VEGF induces airway epithelial cell proliferation in human fetal lung in vitro

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Brown, Karen R. S., Katherine M. England, Kelli L. Goss, Jeanne M. Snyder, and Michael J. Acarregui. VEGF induces airway epithelial cell proliferation in human fetal lung in vitro. Am J Physiol Lung Cell Mol Physiol 281: L1001–L1010, 2001.—Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen involved in normal and abnormal angiogenesis. VEGF mRNA and protein are abundant in distal epithelium of midtrimester human fetal lung. In the present study, we identified immunoreactivity for KDR, a major VEGF-specific receptor, in distal lung epithelial cells of human fetal lung tissue, suggesting a possible autocrine or paracrine regulatory role for VEGF in pulmonary epithelial cell growth and differentiation. Addition of exogenous VEGF to human fetal lung explants resulted in increased epithelium volume density and lumen volume density in the tissues, both morphometric parameters of tissue differentiation. Cellular proliferation demonstrated by bromodeoxyuridine uptake was prominent in distal airway epithelial cells and increased in the VEGF-treated explants. VEGF-treated explants also demonstrated increased surfactant protein (SP) A mRNA, SP-C mRNA, and SP-A protein levels compared with controls. However, SP-B mRNA levels were unaffected by VEGF treatment. [3H]cholesterol incorporation into total phosphatidylcholine was increased by VEGF treatment, but incorporation into disaturated phosphatidylcholine was not affected by exogenous VEGF. Based on these observations, we conclude that VEGF may be an important autocrine growth factor for distal airway epithelial cells in the developing human lung.

vascular endothelial growth factor; KDR; neuropilin-1; lung epithelium; surfactant proteins; cell proliferation

VASCULAR ENDOTHELIAL GROWTH factor (VEGF) is a potent endothelial cell mitogen that also promotes endothelial cell chemotaxis and capillary permeability, properties important for normal and pathological angiogenesis (13). Although VEGF is expressed by many different nonendothelial cells, VEGF mRNA and protein are found in greatest abundance in the distal airway epithelial cells of the developing and adult lungs (4, 26, 34). The abundance of VEGF in the developing lung suggests a role for this growth factor in the development of the pulmonary capillary bed, whereas expression of VEGF in the distal lung of the adult suggests that VEGF may play a role in maintaining the integrity of the already-established air-blood barrier.

Human VEGF mRNA exists as five different isoforms, each derived from alternative splicing of mRNA transcribed from a single VEGF gene located on chromosome 6p21.3 (40). Three receptors have been identified for VEGF. These are Flk-1 (also known as VEGFR-1), KDR (also known as VEGFR-2; Flk-1 is the mouse homolog of KDR), and neuropilin-1 (also known as VEGF165R). Flk-1 is thought to mediate the role of VEGF to cause the organization of endothelial cells into vascular structures (14). Activation of Flt-1 also results in the migration of some cell types (3). KDR is the VEGF receptor responsible for endothelial cell mitogenesis (41) and migration (46). The recently described VEGF receptor neuropilin-1 binds only the 165-amino acid isoform of VEGF (37). The binding of VEGF165 to KDR is enhanced by the presence of neuropilin-1, whereas cells that express neuropilin-1 alone fail to respond to the mitogenic and chemotactic effects of VEGF, suggesting that neuropilin-1 functions solely as a coreceptor (37).

Human fetal lung explants maintained in vitro are a well-characterized model for studying lung development. The distal epithelium in human fetal lung tissue, derived from midtrimester abortuses and maintained in explant culture in serum-free defined medium, differentiates spontaneously from an undifferentiated columnar epithelium to an epithelium composed of differentiated type II pneumocytes (36). We have recently demonstrated the expression of VEGF mRNA and protein in the distal airway epithelial cells of midtrimester human fetal lung. VEGF mRNA and protein levels increased in human fetal lung tissues incubated in serum-free medium in association with epithelial cell differentiation into type II pneumocytes (2). Interestingly, VEGF produced by the epithelial cells localized to the basement membrane subjacent to the epithelium when the tissue was placed in explant culture for 4 days. These data support the hypothesis that VEGF production by distal airway epithelium may be important in driving the develop-
ment of the pulmonary capillary bed and ultimately the air-blood barrier.

In preliminary studies, we observed the presence of KDR protein in the distal airway epithelial cells of midtrimester human fetal lung, data suggestive that VEGF may play an autocrine or paracrine regulatory role in epithelial cell growth and differentiation during lung development. Because VEGF is generally considered to be an endothelial cell growth factor, the goal of the current investigation was to determine if exogenous VEGF has an effect on distal airway epithelial cell proliferation and/or function in the developing lung. These studies were carried out in midtrimester human fetal lung explants so that relevance to the developing human lung could be assumed.

MATERIALS AND METHODS

Lung tissues and organ culture. Lung tissue was obtained from human abortuses of 16–20 wk gestation from local sources or from Advanced Biosciences Resources (Alameda, CA), as approved by the University of Iowa Human Subjects Review Committee. As previously described (1), the fetal lung tissue was dissected free of major blood vessels and airways, minced into 1- to 2-mm³ pieces with a sterile scalpel blade, and placed in organ culture. Lung tissues from one or two fetuses were used for each experiment. Cultures were maintained in serum-free Waymouth's MB752/1 medium (GIBCO, Grand Island, NY) in the absence or presence of human recombinant VEGF₁₆₅ (50 or 100 ng/ml; R&D Systems, Minneapolis, MN) for 4 days. Media were replaced daily. The explants were maintained at 37°C in an incubator containing a humidified atmosphere of 95% air-5% CO₂. Incubated tissue sections were harvested, frozen in liquid N₂, and stored at −70°C or harvested in 10% neutral buffered formalin (Surgipath Medical Industries, Richmond, IL) and stored at 4°C until they were embedded in paraffin. Additional explants were incubated, as described above, in the presence of bromodeoxyuridine (BrdU; 2 mM) for the final 24 h of incubation to label the nuclei of dividing cells (21).

Immunostaining. Human fetal lung tissue and lung explants were fixed overnight at 4°C in freshly prepared 10% neutral buffered formalin before being embedded in paraffin and sectioned on glass slides. The sections were deparaffinized, rehydrated through an ethanol-water series, and then incubated with primary antibody (mouse monoclonal anti-KDR directed against the extracellular domain (1:500, clone KDR-1; Sigma, St. Louis, MO), mouse monoclonal anti-KDR directed against the intracellular domain (1:100, clone A-3; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-KDR directed against the intracellular domain (1:50; Santa Cruz Biotechnology), polyclonal rabbit anti-neuropilin-1 (1:100; Santa Cruz Biotechnology)) for 1 h at room temperature. The sections were then rinsed two times for 10 min/rinse in PBS. Nonspecific binding sites were blocked with 2% normal goat serum at room temperature and then the sections were stained using a VECTASTAIN Elite kit (Vector Laboratories). The tissue sections were incubated for 30 min with an appropriate (anti-mouse, anti-rabbit) biotinylated secondary antibody at room temperature and rinsed two times in PBS for 5 min/rinse. The sections were then incubated for 45 min in avidin-peroxidase reagent. After being rinsed two times in PBS, 5 min/rinse, the sections were incubated in diaminobenzidine (700 μg/ml) for 1–3 min. Sections were rinsed in PBS for 5 min, counterstained with hematoxylin for 30 s, rinsed quickly in distilled water, and then dehydrated and mounted on glass coverslips. Negative controls for detection with mouse monoclonal antibodies were tissue sections incubated for 1 h with nonimmune mouse IgG (2 μg/ml) instead of primary antibody or nonimmune rabbit serum instead of primary rabbit antibody. Sections were viewed and photographed with a Nikon FX photomicroscope.

Morphological studies. Human fetal lung explants incubated in the absence or presence of VEGF were fixed overnight at 4°C in freshly prepared 10% neutral buffered formalin (Surgipath Medical Industries), and immunoreactivity for KDR was assessed as above. Representative sections were then used for morphometric analysis to determine the effect of exogenous VEGF exposure on the volume densities of the lumen, epithelium, and connective tissue in the explants, as described previously (27). Volume densities of the lumen, epithelium, and connective tissue were calculated as the percentage of the total tissue volume that was occupied by each component. The relative proportion of epithelium in the cellular content of the explants was calculated by dividing the volume density of the epithelium by the volume density of the total non-lumen tissue components. Three experiments were analyzed using lung tissues from different abortuses.

Cellular proliferation. Paraffin-embedded tissues that had previously been incubated in the absence or presence of VEGF and in the presence of BrdU (2 mM) were cut and mounted on glass slides. Immunoreactivity for BrdU was detected according to the manufacturer’s instructions using a commercially available kit (Zymed Laboratories, South San Francisco, CA). The proportion of BrdU-labeled epithelial cells was determined by counting the number of BrdU-positive cells within a particular prealveolar duct and dividing by the total number of epithelial cells in the prealveolar duct. In two different experiments with tissue from two different donors, a total of 13–25 representative ducts were counted for each condition. Data were analyzed using an unpaired, two-tailed Student’s t-test.

RNA isolation and Northern blot analysis of surfactant protein A, surfactant protein B, and surfactant protein C mRNA. Frozen tissues that had been incubated in the absence or presence of VEGF were thawed in 4.0 M guanidinium thiocyanate and homogenized. Total RNA was isolated (10) and quantitated by determining the absorbance at 260 nm. Ten micrograms of total RNA for each sample were separated on a 1.2% agarose-formaldehyde gel and then transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary transfer for Northern blot analysis for surfactant protein (SP) A, SP-B, and SP-C mRNA. The absence of ethidium bromide in the gels after transfer confirmed complete transfer. The membranes were baked for 20 min at 80°C, followed by cross-linking with ultraviolet irradiation. Membranes were prehybridized at 42°C for 2–4 h in ULTRAhyb (Ambion, Austin, TX) and then hybridized at 42°C overnight in buffer containing 1 × 10⁶ counts/min·ml⁻¹ of 32P-labeled human cDNAs specific for either SP-A, SP-B, or SP-C mRNA (human cDNAs were kind gifts of Dr. Jeffrey A. Whitsett, University of Cincinnati, Cincinnati, OH; see Refs. 19, 20, and 43). After hybridization, the blots were washed two times for 5 min at 42°C in 2× SSC (300 mM NaCl and 30 mM sodium citrate, pH 7.0) plus 0.1% SDS, two times for 30 min at 42°C in 0.1× SSC-0.1% SDS, and two times for 5 min at room temperature in 0.1× SSC. Blots were exposed to X-ray film with an intensifier screen at −70°C for 24–72 h. After the blots were hybridized to the SP cDNAs, they were stripped and reprobed with a radiolabeled cDNA for human 18S ribosomal RNA (American Type Culture Collection, Manassas, VA). Scanning densitometric values were corrected for RNA loading and
transfer errors by adjustment to the relative amount of 18S rRNA.

**Immunoblot analysis.** Midtrimester human fetal lung tissues that had been maintained in explant culture for 4 days in the absence or presence of VEGF were homogenized in ice-cold water containing phenylmethylsulfonyl fluoride (1 mM). The homogenates were centrifuged at 600 g for 5 min. Supernatant fractions were collected and assayed for total protein (6). Total protein (40 μg) was separated by electrophoresis on a 10% polyacrylamide gel using a Tris-glycine-SDS buffer. The separated proteins were transferred to Immobilon membranes (Millipore, Bedford, MA) by electrophoresis. The membranes were blocked with 5% nonfat dry milk (wt/vol) in Tris-buffered saline (TBS; 0.1 M, pH 7.5) that contained 0.05% (vol/vol) Tween 20 and were then incubated overnight at 4°C. SP-A was detected after incubation of the blots with guinea pig anti-human SP-A antiserum as previously described (15).

**[3H]choline incorporation into total phosphatidylcholine and disaturated phosphatidylcholine.** Fetal lung explants incubated for 4 days in the absence or presence of VEGF (50 ng/ml) were exposed to [3H]choline (1 μCi/culture dish) for the final 24 h of culture. The tissues were homogenized and then frozen in liquid N₂ and stored at −70°C. The protein content of the homogenate was determined by the method of Bradford (6). Lipids were extracted from equal amounts of protein by the method of Bligh and Dyer (5). The lipid extracts were dried under a nitrogen stream, suspended in 50 μl of chloroform-methanol (2:1 vol/vol), and spotted on silica LK5D gel plates (Whatman International, Maidstone, UK). Phospholipids were separated in chloroform-methanol-petroleum ether-acetic acid-boric acid (40:20:30:10:1.8 vol/vol/vol/wt/wt) as described by Gilfillan et al. (17). The radioactivity of the area corresponding to phosphatidylcholine (PC), as determined by comigration with known phospholipid standards (Sigma Chemical), was measured with an RTLC scanner (Radiometric Instruments, Tampa, FL). The incorporation of [3H]choline into disaturated phosphatidylcholine (DSPC) was also determined by the method of Gilfillan et al. (17). PC was separated in the first dimension in silica LK5D plated in chloroform-methanol-7 M ammonium hydroxide (65:35:5 vol/vol/vol). After visualization and marking of the areas corresponding to the migration of a PC standard, the plates were developed with acetone to 10 cm to remove excess iodine. The areas corresponding to PC were scraped from the silica gel and placed in 16 × 100-mm test tubes. The lipids were eluted from the silica gel with the use of chloroform-methanol-water-HCl and subsequently dried under a nitrogen stream. PC was oxidized with 100 μl of 5% osmium tetroxide for 3 min; excess osmium was then removed in the fume hood under a stream of nitrogen. The lipids were resuspended in 50 μl of chloroform-methanol 2:1 (vol/vol) and applied to silica LK5D plates. The plates were then developed to 10 cm in chloroform-methanol-7 M ammonium hydroxide (65:35:5 vol/vol/vol) in the second dimension. The DSPC species was readily identified by comigration with a DSPC standard (Sigma). This area was scraped and processed for counting using an RTLC scanner. Human fetal

![Fig. 1. Photomicrographs of immunoreactivity for KDR (indicated by the brown staining) in midtrimester human fetal lung tissue and explants cultured for 4 days. A and B: immunoreactivity detected by the Santa Cruz antibody (A) and by the Sigma antibody (B) in human fetal lung tissue. C and D: immunoreactivity for KDR in lung explants detected by the Santa Cruz antibody (C) and by the Sigma antibody (D). E: KDR immunoreactivity in mesenchymal and arterial endothelial cells. F: control tissue incubated with nonimmune mouse IgG instead of primary monoclonal antibody against KDR demonstrates the specificity of the antibody. Examples of immunoreactive distal airway epithelial cells (filled arrowheads), mesenchymal cells (open arrowheads), and a small artery (*) are indicated. Bar = 50 μm.](image-url)
lung explants from five different experiments were assayed for the effect of VEGF on phospholipid synthesis.

**Statistical analysis.** Statistical comparisons of the densitometric mRNA data were made using a two-tailed Dunnett’s test (47). Other data were analyzed by one-way ANOVA. Comparisons between individual groups were made with Student’s t-test. Differences were considered significant at $P < 0.05$.

**RESULTS**

Detection of the VEGF receptor, KDR, in midtrimester human fetal lung. Immunoreactivity for KDR in midtrimester human fetal lung tissue was detected in the distal airway epithelium and in scattered mesenchymal cells by two different monoclonal antibodies (Fig. 1, A and B). Explants incubated for 4 days continued to exhibit immunoreactivity for KDR in distal airway epithelial cells (Fig. 1, C and D). A significant degree of immunoreactivity for KDR was observed to localize to the nuclei of selected epithelial cells (Fig. 1, A, C, and D), although nuclear localization was absent in tissues before incubation that were stained with the Sigma monoclonal antibody, clone KDR-1 (Fig. 1B). In addition to KDR immunoreactivity in selected mesenchymal cells, staining was also observed in the endothelial cells of blood vessels (Fig. 1E). Immunoreactivity for KDR in lung tissue explants treated with VEGF was not observed to be different from that in control explants (Fig. 2). Because neuropilin-1 has previously been demonstrated to be a coreceptor with KDR for the isoform-specific binding of VEGF165 (37), we performed immunostaining for neuropilin-1 in human fetal lung and lung tissue explants. Consistent with the finding of immunoreactivity for KDR in the distal airway epithelium, we observed immunoreactivity for neuropilin-1 in selected distal airway epithelial cells of both the midtrimester lung and the lung tissue explants (Fig. 3).

Effect of VEGF on morphology of human fetal lung in vitro. Human fetal lung explants incubated in the presence of VEGF (100 ng/ml) for 4 days exhibited a decrease in the relative amount of connective tissue and an increase in the size of the ductal lumens compared with untreated explants (Fig. 2). These observations were quantified by morphometric analysis using two previously described indexes of tissue differentiation, lumen volume density and epithelium volume density (Fig. 4; see Ref. 27). Both lumen volume density and epithelium volume density were increased significantly in the VEGF-treated lung tissues. Lumen volume density increased from $\sim 35\%$ in control tissue to $\sim 50\%$ in VEGF-treated tissue, whereas the epithelium volume density increased from $\sim 50\%$ in the controls to $\sim 65\%$ in the VEGF-treated tissue (Fig. 4). Thus VEGF treatment resulted in an increase in the volume density of epithelial cells and dilatation of the distal airway ducts in human fetal lung explants maintained in vitro.

BrdU uptake and immunoreactivity of proliferating cells. To determine if treatment of human fetal lung explants with VEGF causes the proliferation of cells
within the human fetal lung explants, tissues were incubated in the presence of BrdU, a thymidine analog, during the final 24 h of culture. The number of BrdU-immunoreactive cells was increased in VEGF-treated tissues (Fig. 5). A significant increase in the proportion of BrdU-labeled epithelial cells was observed in the VEGF-treated tissue (50 and 100 ng/ml), 70 and 75%, respectively, compared with the proportion of BrdU-labeled epithelial cells in control tissues (45%; Fig. 6). BrdU immunoreactivity was observed primarily in the epithelial cells of the lung tissue explants, data suggestive of active proliferation of epithelial cells under the influence of VEGF.

**Effect of VEGF on SP-A, SP-B, and SP-C mRNA levels and on levels of SP-A protein.** To determine if exogenous VEGF influences type II cell differentiation in human fetal lung in vitro, we evaluated mRNA levels for the surfactant-associated proteins SP-A, SP-B, and SP-C by Northern blot analysis of total mRNA. SP-A mRNA levels were increased compared with those in controls in the presence of VEGF by ~20 and 90% in explants treated with 50 or 100 ng/ml of VEGF, respectively (Fig. 7A). Statistical significance was reached between the control and VEGF (100 ng/ml) treatment. There was no apparent effect of VEGF treatment on SP-B mRNA levels (Fig. 7B). In contrast, SP-C mRNA levels appeared to be increased in VEGF-treated tissues, however, not significantly (Fig. 7C). SP-A protein levels, as evaluated by immunoblot analysis of total protein, compared with control explants, were increased by ~50% in tissues treated with 100 ng/ml of VEGF (Fig. 8).

**Effect of VEGF on choline incorporation into phospholipids.** To examine another parameter of type II cell differentiation, human fetal lung tissue was incubated with [3H]choline in the absence or presence of VEGF, and choline incorporation into PC and DSPC was determined. VEGF treatment resulted in an ~50% increase in choline incorporation into PC. However, com-
pared with controls, there was no significant difference in choline incorporation into the more type II cell-specific DSPC (Fig. 9).

**DISCUSSION**

The developing lung requires the formation and maintenance of an extensive capillary network in close proximity to a thin layer of alveolar epithelial cells to provide for efficient gas exchange. VEGF is a potent inducer of endothelial cell growth that has been detected in abundance in the distal epithelium of the fetal lung (2, 34). This observation is suggestive that fetal lung epithelial cell VEGF may play an important role in pulmonary microvascular organization, development, and maintenance.

However, the results of the present study suggest a role for VEGF in addition to that of vasculogenesis, namely that of an epithelial cell growth factor. We have demonstrated that exogenous VEGF increases parameters of tissue differentiation in explants of midtrimester human fetal lung and that distal airway epithelial cells proliferate in response to exogenous VEGF. Epithelial cells in human fetal lung explants spontaneously differentiate in vitro, resulting in an epithelium with the typical ultrastructural morphology of type II pneumocytes (36). This differentiation pattern has
been confirmed by the increased production of several surfactant components, including SP-A and DSPC. Human fetal lung explants treated with VEGF had increased epithelial cell proliferation and decreased amounts of connective tissue, suggesting that VEGF is a mediator of fetal lung epithelial cell differentiation in vitro.

We have recently demonstrated the presence of abundant VEGF mRNA and protein in type II pneumocytes in human fetal lung explants maintained in vitro and the localization of VEGF to the basement membrane of the distal airway epithelial cells (2). In the present study, we have detected the VEGF receptor KDR in mesenchymal cells and in the distal lung epithelium of midtrimester human fetal lung tissue. However, given the surprising finding of KDR immunoreactivity in the epithelial cells of the developing lung, we went through considerable effort to verify this result. We initially employed a rabbit polyclonal antibody from Santa Cruz Biotechnology for these studies, finding results identical to those presented in Fig. 1 (data for the polyclonal antibody are not shown). To verify our findings, we again performed immunohistochemical analysis for KDR with two distinct monoclonal antibodies, each raised against different portions of the KDR protein. Appropriate controls demonstrated the specificity of the antibodies that we employed. Interestingly, we observed not only cytoplasmic staining in epithelial cells but nuclear staining as well in many of the distal airway epithelial cells. Nuclear immunolocalization of KDR has previously been demonstrated in NRK-52E cells, a nontransformed epithelial cell line derived from normal rat kidney (22) and also in endothelial cells (11, 39). The meaning of this finding is unclear. However, Feng et al. (11) demonstrated the nuclear translocation of KDR and endothelial nitric oxide synthase within the calveolae of bovine-derived endothelial cells in response to exogenous VEGF. The authors hypothesized that such VEGF-induced translocation may represent a mechanism for targeting nitric oxide production to the nuclear compartment, with resultant activation of transcription factors and cell growth. Further support for a role for VEGF in developing distal airway epithelium comes from our observation of immunolocalization of neuropilin-1 in the epithelial cells. Neuropilin-1 has recently been demonstrated to act as a coreceptor with KDR to specifically bind only the 165-amino acid isoform of VEGF (37). Neuropilin-1 has not previously been demonstrated in developing lung. These findings, along with our previous observation of distal airway epithelial cell production of VEGF, suggest a possible autocrine role for VEGF in human alveolar epithelial cell proliferation and differentiation.

Although VEGF has traditionally been considered an endothelial cell-specific growth factor, other recent reports also suggest that VEGF may be an important growth factor for epithelial cells. For example, the tubular epithelium of the developing kidney proliferates in vitro in response to exogenous VEGF, and the VEGF-specific receptor Flt-1 localizes to tubular epithelial cells during early stages of renal development (39). Similarly, VEGF induces differentiation and proliferation of pancreatic ductular epithelium in vitro, with immunolocalization of the VEGF-specific receptor KDR to the apical surface of the ductular epithelial cells (31). Both VEGF and its receptor Flt-1 have been identified in epithelial cells of the thyroid, suggesting that VEGF may contribute to the development and function of thyroid epithelial cells (42). VEGF is produced by epithelial and glandular epithelial cells of the endometrium (24). Interestingly, the intensity of immunoreactivity for the VEGF receptors Flt-1 and KDR varies in glandular epithelium during the menstrual cycle, data suggestive of a role for VEGF as an autocrine growth factor in the glands of the endometrium. Siafakas et al. (35) demonstrated significant levels of VEGF in human breast milk and the presence of Flt-1

![Graph and representative immunoblot of SP-A protein levels in midtrimester human fetal lung explants cultured in the absence and presence of VEGF, either 50 or 100 ng/ml. Values represent the means ± SE for 3 separate experiments. *P < 0.05 compared with the control condition.](image)

![Graph of the effect of VEGF on [3H]choline incorporation into total phosphatidylcholine (PC) and disaturated phosphatidylcholine (DSPC) by human fetal lung explants. Human fetal lung explants were incubated in the absence or presence of VEGF (50 ng/ml) for 4 days, with [3H]choline (1 µCi/ml) added for the final 24 h of culture. A: [3H]choline incorporated into total PC. B: [3H]choline incorporated into DSPC. Values represent means ± SE for 5 independent experiments. *P < 0.05 compared with the control condition.](image)
on Caco-2 cells, an intestinal epithelial cell line. Although these authors were unable to demonstrate a proliferative effect of VEGF on this cell line, the significant levels of VEGF in human breast milk during the first week of lactation suggest that VEGF may play an important role in intestinal epithelial cell growth and function in the newborn.

Additional nonendothelial cell activity of VEGF is suggested by the finding of receptors for VEGF in uterine smooth muscle cells (7), monocytes (3), osteoblasts (22), and several tumor cell lines (18, 29). Additional tumor cell lines that proliferate in response to VEGF include choroidal carcinoma and human retinal pigment epithelial cells that express Flt-1 and KDR (9, 16). Taken together, these reports support a nonendothelial cell role for VEGF and complement our current investigation demonstrating a proliferative effect of VEGF in developing pulmonary epithelium.

Because high levels of VEGF mRNA and protein have been localized to distal airway epithelial cells in midtrimester human fetal lung, and given the known effects of VEGF as a potent inducer of endothelial cell growth in vitro and angiogenesis in vivo, it follows that capillary development in the lung may be modulated through secretion of VEGF by alveolar epithelial cells. The absolute requirement of VEGF for vasculogenesis has been demonstrated by inactivation studies of VEGF alleles (8, 12) and of the VEGF receptors Flt-1 (14) and KDR (33). In each of these studies, inactivation of the targeted gene resulted in lethal phenotypes characterized by deficient organization of endothelial cells and endothelial cell precursors. Because the inactivation of VEGF alleles and VEGF receptor genes resulted in early embryonic death, no information was gained specific to the role of VEGF in the development of the pulmonary capillary bed. The role of VEGF in pulmonary vascular development has been suggested directly in a recent report by Zeng et al. (48) in which VEGF was overexpressed in the distal airway epithelial cells of transgenic mice. VEGF was overexpressed throughout development of the lung and resulted in the enlargement of pulmonary blood vessels, with disruption of the normal interface between pulmonary epithelial and capillary endothelial cells. Additionally, overexpression of VEGF resulted in dilated pulmonary tubules lined by immature type II cells, with decreased differentiation into type I alveolar pneumocytes. Therefore, overexpression of VEGF by the developing pulmonary epithelium had effects on both pulmonary vasculature and the epithelial cells. Consistent with Zeng et al.’s observations, the present study indicates a role for VEGF in the developing lung in addition to its effects on small vessel development. Our findings of increased epithelial cell proliferation in the presence of exogenously administered VEGF in human fetal lung explants and the localization of the VEGF receptor KDR to the epithelium of these tissues suggest a possible autocrine role for VEGF in distal airway epithelial cell growth during lung development. Other growth factors that have been localized to distal airway epithelial cells in human fetal lung include epidermal growth factor (32), transforming growth factor-α (32, 38), and neuregulin (30). In each of these cases, the receptors for these ligands have also been localized to the epithelial cells, and the effect of addition of the ligands has resulted in either increased maturation of distal airway epithelium to type II cells or the proliferation of epithelial cells (30, 44).

We observed that an effect of VEGF is to increase mRNA levels for the surfactant-associated proteins SP-A and SP-C but not for SP-B in lung explants. It is unclear whether these findings represent a possible role for VEGF in the maturation of distal epithelial cells to type II cells or a role primarily in increasing distal epithelial cell numbers. This is a difficult point to examine, since the human fetal lung explant system differentiates spontaneously in culture and concurrently exhibits increases in SP-A and SP-B mRNA and decreases in SP-C mRNA levels (23, 28). Furthermore, mRNA expression for the SPs is regulated differentially by various factors in human fetal lung in vitro. For example, mRNA levels for SP-A and SP-B are increased by cAMP, whereas SP-C mRNA levels are unaffected (23, 28). SP-A and SP-C but not SP-B mRNA levels are modulated by oxygen (1), and SP-A, SP-B, and SP-C mRNA levels are differentially regulated by the synthetic glucocorticoid dexamethasone, with changes in mRNA levels being dependent upon the dose of dexamethasone employed (27, 45). Therefore, it is possible that VEGF differentially regulates SP mRNA levels independently of effects on cell numbers or degree of differentiation. Additional investigation will be necessary to determine the exact nature of the observed effect of VEGF on SP-A and SP-C levels; however, our data demonstrating [3H]choline incorporation into PC in VEGF-treated explants may provide a clue. We found that exogenous VEGF resulted in increased incorporation of [3H]choline into PC but not into DSPC, data suggestive that epithelial cell numbers are increasing but may not be maturing into type II cells in VEGF-treated lung explants.

Overall, our data are the first to demonstrate a possible autocrine and paracrine role for VEGF in the distal airway epithelium of the developing human lung. Although previous studies have indicated a role for VEGF in endothelial cell growth, further studies will be needed to delineate the absolute role of VEGF in developing lung. Our previous data demonstrating elaboration of VEGF by distal airway epithelial cells to the basement membrane subjacent to these cells continues to indicate a role for VEGF in vascular development (2). This role presumably occurs in a highly regulated fashion, as evidenced by the observation of Zeng et al. (48) of abnormal pulmonary morphogenesis in the presence of VEGF overexpression. At the present time, little is known about the mechanisms governing development of the air-blood barrier. Further investigation into the effects on VEGF on both epithelial and endothelial cells in the developing lung is likely to yield important information regarding these mechanisms.
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