Differential expression of VEGF mRNA splice variants in newborn and adult hyperoxic lung injury

RICHARD H. WATKINS,1 CARL T. D’ANGIO,1 RITA M. RYAN,1 ALKA PATEL,2 AND WILLIAM M. MANISCALCO1

1Department of Pediatrics, Strong Children’s Research Center, University of Rochester School of Medicine, Rochester 14642; and 2Department of Pharmacology and Toxicology, State University of New York at Buffalo, Buffalo, New York 14214

ANGIOGENESIS, the growth of new blood vessels, is important in the development and repair of the lung. The gas-exchange function of the lung requires the development and maintenance of an extensive capillary network of endothelial cells in close proximity to a thin layer of alveolar epithelial cells. Capillary regeneration is also essential for repair of oxygen-induced lung injury. Acute hyperoxic lung injury is characterized by damage to endothelial and epithelial cells, interstitial edema, and inflammation (8, 13, 18, 32). Impaired function or loss of microvascular endothelial cells contributes to serum protein leakage and edema. Without timely repair and replacement of endothelial cells, alveolar fibrosis or death may result.

Numerous regulators of angiogenesis have been identified, including basic fibroblast growth factor, transforming growth factor-β, interleukin-8, and vascular endothelial growth factor (VEGF) (12, 22). Of these, VEGF, a homodimeric glycoprotein of 34–45 kDa, appears to play a pivotal role. The loss of even a single VEGF allele is lethal to the mouse embryo, indicating an essential role in the development of the vascular system (10). VEGF is mitogenic almost exclusively for endothelial cells. Besides being a mitogen for endothelial cells, VEGF is a vascular permeability factor that induces fenestrations in endothelial cells (30). VEGF also induces endothelial cell expression of several proteolytic enzymes that promote extracellular matrix (ECM) degradation, essential for endothelial cell migration and sprouting (11).

In the normal lung, VEGF is quite abundant, similar to other highly vascularized tissues (3). Lung VEGF is expressed primarily by alveolar epithelial cells, which are in close proximity to microvascular endothelial cells (24, 25). After hyperoxic injury, the expression of VEGF message declines in both newborn and adult lungs (23), coincident with the loss of endothelial cells (14). During recovery, when endothelial cells proliferate, VEGF mRNA becomes very abundant in alveolar type II cells (24). The level of VEGF protein in the parenchyma of the newborn lung follows the same time course as type II cell VEGF expression (23).

VEGF protein exists as several isoforms that are produced by alternative splicing of the primary transcript or by limited proteolysis. The precise functional differences among the isoforms are not known, but they differ in solubility, receptor affinity, and mitogenic potency. The primary VEGF transcript derives from a single VEGF gene that contains eight exons (35). Variable splicing involving exons 6 and 7 results in up to five isoforms containing 121, 145, 165, 189, and 206 amino acids after removal of a common signal peptide (11, 29). Exons 6 and 7 each contain a region of basic amino acids with a high affinity for heparin. The presence or absence of these heparin-binding regions influences the ECM binding and solubility of each isoform. Thus isoforms containing both exons (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206) bind tightly to the ECM or cell surface heparan sulfates. VEGF121, which lacks both exons, does not bind heparin and is highly diffusible. VEGF165, with one heparin-binding region, is moderately diffusible (26). Limited diffusability may spatially limit the action of ECM-binding isoforms such as VEGF189, whereas highly diffusible isoforms such as VEGF121 appear to spread more widely and are more likely to act systemically.
VEGF may have widespread action. Affinity for heparin may play an important role in VEGF binding to its receptors (15, 16, 29), accounting for the lower receptor affinity of VEGF compared with that of VEGF. The isoforms also differ in their mitogenicity. VEGF is the most mitogenic, and VEGF is much less mitogenic (21). A receptor that is specific for VEGF, which may enhance binding to the mitogenic VEGF receptor Flk-1, has been described (33). VEGF does not bind to Flk-1 and may be an ECM storage form (28). Thus although the specific roles of VEGF isoforms are not known, their biological properties suggest differing functions.

The solubility and mitogenicity of VEGF are also regulated by limited posttranslational cleavage of the larger isoforms (20). For example, VEGF can be cleaved by the urokinase-type plasminogen activator (uPA) to create a peptide that is similar to VEGF. In its solubility, mitogenicity, and receptor binding (28). Both VEGF and VEGF can be cleaved by plasmin to create a peptide that is similar to VEGF in solubility and mitogenicity (21). Settings with increased plasmin activity, such as tissue injury, may favor conversion of ECM-bound isoforms to soluble isoforms that can affect more distant endothelial cells. Little is known about the relative abundance of the different VEGF splice variants in the normal lung or during development, injury, and recovery. Whereas VEGF mRNA was noted to be relatively abundant in the adult rat lung, VEGF mRNA has the highest level of expression in most other rat tissues (2). We hypothesized that during development or recovery from injury, i.e., times of active endothelial cell proliferation, the messages for isoforms with high mitogenicity or solubility (VEGF and VEGF) would be most abundant. Conversely, in the normal adult lung, we hypothesized that the messages for the storage isoforms (VEGF and VEGF) would be prevalent. Our findings show that the relative proportion of VEGF mRNA is greater in adult lungs than in several other tissues. During lung development, the relative proportion of VEGF declines significantly, whereas the proportion of VEGF mRNA increases. The relative proportion of VEGF mRNA declines significantly during hyperoxic injury in both newborn and adult lungs but returns to control values during recovery. We also found that immunoreactive VEGF protein in lung lavage fluid increases up to 40-fold during recovery.

**MATERIALS AND METHODS**

Animals and hyperoxic exposure. The use of animals for this study was approved by the University of Rochester (Rochester, NY) Committee on Animal Resources. Newborn New Zealand White rabbits were separated from their mothers within 24 h of birth, placed in Plexiglas chambers, and exposed to either humidified >95% oxygen or air at 5 l/min as previously described (23). Newborns were maintained in hyperoxia for 2, 4, 6, 8, or 9 [50% lethal dose (LD)] days. Additional newborns were exposed to >95% oxygen for 9 days and then allowed to recover from the acute lung injury in 60% oxygen for 1, 3, 5, or 13 days. Attempts at recovering newborns in air resulted in high mortality. Both the hyperoxia-exposed and age-matched control animals were killed with an intraperitoneal injection of 200 mg/kg of pentobarbital sodium.

Adult male New Zealand White rabbits were exposed to >95% oxygen in Plexiglas chambers for up to 64 h and allowed to recover in air for up to 7 days as previously described (24). The animals were killed with an intravenous injection of pentobarbital sodium after 0, 24, 48, and 64 h of oxygen exposure and at 1, 2, 3, 5, and 7 days of recovery. Approximately 80% of the animals survived recovery in air.

Fetal rabbits were obtained by hysterotomy from timed-pregnant New Zealand White rabbits at 22, 25, and 28 days gestation (term = 31 days). The lungs were removed and flash-frozen at −70°C for later RNA isolation.

RNA and lavage preparation. In animals used for RNA preparations, a thoracotomy was performed, the right main stem bronchus was clamped, and the right lung was removed and flash-frozen in liquid nitrogen. Samples of skin, kidney, and placenta were also obtained from normal adult rabbits and flash-frozen. Total RNA was isolated from each rabbit lung with the method of Chomczynski and Sacchi (7).

The lungs for lavage were exposed by thoracotomy, perfused in situ with balanced salt solution (BSS), and removed. Normal lungs were lavaged with five aliquots of BSS of sufficient volume to fully distend the lung. Volume of the lavage fluid for newborns was 250 ml/kg, and absolute volumes varied between 5 and 40 ml depending on weight. For each time point, age-matched control animals were done for reference. Adult rabbits were lavaged with eight 50-ml aliquots of BSS. The lavage fluids for each animal were then pooled, and the cells were sedimented at 300 g for 6 min. The lavage supernatants were stored at −70°C.

Alveolar type II cell isolation. Type II cells from 4-day-old normal rabbits were isolated as previously described (31). Briefly, the pulmonary vasculature was perfused in situ, and the lungs were removed and lavaged with BBS. The lungs were digested with protease solution (trypsin, DNase I, and elastase), minced, and filtered to obtain a single cell suspension. Type II cells were purified on a discontinuous Percoll density gradient and counted with a hemocytometer, and viability was assessed by trypan blue exclusion. Purity and viability of type II isolated from 99% to 90%. Cells were flash-frozen at −70°C for future RNA isolation.

RT-PCR amplification and cloning of rabbit VEGF cDNA. cDNA was synthesized from total RNA with murine leukemia virus reverse transcriptase and oligo (dT) primers (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. Amplification for the purpose of cloning and sequencing the coding region of the different splice variants was performed with human VEGF-specific primers for the first and eighth exons: sense primer 5'-TGGATCCCATGAACTTTCTGCTGTCTT-3' (including the underlined Translation start site) and antisense primer 5'-CCTGGAATTTCTACCGCAGGTCTTGTCAC-3' (including the underlined Translation stop site). Restriction enzyme sites for BamHI and EcoRI were added to the primers for ease of cloning. Amplification was performed through 35 cycles (30 s at 94°C, 1 min at 59°C, and 1 min at 72°C). Amplified sequences were cloned into pBluescript SK II+ (Stratagene, La Jolla, CA) and sequenced with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus).

Semiquantitative RT-PCR of VEGF splice variants. RT-PCR was performed to determine the relative proportions of each splice variant in different tissue and cell samples. We used a sense primer located in exon 4 at nucleotide 355 from the translation start site (5'-CAGTGAATTTGAGATGAG-8' primer 5'-CAGTGAATTTCTGCTGTCTT-3' primer 5'-CCTGGAATTTCTACCGCAGGTCTTGTCAC-3' and antisense primer 5'-CCTGGAATTTCTACCGCAGGTCTTGTCAC-3' (including the underlined Translation start site) and antisense primer 5'-CCTGGAATTTCTACCGCAGGTCTTGTCAC-3' (including the underlined Translation stop site). Restriction enzyme sites for BamHI and EcoRI were added to the primers for ease of cloning. Amplification was performed through 35 cycles (30 s at 94°C, 1 min at 59°C, and 1 min at 72°C). Amplified sequences were cloned into pBluescript SK II+ (Stratagene, La Jolla, CA) and sequenced with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus).
CTTCCTACAGCAC-3') and the same antisense primer as described in RT-PCR amplification and cloning of rabbit VEGF cDNA. PCR products from these primers were smaller, but the relative size differences were maximized, resulting in improved separation on gel electrophoresis. [32P]dCTP (2 × 10^6 dpm/µg) was added to each PCR reaction. To determine whether the relative amplification efficiency of different VEGF splice variant cDNAs by PCR was equivalent, we amplified with PCR three VEGF clones (coding for VEGF189, VEGF165, and VEGF121) individually and mixed in varying ratios. The three clones amplified with equal efficiency, and the ratios of the amplified products reflected the starting ratios. We therefore concluded that differences in the relative ratios of the amplified splice variant products would reflect actual differences in the relative ratios of the splice variant mRNAs. We also amplified rabbit lung cDNA through 25, 30, and 35 cycles to determine whether nearing an amplification plateau would have any effect on the relative ratios of the splice variants present in a sample. The ratios remained consistent through the different levels of amplification. In all subsequent samples, we used 30 cycles of amplification. Amplified products were electrophoresed on a denaturing 8 M urea-40% formamide-5% polyacrylamide gel. Highly denaturing conditions were found to be essential to prevent cross-hybridization among the splice variants. The products were quantified by phosphorimaging and computer image analysis (ImageQuant, Molecular Dynamics, Sunnyvale, CA). To account for the differences in signal intensity due to size differences, the relative amounts of the amplified splice variants were converted to molar ratios. All semiquantitative RT-PCR data are expressed in terms of relative molar proportions of the splice variants.

RT-PCR performed on total RNA from adult rabbit lung, liver, spleen, and kidney demonstrated tissue-specific variations in the relative proportions of the VEGF splice variants. Amplification yielded cDNAs of predicted sizes that were consistent with alternative splicing of the sixth and seventh exons (Fig. 1A). To clone and sequence the coding regions of the splice variants, we used primers from the first and eighth exons, which generated major products of 648, 630, 576, and 444 bp in length. Sequencing showed the cDNAs to be the coding regions of VEGF189, VEGF183, VEGF165, and VEGF121, respectively (Fig. 1B). Sequence analysis of the rabbit VEGF189 cDNA revealed 94% nucleotide and amino acid homology with human VEGF189 (Fig. 1C). Similar to other species, rabbit VEGF165 lacks the sixth of eight exons spanning the coding region and VEGF121 lacks the sixth and seventh exons. Rabbit VEGF183 is identical to VEGF189; except it is lacking 18 bp at the 3'-end of exon 6. It is expressed at a low level in the lung. A fifth, minor cDNA seen on RT-PCR (Fig. 1A) may represent the splice variant VEGF145 as previously reported (19, 29). This product constituted ~5% of the total variants and did not appear to change with hyperoxia. Clones for this minor cDNA were not isolated, and, therefore, we cannot confirm its identity by sequence analysis. We detected no VEGF206 mRNA, consistent with the very restricted expression of this splice variant.

VEGF189 mRNA is more abundant in the lung than in other rabbit tissues. RT-PCR performed on total RNA from adult rabbit lung, liver, spleen, and kidney demonstrated tissue-specific variations in the relative proportions of the VEGF splice variants (Fig. 2). As noted in Cloning and sequencing of rabbit VEGF splice variants, the primary splice variants detected in the normal lung were VEGF189, VEGF165, and VEGF121. Because VEGF189 is difficult to quantify apart from VEGF189 on a gel, we have included both signals in quantifying VEGF189. For the purpose of studying changes in the bioavailability of VEGF, it is reasonable to group the measurement of these variants because they contain both of the heparin-binding regions present in the sixth and seventh exons. Their bioavailability characteristics are therefore distinct from those of VEGF165 and VEGF121. The relative percentage of each splice variant present was determined with the sum of the measured variants as the denominator. In the normal adult lung, the proportion of total VEGF mRNA that was composed of VEGF189 (41 ± 1% (SE)) was significantly greater than that in the liver, spleen, or kidney, which had 16% or less VEGF189 (P < 0.001 by Student’s t-test with Bonferroni correction). In contrast, the proportion of VEGF189 was significantly less in the lung than in the other tissues (39 ± 1 vs. 60–63%; P < 0.001).
Relative abundance of lung VEGF splice variants changes during development. Lung VEGF mRNA increases approximately threefold during gestation (Watkins and Maniscalco, unpublished observations) and is abundant in newborns. Alveolar epithelial type II cells are the primary VEGF-expressing cells in newborn rabbits (23). To investigate further VEGF expression in lung development, we measured the relative ratios of the splice variants in fetal and newborn lungs. RNA from 22-, 25-, and 28-day fetal rabbit lungs (term = 31 days) and from 1-, 4- and 10-day-old newborn rabbit lungs was isolated and amplified by RT-PCR (Fig. 3). The relative abundance of VEGF189 steadily increased from 8 ± 1% at 22 days gestation to 40 ± 2% in 10-day-old newborns (P < 0.0001; r² = 0.84 by linear regression). The proportion of VEGF165 was high in the 22-day fetal lung (70 ± 2%) and decreased significantly during development, declining to 42 ± 5% by 10 days postnatal age (P < 0.003; r² = 0.51 by linear regression). There were no consistent changes in the proportion of VEGF121 in lung development. In RNA isolated from 4-day newborn type II cells, the molar ratios of the splice variants were similar to those in the whole lung at this time. At 10 days postnatal age, the relative proportions of the VEGF splice variants were similar to the values in the adult lung. Thus the proportion of VEGF189 increased fivefold from 22-day fetal lungs to 10-day-old newborn lungs, and VEGF165 decreased proportionately.

Fig. 1. Rabbit lung vascular endothelial growth factor (VEGF) splice variants and nucleotide and deduced amino acid sequences of coding region of rabbit 189-amino acid VEGF (VEGF189). A: RT-PCR amplification of rabbit lung VEGF mRNA with primers from 4th and 8th exons yielded 4 primary splice variant cDNAs of 314, 296, 242, and 110 bp, corresponding to VEGF189, VEGF183, VEGF165, and VEGF121, respectively. A low level of a 5th variant was also detected at 182 bp. This product was ~5% of the total and was not sequenced, but size is consistent with VEGF145.

B: exon composition of VEGF splice variants cloned from rabbit lung. Boxes, various exons not drawn to scale. Nos. on top, length in bases. C: sequence of coding region of rabbit VEGF189. First amino acid of signal peptide is numbered +22. First amino acid of mature polypeptide is numbered +1. PCR primer sites (human) are underlined. Vertical lines divide exons. Arrow, alternative splice site in exon 6 for VEGF183. Sequences for RT-PCR bands corresponding to VEGF189, VEGF183, and VEGF121 were consistent with exon sequences noted here.
VEGF variant differentially decreases during hyperoxic lung injury in newborn rabbit and increases during recovery. After 9 days of exposure to >95% oxygen, newborn rabbit lungs exhibit a decrease in total VEGF mRNA that is then reversed during 5 days of recovery (23). To determine whether hyperoxic injury also changes the ratio of VEGF mRNA splice variants, we performed RT-PCR on RNA from 9-day oxygen-exposed newborn rabbit lungs, age-matched control lungs, and 1-, 3-, and 5-day recovered lungs. We found that the proportion of VEGF189 to total VEGF splice variant mRNA dropped from $38 \pm 2$ to $8 \pm 1\%$ with exposure to hyperoxia ($P < 0.005$ by Student’s $t$-test). The proportions of both VEGF165 and VEGF121 increased significantly at this time ($P < 0.01$). During recovery, a time of endothelial cell proliferation, VEGF189 increased to $30 \pm 2\%$ of the total VEGF at 1 day and to $35 \pm 1\%$ at 3 days and reached the control value ($39 \pm 3\%$) at 5 days of recovery (Fig. 4). During this time, the proportions of VEGF165 and VEGF121 declined to control values. Although we have not measured the absolute amounts of the VEGF variants present, it is clear that with hyperoxic injury the level of VEGF189 message declines substantially because both the amount of total VEGF mRNA and the proportion that is VEGF189 decrease.

To confirm these results, we also performed RPAs to measure changes in the splice variant ratios during hyperoxic exposure and recovery in newborn rabbits.
Antisense 32P-labeled VEGF189 hybridized to total RNA was digested with a mixture of RNases A and T1 at a dilution that digested all single-stranded overhangs and loops but did not significantly nick RNA opposite a loop site. It was anticipated that the VEGF189 riboprobe would generate protected fragments of 649 bases when hybridized to VEGF189 RNA, 477 and 154 bases when hybridized to VEGF183, 423 and 154 bases when hybridized to VEGF165, and 423 and 22 bases when hybridized to VEGF121. To test our digestion conditions and demonstrate that the anticipated bands were seen, RPA with a mixture of all three sense RNAs gave the combination of bands expected. RPAs performed with RNA samples from newborn rabbit lungs demonstrated a change in the ratios of the splice variants during injury and recovery that closely matched the changes seen with RT-PCR (Fig. 5A). Relative molar percentages were calculated with the sum of the two densities as the denominator after adjustment for the differences in band size. Scanning densitometry indicated that the 649-base fragment was 35% of the total density in control lungs but only 9% in the injured lungs. During recovery, the molar percentage of VEGF189 increased gradually from 24% at 1 day of recovery to 35% at 5 days of recovery. These data are consistent with those obtained with RT-PCR.

Hyperoxic injury in the adult rabbit lung results in changes in the VEGF variants similar to those seen in newborn hyperoxic lung injury. Adult rabbits were exposed to 95% oxygen for 64 h and allowed to recover in room air for up to 7 days. VEGF RNA from the lungs was amplified by RT-PCR and quantified (Fig. 6). VEGF189 mRNA comprised 41.6% of the total VEGF present in control lungs, but after 64 h of oxygen, the proportion of VEGF189 dropped to 20.6% of the total VEGF (P < 0.005 by Student’s t-test), whereas VEGF165 and VEGF121 increased significantly (P < 0.01). Normal ratios were reestablished during recovery.

VEGF protein in lung lavage fluids increases during recovery from hyperoxic injury. A previous study (23) demonstrated by immunohistochemical analysis that VEGF protein in the parenchyma of the newborn lung
declined to nearly undetectable levels during hyperoxic injury but reappeared during recovery, coincident with increased VEGF mRNA. Because lung VEGF is expressed primarily by alveolar epithelial cells, we reasoned that it could be detected in lung lavage fluid. An anti-human VEGF ELISA was used to measure immunoreactive VEGF protein in the lavage fluid of newborn and adult lungs. The antibodies used recognized all isoforms of VEGF. Lavage fluids of hyperoxic lungs yielded VEGF protein concentrations slightly greater than those in control lungs (Fig. 7A). By 9 days of hyperoxia (LD50), however, VEGF was barely detectable. During the first 5 days of recovery, the lavage fluid VEGF increased 10-fold over age-matched control levels. By 13 days of recovery, the level declined to the control value in adult rabbits, a similar pattern was observed (Fig. 7B). Low levels of VEGF protein were measured in the control lungs. During hyperoxic exposure, the VEGF level rose approximately twofold. The lowest level of VEGF was seen at 1 day of recovery, but subsequent concentrations at 3 and 5 days of recovery increased up to 40-fold over the control level. By 7 days of recovery, the VEGF concentration decreased to just twofold above the control value.

DISCUSSION

The proliferation and migration of endothelial cells, the sprouting and formation of new blood vessels, and the remodeling of extracellular matrix characterize the complex process of angiogenesis, essential for the development and repair of the lung. Several growth factors, including basic fibroblast growth factor, transforming growth factor-β, and VEGF, can modulate this process. VEGF is particularly important because it is mitogenic mainly for endothelial cells. Significant variation in VEGF mitogenicity, receptor affinity, and ECM binding result from alternative splicing of the primary VEGF transcript. Unlike several other organs, adult lungs had a significantly greater proportion of VEGF189 mRNA, which codes for a highly ECM-bound isoform, whereas the other tissues had a significantly greater proportion of VEGF165, which codes for a more soluble isoform. In 22-day-gestation fetal rabbit lungs, the proportion of VEGF189 was low (8%), whereas VEGF165 mRNA was highly expressed (~70%). VEGF189 increased significantly during fetal rabbit development, and VEGF165 became less prominent. With oxygen injury, the relative proportion of lung VEGF mRNA splice variants changed: VEGF189 mRNA declined and the proportions of VEGF165 and VEGF121 increased. Control values were reestablished during recovery. Total VEGF protein in lung lavage fluid increased up to 40-fold during recovery.

The sequences of VEGF splice variants found in the rabbit lung are quite similar to those found in human mRNA. Rabbit VEGF121, VEGF165, and VEGF189 share ~94% homology with their human counterparts. We also cloned a VEGF183 message that lacks 18 bp from the 3′-end of exon 6. The 5′-end of this 18-bp fragment begins with GT, the consensus sequence for the 5′ splice donor necessary for splicing. Thus the variability of the 5′ donor splice site between exons 6 and 7 may generate several isoforms, including VEGF183, VEGF185, and VEGF206. VEGF183, although missing 18 bp, still contains the heparin-binding site of exon 6. It seems likely, therefore, that its affinity for heparan sulfate is high and that it would be similar in its binding characteristics to VEGF189. For this reason, we included the contributions of VEGF183 in our measurements of VEGF189.

Because the pulmonary endothelium in the normal adult lung has a low proliferation rate, the production of VEGF by the normal lung suggests that its role may be endothelial cell maintenance. The intact VEGF189 isoform may be inactive as a mitogen because of its inability to bind efficiently to the high-affinity VEGF receptor Flk-1, which transmits the signals for mitogenesis and chemotaxis (28). VEGF189 is likely a storage form, but it may be activated by proteolytic cleavage. Two serine proteases that cleave and activate VEGF189...
are uPA and plasmin (28). Although these proteases may be present in the normal lung (17), it is not known whether stored VEGF189 is released by proteolytic cleavage in the normal lung or whether intact VEGF189 has a function in the maintenance of the pulmonary vasculature. Intact VEGF189 binds to a second high-affinity VEGF receptor, flt-1, which is present in endothelial cells (28). The role of flt-1 is not clear, although it does not appear to transduce mitogenic signals in endothelial cells (36).

Our findings that VEGF189 increases in lung development and is more abundant in the lung than in several other tissues suggest that it may have a unique role in the development and maintenance of the normal pulmonary endothelium. For example, alveolar capillaries in the mature lung are directly subjacent to the alveolar epithelial basement membrane, whereas the alveolar capillaries in fetal lung are located centrally in the alveolar septa (4). Increased expression of a highly ECM-bound VEGF such as VEGF189 by developing alveolar epithelial cells may regulate the spatial orientation of the microvasculature.

The mechanisms governing the changes we observed in the relative amounts of the VEGF splice variants during lung development and injury are unknown. Although different cell types may express different VEGF splice variants (6) and organ-specific differences in the splice variants exist, there are no data on the differences among cells within an organ. It is possible that a change in the total VEGF mRNA level of one cell type in the lung may result in a change in the ratio of VEGF variants observed in the whole lung. For example, a previous study (23) showed that VEGF-expressing cells, including neonatal type II cells, were most prominent in the alveolar epithelium. The present study showed that whole lung splice variant ratios were very similar to type II cells from 4-day-old rabbits. It is possible that the increase in whole lung VEGF189 during fetal development represents an increase in the number or differentiation state of type II cells. Similarly, a shift in the pattern of whole lung VEGF splice variant expression during lung injury and recovery may reflect changes in the contribution of type II cells. Because type II cells have increased VEGF expression during recovery from hyperoxia (24), it is likely that these cells make a major contribution to the splice variant patterns during recovery. It is unlikely that our data resulted from dying animals because extremely ill animals (cyanotic or lethargic) were culled and not used for analysis.

Another explanation for the changes in the splicing patterns of VEGF is that extracellular and intracellular signals cause changes in VEGF variant ratios within expressing cells. Molecules that play a role in splicing are regulated in a variety of ways. For example, the activity and selectivity of serine-arginine-rich (SR) proteins, which mediate the selection and joining of splice sites, are influenced by phosphorylation, concentration of pre-mRNA, regulator proteins, and RNA enhancer regions (5). Although any of the SR proteins efficiently splice constitutive splice sites (such as those in VEGF exons 1–5), specific SR proteins in concert with other factors determine which variable splice sites (as in VEGF exons 6–8) are utilized. It remains to be determined what factors cause the changes in the VEGF splice variant ratios during development and injury.

The decline in total VEGF expression or a particular splice variant during hyperoxic injury may contribute
to the endothelial loss associated with hyperoxic lung injury. For example, hyperoxia in the rat retina results in decreased VEGF expression and the subsequent apoptosis of endothelial cells (1). Intraocular addition of exogenous VEGF before the hyperoxic period prevents the apoptosis. In vitro, VEGF ameliorates the apoptotic effects of tumor necrosis factor-α on endothelial cells (34). These data imply that VEGF may have a maintenance function for endothelial cells. A decrease in normal VEGF expression or a change in normal splice variant ratios may contribute to endothelial cell loss in hyperoxic or inflammatory injury. It is not known, however, whether different VEGF isofoms have different effects on endothelial cell survival.

Our original hypothesis was that there would be an increase in the proportion of mRNA for soluble VEGF isoforms during recovery. We observed that the relative proportions of the VEGF mRNA splice variants returned to control values in recovery. However, we also found that total VEGF protein in lung lavage fluid increased up to 40-fold during recovery. Although the relative proportions of the splice variants returned to control values in recovery, the large increase in VEGF protein suggests that both soluble and ECM-binding isoforms would be highly abundant in recovering lung tissue. Another potential mechanism that may increase VEGF solubility in recovery is proteolytic processing. With injury and inflammation, proteases such as uPA and plasmin are induced in endothelial cells, alveolar epithelial cells, and activated macrophages (9, 17). Limited proteolysis results in the conversion of VEGF189 and VEGF165 to an active, soluble isofom similar to VEGF121 (21, 28). Proof that VEGF is processed in lung injury, however, will require further study.

A limitation of this study was the inability to verify that the VEGF mRNA splice variants were translated into corresponding proteins. Cells transfected with individual splice variant cDNAs express the appropriate protein isofom in vitro (27), but correlation of splice variant message abundance with isoform levels for organs or tissues in vivo has not been reported. There are no data suggesting that any of the splice variants are not translated or that they have differing translational efficiencies. Because all VEGF isoforms can be proteolytically processed to a smaller, non-heparin-binding isofom (20, 28), determining the total amount of protein translated from a splice variant mRNA would be very difficult, particularly in a setting of plasmin activation. Such an analysis would require measurement of both the intact isofom and any proteolytic products. Currently, there is no method to trace the lineage of a cleavage product.

In summary, we found that the rabbit VEGF splice variant sequences are quite similar to human VEGF variants. An additional variant, VEGF183, was identified. We found that the lung, unlike some other tissues, had VEGF189 as a major splice variant. The proportions of VEGF mRNA splice variants changed in lung development: VEGF189 increased significantly, whereas VEGF165 declined. In hyperoxic injury of both newborn and adult rabbits, the relative proportion of VEGF189 decreased, whereas that of VEGF165 increased. Normal proportions were reestablished in recovery. VEGF protein levels in lung lavage fluid from recovering animals increased many-fold over the levels in control animals. These results are suggestive of differing and flexible roles for VEGF splice variants in the development, maintenance, and repair of the lung. The precise roles of the VEGF splice variants in developing and injured tissues, however, remain to be clarified.

We gratefully acknowledge the expert technical work of Michael LoMonaco and Anna Paxhia.

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) Specialized Center of Research (SCOR) Grant HL-36543 (to W. M. Maniscalco); NHLBI Grant RO1-HL-54632 (to W. M. Maniscalco); NHLBI National Research Service Award SF-32-HL-09022 (to C. T. D’Angio); a March of Dimes grant (to R. M. Ryan); and NHLBI Clinical Investigator Award HL-02630 (to R. M. Ryan).

Address for reprint requests and other correspondence: W. M. Maniscalco, Box 651, Dept. of Pediatrics, Univ. of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642 (E-mail: william_maniscalco@urmc.rochester.edu).

Received 23 March 1998; accepted in final form 25 January 1999.

REFERENCES


