VEGF Attenuates Hyperoxic Injury through Decreased Apoptosis in Explanted Rat Embryonic Lung

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ABSTRACT: Ambient oxygen concentration and vascular endothelial growth factor (VEGF)-A are vital in lung development. Since hypoxia stimulates VEGF-A production and hyperoxia reduces it, we hypothesized that VEGF-A down-regulation by exposure of airways to hyperoxia may result in abnormal lung development. An established model of *in vitro* rat lung development was used to examine the effects of hyperoxia on embryonic lung morphogenesis and VEGF-A expression. Under physiologic conditions, lung explant growth and branching is similar to that seen *in vivo*. However, in hyperoxia (50% O_2) the number of terminal buds and branch length was significantly reduced after 4 d of culture. This effect correlated with a significant increase in cellular apoptosis and decrease in proliferation compared with culture under physiologic conditions. mRNA for *Vegf*164 and *Vegf*188 was reduced during hyperoxia and addition of VEGF165, but not VEGF121, to explants grown in 50% O_2 resulted in partial reversal of the decrease in lung branching, correlating with a decrease in cell apoptosis. Thus, hyperoxia suppresses VEGF-A expression and inhibits airway growth and branching. The ability of exogenous VEGF165 to partially reverse apoptotic effects suggests this may be a potential approach for the prevention of hyperoxic injury. (*Pediatr Res* **63: 20–25, 2008**)

The lung develops by outgrowth, elongation, and reiterated subdivision of the distal embryonic lung bud (1). Rodent embryonic lung explants appear to retain this stereotypic pattern of growth, thus allowing investigation of the pathways that can regulate lung development and how lung maturation may be disrupted under conditions of cellular stress.

In the mouse, lung branching morphogenesis begins at embryonic day 9.5 (E9.5) (2) and is regulated by growth factors, cytokines, and the ambient oxygen concentration (3). Low oxygen tensions are a persistent feature of embryonic life (4) and experiments in explanted lungs reveal that oxygen concentrations between 3% and 5% stimulate the process of bud branching and cell proliferation compared with ambient oxygen tension (5). Under similar conditions, cultured developing kidneys exhibit enhanced growth and greater numbers of tubules and blood vessels (6).

The ability of hypoxia to stimulate organ development has been attributed to the transcriptional up-regulation of hypoxia-inducible factor (HIF)-dependent pathways such as *Vegf-A* (7) and its receptors 1 (*Vegf-r1 or Flt-1*) and 2 (*Vegf-r2 or Flk-1*) (8). *VEGF-A* is secreted by multiple cell types, including the airway epithelium, and activates VEGF receptors on nearby endothelial cells, stimulating vascular growth. Both *Vegf-r1* and *Vegf-r2* have been localized to endothelial cells and are expressed throughout development. Recent studies have shown that *Vegf-A* can also play a role in epithelial cell morphogenesis in both the lung and the kidney (9,10). This may be due to an indirect effect of VEGF-stimulated vascular development on adjacent epithelial structures, or a direct effect of VEGF-A on the epithelial cells themselves.

Alternative splicing of the murine *Vegf* results in three major isoforms [*Vegf*120, 164, and 188 (11); corresponding to the human isoforms 121, 165, and 189 (12)]. *Vegf*120 is diffusible, whereas *Vegf*164 and 188 can bind to heparan sulfate moieties on the cell surface and in the extracellular matrix (13). These isoforms are expressed in distinct temporospatial patterns, suggesting that each may serve a specific developmental function. In the mouse, *Vegf*164 is the most abundant during early lung development (E 9.5–E 16), but the levels of both *Vegf*164 and *Vegf*120 decrease as development progresses, and remain relatively low in the adult lung. In contrast, *Vegf*188 becomes the predominant isoform after E16, remaining high throughout adulthood (12). Furthermore, mice that produce only *Vegf*120, but not *Vegf*164 or *Vegf*188, demonstrate pruned vasculature and delayed airway development, suggesting that *Vegf*164 and/or *Vegf*188 are required for normal airway maturation (14).

In contrast to the hypoxia-induced increase of *Vegf*-A expression in alveolar cells (7), *Vegf* expression is reduced by hyperoxia, mainly due to suppressed expression by alveolar type II cells (15). Consistent with these *in vitro* studies, VEGF-A expression is reduced in airway aspirates of children with bronchopulmonary dysplasia (16) and respiratory distress

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; **DAPI,** 4′, 6-diamidino-2-phenylindole; **E,** embryonic day; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **RT,** reverse transcription

syndrome (17), conditions in which the airways are exposed to hyperoxia.

Based on these observations, we hypothesized that hypoxia-induced VEGF expression is important for normal airway development and that down-regulation of VEGF expression in the setting of hyperoxia may play a significant role in the failure of normal arborization of the airways. To test this hypothesis, we used an established model of explanted embryonic lung for the quantitative study of branching morphogenesis (18) to investigate the effects of hyperoxia and VEGF on lung growth, branching morphogenesis, and airway cell proliferation and survival.

MATERIALS AND METHODS

Lung explant culture. Pregnant Sprague-Dawley rats (Harlan Bioproducts for Science, Indianapolis, IN) on E12 were euthanized and lung embryos were cultured as explants and placed on Transwell permeable supports that had DMEM containing 10% fetal bovine serum and penicillin 12.5 U/mL/ streptomycin 12.5 μg/mL (Invitrogen, Carlsbad, CA). Explants were cultured for up to 96 h at 37°C in sealed chambers (Billups-Rothenberg, Del Mar, CA) equilibrated to a humidified atmosphere of 5% CO_2 with defined oxygen concentrations, balanced by nitrogen. Medium was replaced every 48 h. In some experiments, human VEGF165 or VEGF121 (R & D Systems, Minneapolis, MN) were added to the medium at a concentration of either 50 or 100 ng/mL as described by other investigators (9). All experiments were conducted under approved Institutional Animal Care and Use Committee guidelines at Yale University.

Quantification of lung branching. Explants were randomly assigned to 3% O_2 or high oxygen (50% or 60% O_2) culture ± VEGF. Some experiments consisted of exposure to 50% O_2 for 48 h followed by 3% O_2 for another 48 h. Lungs were digitally photographed at 0, 48, and 96 h using a Nikon TE200 microscope. The images were used to determine the number of terminal bud branches (structures with blind ends) and to quantitate total branch length (sum of straight lines beginning at the point of branching and ending at the branch tip) (Fig. 1*A*). Each experiment used 9–10 explants with two to three lungs in each experimental group. The results for each explant in the experimental group were averaged and counted as n of 1. The entire experiment was repeated on at least three to five occasions.
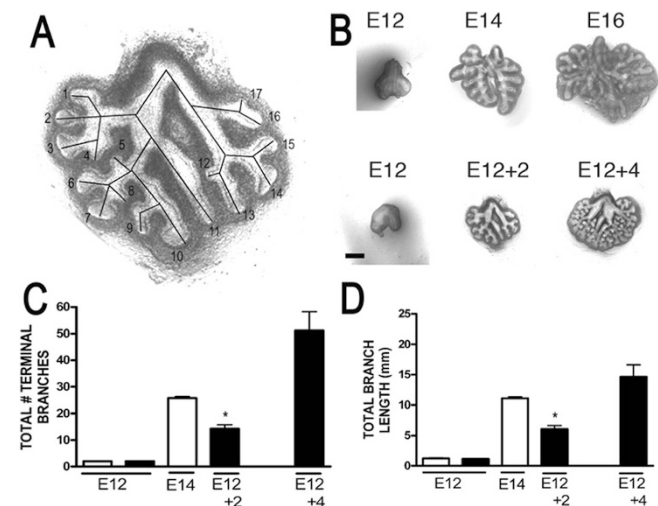
Figure 1. Comparison of morphogenic lung development *in vitro* and *in vivo*. (*A*) Cultured embryonic lungs adopt a flattened morphology that allows quantification of terminal branches (17 for this example) and branch length. (*B*) Lungs harvested at E12, E14, and E16 (*upper panel*) are compared with lungs harvested at E12 and cultured in 3% oxygen for 0, 2, or 4 d (*lower panel*). (*C*) Terminal branches and (*D*) branch length were quantitated in lungs exposed to 3% oxygen (■) for 0, 2, or 4 d and compared with harvested lungs (□) at E12 and E14. n = 5. Photographs at 4× magnification. *$p < 0.05$ *vs* E14. Bar = 1 mm.

Cell proliferation assay. Explants were incubated in 5 mM of 5-bromo-2-deoxyuridine (BrdU) (BD PharMingen, San Diego, CA) and cut in 1-μm sections. These were washed in PBS followed by antigen retrieval. The sections were permeabilized in 0.2% Triton for 1 h and then washed three times and nonspecific binding blocked for 1 h. Sections were incubated overnight with monoclonal anti-BrdU (1:100, Sigma Chemical Co., St. Louis, MO) and incubated with goat anti-mouse antibody conjugated to Alexa 448 (1:200). Sections were mounted with VectaShield containing 4′, 6-Diamidino-2-phenylindole (DAPI). The proportion of BrdU-labeled epithelial cells was determined by counting the number of BrdU-positive cells (typical punctate pattern throughout the nucleus) from three to four representative lung branches and dividing by the total number of cells (DAPI positive) in that branch, using an average of 12 views per experiment. Over 850 cells were counted for each condition. The cell proliferation index was measured as the percentage of BrdU-positive epithelial cells compared with the total number of epithelial cells in the airway.

Cell apoptosis assay. Analysis of apoptosis was performed by the TUNEL In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN). Sections were counterstained with DAPI. A minimum of 12 views of three to four airway structures were analyzed for each explant and three explants were analyzed per experiment (averaged to comprise an n of 1 for each experiment) with a total of three experiments per condition. Cells were included only if they were contained within the airway branch and were defined as apoptotic only if the entire nucleus was stained by TUNEL. Over 1000 cells were counted for each condition. The apoptosis index was defined as the percentage of TUNEL-positive epithelial cells/total number of cells in the airway structure.

Identification of airway and nonairway cells by double immunostaining. Sections were washed in PBS followed by antigen retrieval and then blocked for 1 h. To distinguish airway epithelial from mesenchymal compartment cells, the basement membrane component fibronectin was identified by incubating sections with a polyclonal antibody (1:400, Sigma Chemical Co.) and incubated with goat anti-rabbit antibody conjugated to Alexa 594 (1:200). Subsequently, stainings for cell proliferation and cell apoptosis were performed.

Quantification of VEGF mRNA expression. After 2 d of culture, total RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA). Three hundred nanograms of total RNA were used for reverse transcription (RT) and 1 μL of this reaction was used for PCR analysis. The primers were:

*Vegf-A*164 Forward: 5′-GAACAAAGCCAGAAAATCACTG-3′, *Vegf-A*164 and *Vegf-A* 188 reverse: 5′-CACCGCCTTGGCTTGTCACAT-3′, *Vegf-A*188 Forward: 5′-CAGATGTGAATGCAGACCAAA-3′, Glyceraldehyde 3-phosphate Dehydrogenase (*Gapdh*) Forward: 5′-TCACCAC-CATGGAGAAGGC-3′, *Gapdh* Reverse: 5′-GCTAAGCAGTTGGTGGTGCA-3′

Gene expression was measured by MyiQ real-time PCR (Bio-Rad, Hercules, CA). After RT, 47 cycles of amplification were performed (denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s). All reactions had an efficiency between 90 and 100%. To determine the starting quantity (SQ) of each isoform, a standard curve was constructed from a RNA sample with a known concentration and calculated by using the iQ PCR Detection System Software. Results were normalized by dividing the SQ for the isoform by the SQ for GAPDH for that same PCR reaction. The specific amplification of the desired isoform was verified by the correlation coefficient of the standard curve of ≥0.94, the appearance of a single amplified product on agarose gel electrophoresis, and the absence of product in samples lacking RT. The SQ of GAPDH at 3% *versus* 50% oxygen was similar.

Immunoblotting. Twelve lung explants at E12 were cultured in 3% and 50% oxygen and after 2 d, they were homogenized in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM Tris, 0.16 M NaCl, 1 mM EGTA, 1 mM EDTA, 15 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL leupeptin, and 0.5 μg/mL pepstatin A) and immunoblotted for HIF-1 alpha (rabbit polyclonal antibody 1:1000; Novus Biologicals, Littleton, CO). To ensure equal loading, membranes were re-probed with antibody to heat shock cognate 70 (1:5,000) (Stressgen Bioreagents, Assay Designs, Ann Arbor, MI).

Statistical analysis. All data are expressed as means ± SEM. Paired *t* test or one-tailed *t* test were used for calculating statistical significance, where appropriate. A $p < 0.05$ was considered statistically significant.

RESULTS

Branching morphogenesis in cultured embryonic lung explants simulates in vivo ***lung development.*** Initial experiments were performed to determine whether the explanted

lung underwent linear growth and branching morphogenesis that were comparable to that observed *in vivo*. These experiments were performed under 3% oxygen to simulate embryonic *in vivo* lung oxygen concentrations (4,19). Lung branch length and number were quantitated by creating a simple two-dimensional stick diagram of the explanted organ (Fig. 1*A*).

Embryonic lungs cultured in 3% oxygen for 2 or 4 d grew extensively and underwent substantial branching morphogenesis. These lungs reached about 60% of the size of lungs removed from age-matched *in vivo* controls (Fig. 1*B*, E14 lungs compared with E12 + 2 d of culture and E16 lungs compared with E12 + 4 d of culture). Quantification of total terminal bud branches and branch length revealed a marked progression of both processes, again reaching approximately 60% of that seen *in vivo* (Fig. 1, *C* and *D*). Quantification of E16 lungs was not performed due to their tridimensional structure, which prevented accurate visualization of all branches (Fig. 1*B*, *upper right*). These data suggest that lung explants are a valid model for the study of branching morphogenesis in the embryonic lung.

Hyperoxia inhibits **in vitro** ***lung development via enhanced apoptosis and decreased proliferation.*** To examine the effects of hyperoxia on lung development, embryonic lung explants grown under 3% O_2 (control) were compared with those grown in increasing concentrations of oxygen. Lung explants grown for 2 d in 21% O_2 had 17% fewer terminal bud branches and were 5% shorter in total branch length compared with controls grown in 3% O_2, although these differences did not reach statistical significance (data not shown). However, explants grown in 50% O_2 revealed a moderate reduction in terminal branch number and total branch length after 2 d of culture, and significantly reduced growth after 4 d (Fig. 2). Interestingly, a modest recovery in the total lung growth (30%) was observed in lungs that were exposed to 50% O_2 for 2 d and then returned to 3% O_2 for an additional 2 d, compared with lungs grown in 50% O_2 for the entire 4 d. However, this increase in the total branch length was significantly lower than control explants grown in 3% O_2 for 4 d (3% O_2: 12.48 ± 2.6 mm; 50% O_2 for 4 d: 5.17 ± 1.85 mm; 50% O_2 for 2 d and 3% O_2 for 2 d: 6.78 ± 2.49 mm $p < 0.05$ *versus* 3% O_2 alone; $n = 3$). Oxygen concentrations of 60% or higher resulted in the rapid explant death (Fig. 2). These data demonstrate that *in vitro* oxygen concentrations of 50% or greater have a marked inhibitory effect on normal lung development.

To determine the mechanism of hyperoxia-induced inhibition of embryonic lung growth, the rates of cellular apoptosis and proliferation were examined in the lung explants. E12 lung explants were cultured for 2 d in 3% or 50% O_2. The proportion of TUNEL positive cells was significantly higher in explants cultured in 50% O_2 (8.3 ± 1.6%)) compared with those cultured in 3% O_2 (3.2 ± 1.7%), (Fig. 3). Dividing cells were seen in both the airway epithelium and surrounding mesenchyme. However, both the absolute number of dividing cells and the proliferation index were higher in explants cultured in 3% O_2 compared with culture in 50% O_2 (10 ± 4% *versus* 1.98 ± 0.25%, $p < 0.05$) (Fig. 3).

Hyperoxic inhibition of lung development is mediated in part by down-regulation of VEGF expression. It has been
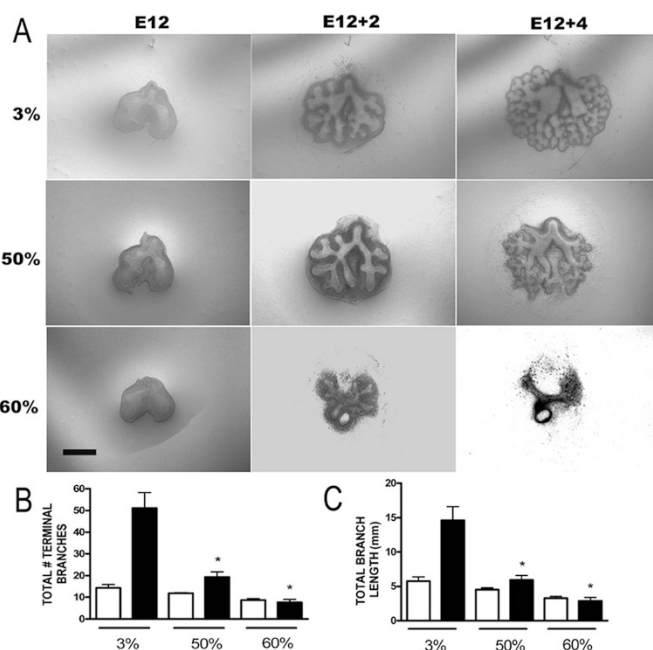

Figure 2. Hyperoxia inhibits lung branching morphogenesis. (*A*) Lung explants cultured for 0–4 d under 3% oxygen were compared with explants exposed to 50% or 60% oxygen. (*B*) Terminal branches and (*C*) branch length were quantitated and compared for each condition. □, E12 + 2; ■, E12 + 4. $n = 4$. *$p < 0.05$ *vs* 3% O_2. Photographs at 4× magnification. Bar = 1 mm.

previously demonstrated that hypoxia increases the transcriptional activation and mRNA stabilization of HIF-1α, leading to increased expression of VEGF. Consistent with this, exposure of the embryonic lung explant to hyperoxia was found to
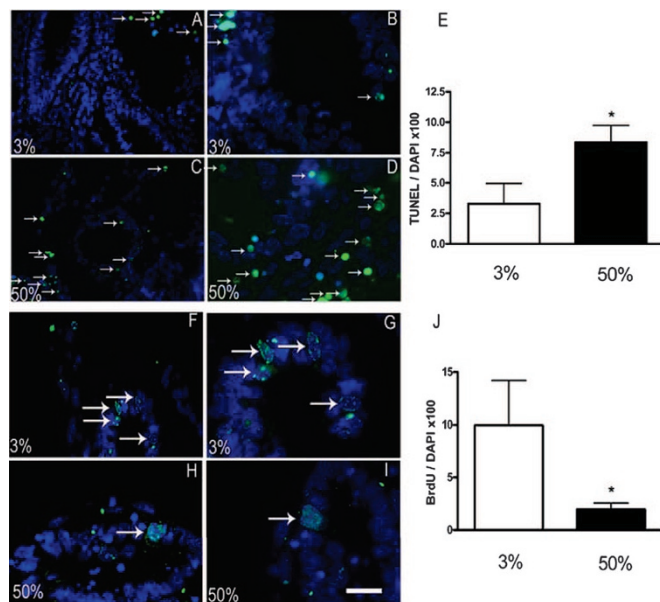
Figure 3. Hyperoxia increases airway cell apoptosis and inhibits airway cell proliferation. E12 lungs were cultured for 48 h under 3% (*A, B* and *F, G*) or 50% oxygen (*C, D* and *H, I*), then cell apoptosis determined by TUNEL staining (*A, B, C, D*) and cell proliferation by BrdU uptake (*F, G, H, I*). (*E* and *J*) Quantitation of number of apoptotic cells and proliferating cells/100 DAPI-positive nuclei. $n = 3$ with 2–3 lungs/condition/experiment. *$p < 0.05$. Arrows demonstrate TUNEL+ cells and BrdU+ nuclei. *Left panels* are 40× and *right panels* are 100× magnification. Bar = 20 μm
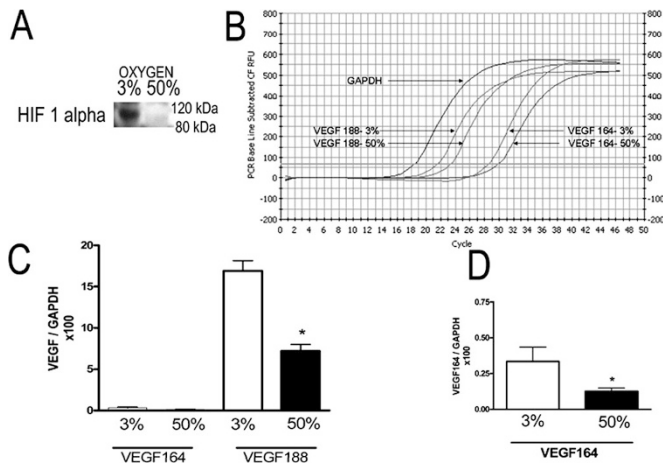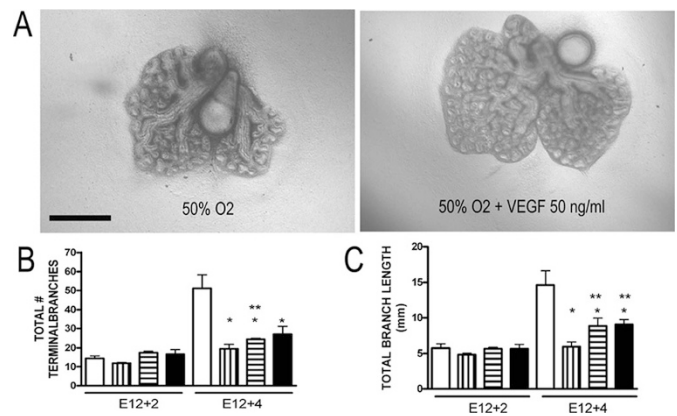
Figure 4. Hyperoxia down-regulates HIF-1α expression and inhibits VEGF mRNA expression. (*A*) HIF-1α protein expression from E12 lungs after 2 d of exposure to 3% or 50% oxygen. (*B*) Real-time PCR for VEGF164, VEGF188, and GAPDH. (*C*) A graphic representation of the SQ of VEGF164 and VEGF188 normalized to the SQ of GAPDH. (*D*) Enlargement of the columns for VEGF164 seen in (*C*). $n = 3$, $*p < 0.05$.

Figure 5. Hyperoxia-dependent inhibition of lung morphogenesis is attenuated by VEGF165. (*A*) E12 lung explants exposed to 50% oxygen ± VEGF165 (50 ng/mL) for 5 d. (*B*) Total number of terminal branches and (*C*) total branch length were quantitated at E12 + 2 and E12 + 4 d. □, 3% oxygen; ▥, 50% oxygen; ▤, 50% oxygen + VEGF165 at 50 ng/mL; ■, 50% oxygen + VEGF165 at 100 ng/mL. $n = 3$, $*p < 0.05$ *vs* 3% alone and $**p < 0.05$ *vs* 50% alone at E12 + 4. Photographs at 4× magnification. Bar = 1 mm.

inhibit HIF-1α protein expression (Fig. 4*A*). To determine the impact of oxygen concentration on VEGF expression, quantitative PCR for *Vegf*164 and *Vegf*188 mRNA was performed. Explants cultured for 2 d in 3% O_2 expressed approximately 50-fold greater amounts of *Vegf*188 mRNA compared with *Vegf*164 (Fig. 4*B*, quantitated in *C*). Culture in 50% O_2 resulted in a 2.5-fold decrease in both *Vegf*188 and *Vegf*164 mRNA expression (Fig. 4*B*, quantitated in *C* and *D*), whereas culture in 21% oxygen yielded a lesser decrease in VEGF expression (data not shown).

To determine whether the decrease in VEGF expression plays a role in hyperoxic inhibition of lung development, human recombinant VEGF165 or VEGF121 were added to the medium of explants grown in 50% O_2. VEGF165 induced a partial but significant reversal of the decrease in terminal bud branching and total branch length at both 50 and 100 ng/mL concentrations, whereas VEGF121 failed to exhibit this effect (Fig. 5). Of note, VEGF165 failed to rescue explants cultured in 60% O_2 (data not shown).

Vegf165 attenuates hyperoxic injury through decreased apoptosis of the airway epithelial cells. To determine whether added VEGF improves lung growth *via* diminution of the toxic effects of hyperoxia on airway cell viability, E12 lung explants were cultured for 2 d at 3% or 50% O_2 ± VEGF (50 ng/mL) followed by TUNEL staining to detect apoptosis or BrdU incorporation to define proliferation. The addition of VEGF165 significantly reduced apoptosis in explants cultured in 50% O_2 (5.96 ± 3.8 *versus* 10.74 ± 2.7%, $**p < 0.05$ 50% O_2 with VEGF *versus* 50% O_2 alone) (Fig. 6, *A, B*, quantitated in *C*). To determine which cell types were responding to the added VEGF, airway epithelial cells and mesenchymal cells were identified based on their location within or outside of the airway basement membrane. Cells in both compartments were found to exhibit a similar degree of hyperoxia-induced apoptosis, based on TUNEL staining following culture in 50% O_2. Interestingly, the beneficial effect of VEGF165 was primarily due to reduced apoptosis in the airway epithelial cells

(6 ± 1.8% *versus* 12.1 ± 5.2%, //$p < 0.05$) (quantitated in Fig. 6*D*), with a lesser effect seen in the mesenchymal cells (7.7 ± 3.3% *versus* 10 ± 4%, $p = $ NS).

Examination of BrdU uptake again confirmed that the absolute number of dividing cells and the proliferation index were higher in explants cultured in 3% O_2 (9.2 ± 1.6%) compared with 50% O_2 (4.9 ± 2.7%) ($*p < 0.05$) (quantitated in Fig. 6*G*). Comparison of airway epithelial cells and mesenchymal cells under these conditions revealed that both cell types underwent division during growth of the explant under 3% oxygen, and that the majority of the inhibition of proliferation seen during culture under 50% oxygen was probably due to a decrease in proliferation in the mesenchymal cell compartment (4.8 ± 4.6% in 50% O_2 *versus*. 8.2 ± 1.1 in 3% O_2) (data not shown). Addition of VEGF165 resulted in a modest rescue of cell proliferation in explants grown under 50% O_2, although this did not reach statistical significance in either the epithelial compartment, mesenchymal compartment, or cumulatively (Fig. 6, *E, F,* quantitated in *G* and *H*).

DISCUSSION

In this study, we use a morphometric analysis of the rat lung and in agreement with others, we found that embryonic lung explants grow well in low oxygen concentrations that approximate the *in vivo* environment (3,20). The quantitative results of this work in 3% O_2 culture show that although lung growth is somewhat slowed in the explanted organ compared with development *in vivo*, overall terminal bud branching and branch elongation proceed at rates that are comparable to those seen *in vivo*, making this a useful model for the study of factors that regulate branching morphogenesis.

In contrast to the results in low oxygen culture, we show that high oxygen concentrations are clearly inhibitory for embryonic lung development. A dose response was observed in which exposure to 21% oxygen during the culture appeared to slow growth and morphogenesis modestly (with no statis-
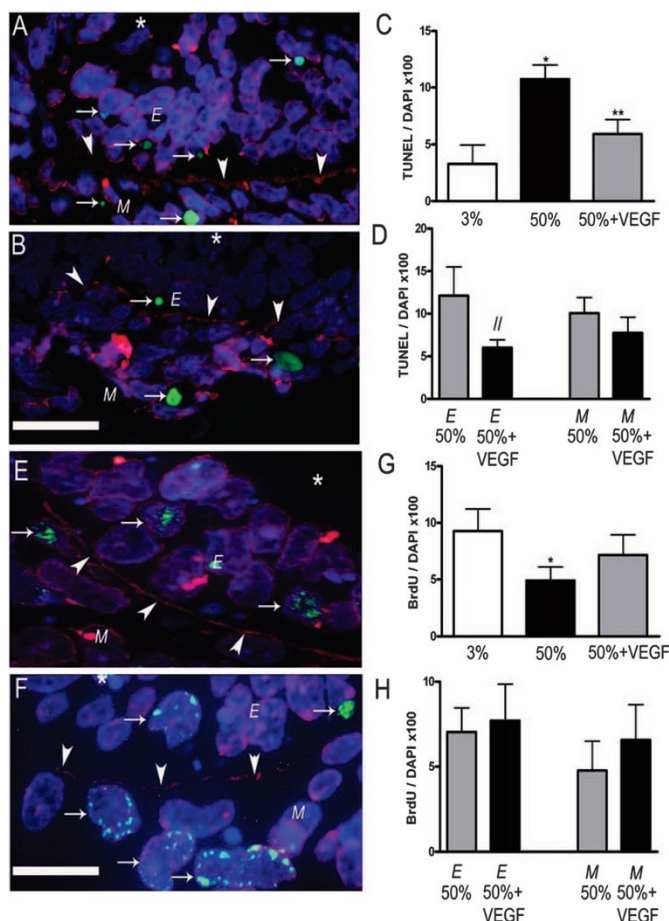
Figure 6. VEGF165 decreases hyperoxia-induced apoptosis in airway epithelial cells. E12 lungs were cultured for 48 h under 50% oxygen (*A, E*) or 50% oxygen + VEGF165 (*B, F*) (50 ng/mL) and arrows either show apoptotic cells detected by TUNEL staining (*A, B*) or proliferating cells detected by BrdU uptake (*E, F*). Fibronectin staining of the basement membrane (*arrowheads*) was used to identify epithelial (*E*, inside the basement membrane) and mesenchymal cells (*M*, outside). (*C* and *G*) Quantitation of total number of apoptotic cells or proliferating cells/100 DAPI-positive nuclei respectively in explants cultured under 3% or 50% oxygen ±VEGF165. (*D* and *H*) Quantitation of apoptosis or proliferating airway epithelial (*E*) and mesenchymal (*M*) cells following culture in 50% oxygen ±VEGF165. $n = 4$ with 2–3 lungs/condition/experiment. *$p < 0.05$ *vs* 3% alone. **$p < 0.05$ *vs* 50% alone. //$p < 0.05$ *vs* E 50% alone. Asterisk = lumen. Photographs at 100× magnification. Bar = 20 μm.

tical difference from 3% oxygen control), whereas 60% oxygen concentrations resulted in complete inhibition of explant growth and development. Exposure of the rat lung explant to 50% oxygen resulted in an intermediate phenotype that allowed us to examine the mechanism of inhibition. Our study agrees with others (21,22) that show that high oxygen concentrations induce accelerated apoptosis in the embryonic lung. The apoptosis is believed to occur primarily due to the generation of reactive oxygen species (ROS) and the subsequent activation of caspases, although Budinger *et al.* (23) demonstrated that hyperoxia-stimulated apoptosis can occur independent of ROS generation. In our experiments, hyperoxia-induced apoptosis was seen in both the epithelial cells as well as the mesenchymal cells surrounding the basement membrane.

In addition to increased apoptosis, lungs cultured in 50% O_2 also demonstrated a decrease in cellular proliferation. This

effect does not appear to be mediated simply by cellular toxicity since many cells were still proliferating under these conditions and the explanted lungs continued to grow and branch, albeit at a slower pace. Combined, these results suggest that hyperoxia might down-regulate an anti-apoptotic and/or pro-proliferative factor that is generated in the explanted lung. Based on its known regulation by oxygen concentration and ability to stimulate lung development (24), we examined the possibility that suppression of VEGF expression was contributing to the growth inhibition seen under hyperoxic culture conditions.

Quantitative determination of VEGF expression revealed that the *Vegf*188 isoform predominates in the rat lung as early as E12 + 2 d of culture. This is somewhat different from previous reports in the mouse lung where *Vegf*164 was the dominant isoform until E16 (12), possibly due to species differences between the rat and mouse or differences in the technique of explant. This isoform switch between *Vegf*164 and *Vegf*188 during development raises the possibility that these two proteins might have distinct functions that are important for specific stages of lung growth, branching, and/or maturation. In conceptual support of this possibility, we have recently shown that murine *Vegf*120 and *Vegf*164 signal in distinct fashions that can independently regulate cell proliferative and morphogenic responses (10), and in the present study we demonstrate that VEGF165 protects against hyperoxia-induced inhibition of lung growth whereas VEGF121 does not. The known ability of VEGF165 to bind heparan sulfate proteoglycans and to signal through neuropilin-1, functions not shared by VEGF121, suggests that either the mechanism of local presentation of VEGF to the airway cell or neuropilin-dependent signaling may be critical for these protective effects of VEGF. To date, there are no known signaling or functional differences between *Vegf*164 and *Vegf*188, but the up-regulated expression of *Vegf*188 late in gestation suggests that this should be investigated once the reagents are available.

Because both airway epithelial cells and surrounding mesenchymal cells are exposed to hyperoxia, it is possible that decreased expression of VEGF in response to hyperoxia prevents normal endothelial cell function, and that a subsequent loss of paracrine functions of the endothelial cell prevents adjacent airway growth and branching. Thus, addition of VEGF might reconstitute this paracrine role of the endothelial cell. Alternatively, VEGF may directly signal through receptors on the epithelial cells themselves. Indeed, in the embryonic kidney we have found that epithelial cells from the ureteric bud express low levels of VEGF receptors, and that these cells can respond to VEGF in a pro-proliferative and pro-morphogenic fashion (10). The observation that VEGF165 only partially rescues hyperoxic growth suppression suggests that pathways independent of VEGF may also be important in mediating the suppressive effects of hyperoxia, but is consistent with the possibility that both VEGF165 and VEGF188 may be required for the maximal protective response.

In conclusion, this study employs a quantitative model for the study of lung branching morphogenesis, and utilizes this approach to demonstrate that hyperoxia suppresses normal lung growth and development in part due to suppression of VEGF

function. Since VEGF has been shown to accelerate lung branching morphogenesis (9), enhance neonatal alveolarization (25), and increase epithelial proliferation and surfactant production (26), further studies are warranted to explore the possible protective role of VEGF as an intervention to promote maturation and maintain airway integrity during exposure to high levels of inhaled oxygen. However, the observation that excessive VEGF can induce vascular leak and disrupt airway development (27) suggests that careful studies in animals will be required before consider VEGF as a therapeutic tool in humans.

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