3 Protein Content and Identification and Localization of Apoptotic Cells and the Effects of iNO Treatment

Lung active caspase-3 protein content in 5-day-old rats increased by 28% from control values, 2 days after SU-5416 treatment (P < 0.05), and then subsided to below control values in 7-day-old rats, 4 days after SU-5416 treatment (Fig. 4A). iNO treatment after SU-5416 injection prevented the increase of lung active caspase-3 protein in 5-day-old rats (Fig. 4B).

Active caspase-3 staining increased in lung cells of SU-5416-treated rats at 5 days of age compared with controls (Fig. 5A). In SU-5416-treated rats, active caspase-3 stained...
primarily the pulmonary vascular endothelial cells, shown by colocalization with vWF staining (Fig. 5B). The increased active caspase-3 staining involved pulmonary vessels of various sizes in SU-5416-treated rats (Fig. 5A). Compared with the SU-5416-treated rats raised in room air, iNO treatment after SU-5416 injection decreased active caspase-3 staining in the pulmonary vascular endothelium (Fig. 5B). To evaluate the potential effects of SU-5416 treatment on AT2 cells, we also performed colocalization studies staining for proSP-C and active caspase-3 in 5-day-old rat lungs. Both control and SU-5416-treated rats demonstrated little active caspase-3 signal in nonvascular parenchymal cells (Fig. 6). Active caspase-3 staining did not localize to proSP-C-positive cells (Fig. 6).
Quantification of Apoptotic Endothelial and AT2 Cells

The apoptotic index of lung endothelial cells in 5-day-old rats increased by eightfold over control values (56.5% vs. 6.3%; $P < 0.0001$) 2 days after SU-5416 treatment (Fig. 7). iNO treatment following SU-5416 injection decreased the apoptotic index of endothelial cells to 18% at 5 days of age ($P < 0.0001$ vs. SU-5416-treated rats in room air; $P = 0.06$ vs. control rats; Fig. 7).

To evaluate the impact of SU-5416 treatment on AT2 cells, proSP-C immunostaining was performed on rat lung sections. AT2 cells were identified by their location, morphology, and positive immunostaining for proSP-C. Throughout the study period, there was no difference in the density (number per high-power field) or distribution of AT2 cells between control and SU-5416-treated rats in room air (micrographs are not shown here). The number of AT2 cells per ×400 field was similar between control and SU-5416-treated rats from 5 days of age through 10 days of age (Fig. 8). From 5 to 7 days of age, the density of AT2 cells decreased in both control ($P < 0.05$) and SU-5416-treated rats ($P < 0.05$) (Fig. 8). From 7 to 10 days of age, the density of proSP-C-positive cells remained steady in both control and SU-5416-treated rats (Fig. 8).

Identification of Proliferating Lung Cells

To evaluate the impact of SU-5416 treatment on lung cell proliferation, Ki67 immunohistochemistry was performed. We
found that the density of Ki67-positive cells was similar between controls and SU-5416-treated rats in room air at 5 days of age (Fig. 9). In 10-day-old rats, the number of Ki67-positive lung cells decreased remarkably in SU-5416-treated rats compared with controls (Fig. 9). Ki67-positive cells in 10-day-old control rats localized to the distal lung interstitium and alveolar septae, including the bases and tips of septal buds (Fig. 9). In contrast, SU-5416-treated rats demonstrated globally scant Ki67-positive lung cells at 10 days of age (Fig. 9). From 5 to 10 days of age, the density of Ki67-positive cells remained steady in SU-5416-treated rats, whereas it increased in control rats (Fig. 9).

DISCUSSION

We found that treatment with a single dose of SU-5416, a VEGF receptor inhibitor, induced apoptosis in endothelial cells of neonatal rat lungs within 2 days after treatment, which then subsided 4 days after treatment. This transient increase in endothelial cell apoptosis preceded the early and persistent disruption of lung growth, as manifested by decreased vessel density and alveolarization, that was first noticeable 4 days after SU-5416 injection and persisted through the first weeks of life. Despite these changes in the distal air space architecture, the number of AT2 cells was not altered after neonatal VEGF receptor inhibition. Since NO may act downstream from VEGF receptor activation in developing lung, we further studied the effects of iNO treatment after SU-5416 injection in neonatal rats. We found that early iNO treatment after neonatal VEGF inhibition significantly decreased apoptosis of pulmonary vascular endothelial cells in this experimental model of BPD. Overall, these findings support our hypotheses that inhibition of VEGF signaling disrupts neonatal lung growth by increasing apoptosis in endothelial cells and that early iNO treatment reduces apoptosis of pulmonary endothelium caused by neonatal VEGF inhibition.

Our findings elucidate mechanisms by which VEGF signaling preserves normal lung vascular development. These results also explain the association between decreased lung VEGF expression and disrupted lung structure in human infants with BPD (6, 24) and in experimental models of BPD (4, 8, 19, 21, 23, 27, 30, 31, 41, 42). In addition, we have previously reported marked downregulation of eNOS expression and decreased NO production in infant rat lungs after neonatal SU-5416 treatment (38). The transient apoptosis of lung vascular endothelial cells in the present study, peaking 2 days after SU-5416 injection, parallels the early decrease in lung eNOS protein after neonatal VEGF receptor inhibition shown in our previous study (38). It suggests that decreased NO signaling may correlate with endothelial cell apoptosis after injury in developing lung. This correlation is further supported by our findings that iNO treatment decreases apoptosis of pulmonary endothelium after neonatal VEGF receptor inhibition. Reduced lung eNOS protein content and decreased pulmonary NO production have been demonstrated in other models of BPD (2, 27, 29), but endothelial cell apoptosis was not assessed in those studies. Our findings in the present study suggest that early endothelial cell apoptosis due to disruption of VEGF signaling in the newborn may impair pulmonary vascular and alveolar growth, leading to persistent structural abnormalities of the distal lung in BPD.

This is the first report demonstrating that NO decreases apoptosis of lung endothelial cells in a model of BPD. Reduction of endothelial cell apoptosis by early iNO treatment may represent one mechanism by which iNO improves lung growth in other experimental models of BPD, such as hyperoxia exposure and chronic ventilation in preterm primates and...
lambs (7, 27, 33). NO itself is a potent modulator of apoptosis; it may act by several mechanisms to protect endothelial cells from apoptosis. NO prevents apoptosis in hepatocytes by inhibiting caspase activity (26), protects alveolar cells of adult rat lungs from hyperoxia-induced apoptosis by attenuating caspase-3 activation (17), and blocks lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells by decreasing NF-κB DNA binding activity (12). Our data suggest that VEGF blockade leads to activation of caspase-3 in endothelial cells in neonatal rat lungs and that the protective effect of iNO after VEGF receptor blockade may be due to inhibition of active caspase-3. It remains to be determined whether there are additional protective mechanisms of NO on endothelial cells in developing lung.

This is the first report demonstrating that VEGF receptor inhibition increases apoptosis of endothelial cells in the developing lung. Previous studies showed VEGF has antiapoptotic effects on human umbilical vein endothelial cells in vitro (14), and VEGF prevents endothelial apoptosis in retinal capillaries of neonatal rats in vivo during hyperoxia (3). VEGF-dependent survival of endothelial cells via PI-3 kinase/Akt signal transduction pathway mediates the inhibition of proapoptotic signals, such as Bad and caspase-9 (15). In addition, VEGF upregulates antiapoptotic proteins, such as Bcl-2 and inhibitor of apoptosis, and inhibits upstream and terminal caspases (14, 43). Prolonged treatment with SU-5416 in combination with chronic hyperoxia increases apoptosis of lung endothelial cells and causes pulmonary hypertension in adult rats, which is paradoxically characterized by exuberant endothelial proliferation (40). In contrast with neonatal rats, the apoptotic effects of SU-5416 treatment was less striking in adult rats when administered without the additional stress of prolonged hyperoxia (40). Other studies utilizing chronic VEGF inhibition in adult mice and rats demonstrate increased apoptosis of alveolar sepal cells (20, 39). In those studies, it was unclear whether VEGF inhibition increased apoptosis in epithelium, endothelial cell, or other cell types.

The impact of VEGF inhibition on apoptosis in developing lung had been controversial. Chronic treatment with a VEGF receptor-1 inhibitor, mFlt Ig, increased apoptosis in the epithelial tubes in embryonic mouse lungs that were grafted into renal capsules of host mice (44). In contrast, a different study suggested that treatment with repeated doses of a VEGF receptor-2 inhibitor, DC101, did not increase apoptosis in the neonatal mouse lung, although radial alveolar counts were decreased (34). In the present study, we found that a single dose of VEGF receptor inhibitor immediately but transiently increased active caspase-3 in endothelial cells and not in AT2 cells of neonatal rat lungs. Our findings confirm that VEGF is a survival factor for vascular endothelium, but not for AT2 cells. This early and transient endothelial cell apoptosis in developing lung following VEGF inhibition might have been missed in the previous studies (34, 44).

Mechanisms linking early and transient endothelial cell apoptosis with persistent impairment of lung growth after neonatal VEGF receptor inhibition and whether similar mechanisms cause abnormal lung structure in other models of BPD require further studies. Interactions between endothelial and other mesenchymal or epithelial cells may play an important role in alveolarization during lung development. For example, differentiation of AT2 cells into alveolar type 1 cells appears to be modulated by capillary growth, perhaps through the loss of epithelial contact with underlying fibroblasts when endothelial cell proliferation increases, as observed during late gestation in fetal rat lungs (1). Endothelial cell apoptosis might interrupt multiple intercellular interactions in developing lung, leading to persistent impairment of lung growth. A previous study using a coculture model of dissociated cells from fetal mouse lungs showed that formation of alveolus-like structures was attenuated by an anti-angiogenic protein, endothelial monocyte-activating polypeptide II, which induces endothelial cell apoptosis (37). In the present study, the decrease in cellular proliferation within the septum and interstitium, as shown by Ki67 immunohistochemistry in rat lungs 7 days after neonatal SU-5416 treatment, suggests that normal growth of non-endothelial lung cells remains decreased despite the fact that transient apoptosis of pulmonary endothelium subsides in the developing lung. Alternatively, apoptotic endothelial cells might release factors, including reactive oxygen species, which could adversely affect the growth of other pulmonary cells.

We conclude that neonatal VEGF receptor inhibition immediately and transiently induces apoptosis in pulmonary vascular endothelial cells, which is followed by persistent impairment of vascular growth and alveolarization in infant rats. In addition, early iNO treatment after VEGF inhibition decreases apoptosis of endothelial cells in neonatal rat lungs, which may account for some of the beneficial effects of iNO treatment in experimental models of BPD (7, 27, 33) and in the clinical settings (5, 22, 36). We speculate that strategies enhancing endothelial cell survival after neonatal lung injury may preserve distal lung growth and improve lung architecture in BPD.

GRANTS

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