Role of VEGF-B in the lung during development of chronic hypoxic pulmonary hypertension

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Louzier, Vanessa, Bernadette Raffestin, Aude Leroux, Didier Branellec, Jean Michel Caillaud, Micheline Levalme, Saadia Eddahibi, and Serge Adnot. Role of VEGF-B in the lung during development of chronic hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 284: L926–L937, 2003. First published January 24, 2003; 10.1152/ajplung.00247.2002.—Angiogenic factors exert protective effects on the lung. To investigate the effect of VEGF-B, a factor coexpressed in the lung with VEGF-A, we assessed chronic hypoxic pulmonary hypertension in VEGF-B knockout mice (VEGF-B+/−) and in rats with lung overexpression of VEGF-B induced by adenovirus transfer. No significant difference in pulmonary hemodynamics, right ventricular hypertrophy, distal vessel muscularization, or vascular density was found between VEGF-B+/− and control mice after 3 wk of hypoxia. When overexpressed, VEGF-B167 or VEGF-B186 had protective effects similar to those of human VEGF-A165. Lung endothelial nitric oxide synthase (eNOS) expression was increased by 5 days of hypoxia or VEGF-A adenovirus vector (Ad.VEGF-A) overexpression, whereas VEGF-B167 or VEGF-B186 had no effect. With hypoxia or normoxia, the wet-to-dry lung weight ratio was increased by 5 days after Ad.VEGF-A administration compared with control (Ad.nul), Ad.VEGF-B167, or Ad.VEGF-B186. Endogenous VEGF-B does not counteract the development of hypoxic pulmonary hypertension. However, when overexpressed in the lung, VEGF-B can be as potent as VEGF-A in attenuating pulmonary hypertension, although it has no effect on eNOS expression or vascular permeability.

ANGIOGENIC FACTORS ARE KNOWN to play an important role in lung development and adaptation to various abnormal conditions. The angiogenic factor VEGF-A is abundantly expressed in the adult lung (30). Chronic hypoxia has been shown to increase the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2 in the rat lung (8, 41). Moreover, we have demonstrated that adenovirus-mediated VEGF-A overexpression in the lung attenuates the development of hypoxic pulmonary hypertension (PH), in part through an improvement in endothelium-dependent function (35). Among other more recently discovered members of the VEGF family, VEGF-B is expressed in the heart and skeletal muscle in adults, as well as in the arterial wall, particularly of the pulmonary arteries (33). In contrast with VEGF-A, its expression is not regulated by hypoxia or cytokines (31, 39). Moreover, whereas VEGF-A binds to both VEGFR-1/Flt-1 and VEGFR-2/kinase insert domain-containing receptor (KDR), VEGF-B binds to VEGFR-1 but not to VEGFR-2 (32), the receptor that seems to mediate the angiogenic effects of VEGF-A (45). Although loss of VEGFR-1 disrupts normal vascular development (13), partial deletion restricted to its tyrosine kinase domain allows normal embryogenic angiogenesis, suggesting that VEGFR-1 may function as an inert decoy by binding VEGF-A, thereby regulating the availability of VEGF-A for VEGFR-2 activation (16). However, this does not rule out a role for specific intracellular signals mediated by VEGFR-1 (5, 15, 19, 20).

The role of VEGF-B is unclear. Whereas targeted inactivation of a single VEGF-A gene allele in mice causes lethal impairment of angiogenesis (6), VEGF-B knockout mice are healthy and fertile (2). However, their hearts are abnormally small and exhibit vascular dysfunction after coronary occlusion and impaired recovery after experimental cardiac ischemia (2). It has also been shown recently that placent growth factor (PIGF), another VEGFR-1 ligand, plays a role in amplifying endothelial cell responsiveness to VEGF-A during the angiogenic switch associated with many disorders (7).

In this study, we tested the hypothesis that endogenous VEGF-B, which is predominantly active on VEGFR-1, may contribute to counteract the development of chronic hypoxic PH. To this end, we assessed pulmonary hemodynamics, right ventricular hypertrophy, pulmonary vascular density, and distal vessel muscularization in mice lacking the VEGF-B gene (VEGF-B−/−) and in wild-type controls (VEGF-B+/+), after exposure to 3 wk of hypoxia.

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We also investigated the effect of VEGF-B overexpression in the rat lung on the development of hypoxic PH. We built an adenovirus vector containing an expression cassette with cytomegalovirus (CMV) promoter/enhancer driving cDNA for either of the two human VEGF-B isoforms, VEGF-B167 (Ad.VEGF-B167) or VEGF-B186 (Ad.VEGF-B186). We compared the protective effects of VEGF-B overexpression with those of VEGF-A overexpression obtained by transfer of the human VEGF-A165 gene in an adenoviral vector (Ad.VEGF-A). We compared the effect of VEGF-A and VEGF-B overexpression on lung permeability and eNOS expression under normoxic and hypoxic conditions.

MATERIALS AND METHODS

Exposure to Hypoxia

Animals were exposed to chronic hypoxia (10% O2) in a ventilated chamber (500-l capacity; Flufrance, Cachan, France). To establish a hypoxic environment, we flushed the chamber with a mixture of room air and N2, and the gas was then recirculated. We monitored the chamber environment using an oxygen analyzer (model OA150; Servomex, Crowborough, UK). CO2 was removed by soda lime granules, and excess humidity was prevented by cooling of the recirculation circuit. The temperature in the chamber was 22–24°C. The chamber was opened on alternate days for 1 h to clean the cages and replenish food and water supplies.

Development of Chronic Hypoxic PH in VEGF-B−/− Mice

VEGF-B−/− mice. VEGF-B−/− mice were generated by homologous recombination on an SV129 genetic background. The generation and genotyping of the mice have been previously described. The wild-type and mutant homozygous VEGF-B−/− mice used in these studies were siblings (8–10 wk of age) obtained by breeding heterozygous mutants. The responses of male VEGF-B−/− and VEGF-B−/− mice to chronic hypoxia were examined. All animal care and procedures were in accordance with institutional guidelines.

Hemodynamic response to chronic hypoxia. Mice (6–10 wk, ~20–30 g) were exposed to normoxia or chronic hypoxia for 3 wk, as described above. After anesthesia with intraperitoneal ketamine (6 mg/100 g) and xylazine (1 mg/100 g), the trachea was cannulated, and the lungs were ventilated with room air at a tidal volume of 0.2 mL and a rate of 90 breaths per min. A 26-gauge needle was introduced percutaneously into the right ventricle via the subxyphoid approach. Systolic right ventricular pressure (RVP) was measured using a Gould P10 EZ pressure transducer connected to pressure modules and a Gould TA 550 recorder (Gould Electronics, Ballainvilliers, France). RVP and heart rate were recorded while the animal was ventilated with room air. The heart rate under these conditions was between 300 and 500 beats per min (bpm). If the heart rate fell below 300 bpm, the measurements were excluded from the analysis.

Effect of VEGF-B expression on pulmonary vascular density and remodeling. After an intraperitoneal injection of pentobarbital sodium (40 mg/kg), the thorax was opened and the mouse exsanguinated. Then, the lungs were removed and fixed in the distended state by formalin infusion at a constant pressure of 30 cmH2O. A midsagittal slice of each lung was processed for paraffin embedding, and 5-μm sections were prepared. After toluene treatment and rehydration, endogenous peroxidase activity was quenched by incubation with 0.3% H2O2 in methanol for 30 min. Then, sections were washed in Tris-buffered saline (TBS) and in TBS+ (CaCl2, MnCl2, MgCl2 0.1 mM) and incubated overnight at 4°C with the peroxidase-labeled lectin *Ulex europaeus* (20 μg/mL; Sigma), which binds to fucosyl residues in the endothelium. After three washes, peroxidase staining of the slides was carried out with 3,3'-diaminobenzidine tetrahydrochloride dihydrate with metal enhancer (Sigma Fast DAB with metal enhancer). We assessed vascular density with an image analysis system using Perfect Image and quantification of lung staining for *U. europaeus* expressed per area of lung parenchyma. Areas with bronchioli or arteries 15 μm or more in diameter were excluded. At least 10 fields per slide were examined (magnification ×25).

To assess the degree of distal vessel muscularization, we stained sections with hematoxylin phloxin saffron, and intraacinar vessels were categorized as muscularized (fully or partially muscularized) or nonmuscularized.

Adenoviral-Mediated Gene Expression in the Lung: Methodological Procedure

Adenovirus vector construction and production. We constructed replication-defective recombinant adenovirus vectors, based on the human Ad5 serotype and containing cDNA of VEGF-A165, VEGF-B167, or VEGF-B186 (Ad.VEGF-A, Ad.VEGF-B167, and Ad.VEGF-B186, respectively). Ad.nul, similar to Ad.VEGF but with no gene in the expression cassette, was used as the control vector. The coding sequences of human VEGF-B167 and VEGF-B186 were cloned by PCR from a commercially available cDNA library (human heart cDNA ref K1003–1; Clontech). The VEGF-A cDNA came from a human placenta RNA library purchased from Clontech (17). The recombinant adenoviruses were constructed by recombination in *Escherichia coli* as previously described (9). VEGF-B and VEGF-A expression was driven by the CMV immediate early promoter (−522/+72), the polyadenylation signal being the polyA late signal of SV40. Adenoviruses were amplified in 293 cells. The supernatant collected 10 days after infection was concentrated by tangential flow filtration (Jean-Marc Guillaume, personal communication). Purification and titer determination (viral particles (VP)/ml) were performed by high-performance liquid chromatography, as described by Blanché et al. (3). All viral stocks were also subjected to restriction analysis to check the integrity of the virus. The ratio of plaque-forming units to VP was <1/100 for each virus. All viral stocks contained <1 replication-competent adenovirus per 1010 VP. The adenovirus titers were 4.7 × 1012, 3.3 × 1012, 4 × 1012, and 3 × 1012 VP/ml for Ad.nul, Ad.VEGF-B167, Ad.VEGF-B186, and Ad.VEGF-A165, respectively.

Delivery of adenovirus vectors to rats. Wistar rats (200–250 g body wt) were used for all rat studies. All procedures and animal care were in accordance with institutional guidelines. Ad.VEGF or Ad.nul as the control was diluted before use with sterile saline, pH 7.4, in a final volume of 150 μL. The rats were anesthetized with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g). Treatment of the lungs was achieved by intratracheal instillation of 150 μl/rat of diluted Ad.VEGF or Ad.nul, using a previously described standard procedure (10).

Evaluation of gene transfer. To evaluate the efficiency of gene transfer, we measured VEGF-B protein levels in bronchoalveolar lavage (BAL) fluid in normoxic rats 5 days after infection with various doses of Ad.VEGF-B167 or Ad.VEGF-B186 (1010–1011 VP), Ad.VEGF-A (1010 VP), or Ad.nul (1011 VP).
ROr Ad.nul (10^10 VP), rats were either exposed to hypoxia (10% O_2) 1 day after being treated or left in normoxia.

To evaluate the inflammatory response after adenovirus infection, we performed histological examination in normoxic rats 5 days after treatment with Ad.nul, Ad.VEGF-B167, Ad.VEGF-B186, or Ad.VEGF-A (10^10, 10^11, and 10^13 VP). Immediately after BAL, the left lung was removed and fixed by infusion of neutral buffered formaldehyde into the trachea. After routine processing and paraffin embedding, multiple sections from each lobe were stained with hematoxylin and eosin. The inflammatory response was analyzed on an empiric semiquantitative scale, as described previously (4). The following were determined: the type of inflammatory cells, their location (alveoli, bronchi, blood vessels), and the presence of edema and hemorrhage. Epithelial damage in bronchi, bronchioles, and/or alveoli was scored 0–4 (none to severe). Extension of inflammation was also scored 0–4 as follows: 0, none; 1, patchy small areas involved; 2, <10%; 3, 10–50%; 4, >50% of section area.

Evaluation of VEGF-B expression on pulmonary edema and induction of endothelial nitric oxide synthase. After administration of Ad.VEGF-B167, Ad.VEGF-B186, Ad.VEGF-A, or Ad.nul (10^10 VP), rats were either exposed to hypoxia (10% O_2) 1 day after being treated or left in normoxia.

After 5 days of exposure to hypoxia or normoxia, the lungs were excised en bloc and dissected from the heart and thymus. The right medial lobe was immediately weighed and placed in a desiccating oven at 37°C for 72 h, at which point the dry weight was measured. The ratio of wet-to-dry weight was used to quantify lung water content.

We extracted total proteins from the right cranial lobe by grinding in a lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM leupeptin, 1 mM PMSF, and 1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate). Protein concentration was determined by a modified Lowry assay (DC protein assay; Bio-Rad Laboratories, Richmond, CA). Then, 150 μg of denatured protein per lane were loaded, separated on 7% SDS-PAGE gels, and transferred to a nitrocellulose membrane. After being blocked in TBS with 0.01% Tween and 5% bovine serum albumin, the membranes were exposed for 1 h to endothelial nitric oxide synthase (eNOS) monoclonal antibody (Transduction Laboratories, Lexington, KY). The membranes were then washed three times and incubated with goat antimouse secondary antibody coupled to peroxidase activity (Calbiochem, San Diego, CA). After three more washes, the membranes were incubated with chemiluminescence detection reagents and exposed to Kodak Xar film. Sample of a normoxic rat lung was used as an internal standard, which was given the arbitrary value of 100. Each lane corresponding to one lung sample was compared with this standard.

Evaluation of VEGF-B expression on nitric oxide-derived products in the lung. Levels of nitrite/nitrates (NOx) were measured in BAL fluid, which was recovered as described previously. A quantity of 100 μl of sample was injected into a reaction chamber containing a mixture of vanadium (III) chloride in 2 M HCl heated to 90°C to reduce NOx to nitric oxide (NO) gas. The NO gas was carried into the analyzer (Sievers, Boulder, CO) via a constant flow to N_2 gas. The analyzer was calibrated by NaNO_3 standard curve.

Effect of Ad.VEGF-B Administration on the Development of Chronic Hypoxic PH

To examine the effect of gene transfer on the development of PH, we administered Ad.VEGF-B167, Ad.VEGF-B186, or Ad.nul (10^10 VP) intratracheally to normoxic rats on the day before the beginning of hypoxia exposure. Hemodynamic measurements and assessments of right ventricular hypertrophy, pulmonary vascular density, and remodeling were performed after 15 days of continuous exposure to hypoxia.

Hemodynamic measurements and assessment of right ventricular hypertrophy. At the end of the 2-wk exposure to hypoxia, the rats were anesthetized with intramuscular ketamine (7 mg/100 g) and xylazine (1 mg/100 g). After exposure of the right jugular vein, a polyvinyl catheter was inserted and manipulated through the right ventricle into the pulmonary artery. A polyethylene catheter was inserted into the right carotid artery. Pulmonary (PAP) and systemic arterial pressures (SAP) were measured under normoxic breathing conditions, immediately after insertion of the catheters, by Gould P 23 ID transducers coupled to pressure modules and a Gould TA 550 multichannel recorder. Only PAP successfully recorded within 30 min of catheter insertion were taken into account. Blood was also sampled from the systemic artery catheter for hematocrit measurements. Finally, after an intraperitoneal injection of pentobarbital sodium (60 mg/kg), the thorax was opened, and the heart was excised and weighed. Right ventricular hypertrophy was assessed on the basis of the ratio of right ventricle free wall weight over septum plus left ventricle free wall weight (RV/LV+SV, Fulton index).

Assessment of pulmonary vascular density and remodeling. After BAL, the lungs were fixed and processed as described above for mice. We analyzed a total of 35–65 intra-acinar vessels from each rat to assess the distribution and degree of muscularization; vessels accompanying alveolar ducts and those accompanying alveoli were assessed separately.

Pulmonary vascular density was evaluated in rats pretreated with Ad.nul or Ad.VEGF-B186 after 2 wk of exposure to various oxygenation conditions. The procedure was the same as described for mice.

Comparison of the effect of Ad.VEGF-B186 and Ad.VEGF-A administration on the development of PH. In a separate set of experiments, Ad.VEGF-A, Ad.VEGF-B186, or Ad.nul (10^10 VP) were administered intratracheally to normoxic rats on the day before the beginning of a 2-wk exposure to hypoxia. The procedure was the same as described above.

Statistical Analysis

All results are reported as means ± SE. Hemodynamic parameters, body weights, and heart weights in various groups of animals were compared by nonparametric Mann-Whitney or Kruskal-Wallis tests. To compare the degree of
pulmonary vessel muscularization between groups, we used a nonparametric Mann-Whitney or a Kruskal-Wallis test after ordinal classification of the vessels as nonmuscular, partially muscular, or fully muscular. Two-way analysis of variance (ANOVA) was performed to compare the effect of Ad.VEGFs vs. Ad.nul pretreatment in normoxic and hypoxic animals, followed by the Fisher test or Kruskal-Wallis test to compare Ad.VEGFs and Ad.nul for each oxygenation condition when interaction was significant.

RESULTS

Development of Hypoxic PH in VEGF-B+/+ and VEGF-B−/− Mice

Right ventricular systolic pressure, hematocrit, and the Fulton index, which were similar in normoxic VEGF-B+/+ and VEGF-B−/− mice, increased similarly in the two groups of mice under hypoxic condition (10% O2 for 3 wk) (Table 1). As shown in Fig. 1, muscularization of distal pulmonary vessels did not differ in normoxic VEGF-B+/+ and VEGF-B−/− mice and increased similarly after exposure to hypoxia. Vascular density, assessed by quantifying the surface area per field that stained for U. europaeus, did not differ between VEGF-B+/+ and VEGF-B−/− mice in normoxic condition and increased similarly after hypoxic exposure (Fig. 2).

Evaluation of Adenovirus Gene Transfer in Rats

Dose-dependent VEGF-B or VEGF-A expression and inflammatory response 5 days after Ad.VEGF-B or Ad.VEGF-A administration to normoxic rats. After administration of Ad.VEGF-B167 or Ad.VEGF-B186, protein was detected in BAL fluid with a dose as low as 10^9 VP. The protein levels increased in a dose-dependent manner with doses ranging from 10^9 to 10^11 VP (Fig. 3). For a similar adenovirus dose, protein expression was higher after Ad.VEGF-B186 than after Ad.VEGF-B167. No VEGF-B167 or very little VEGF-B186 protein (≤1.27 ng/ml) was detected in BAL fluids from control rats pretreated with Ad.nul or Ad.VEGF-A. No human VEGF-B167 or VEGF-B186 was found in plasma from rats treated with Ad.VEGF-B167 or Ad.VEGF-B186 in a dose of 10^10 VP.

The intensity of the inflammatory response after adenovirus transfer varied in a dose-dependent manner. Five days after administration of either Ad.VEGF-B167 or Ad.VEGF-B186 in a dose of 10^9 VP, histological examination revealed normal pulmonary architecture with no inflammation. Lung inflammation was mild with 10^10 VP but severe with doses >10^11 VP (Table 2). Five days after Ad.nul administration, inflammation was absent with 10^9 or 10^10 VP and mild with 10^11 VP (Table 2).

On the basis of these dose-response experiments, the dose of 10^10 VP was chosen for further experiments,
because it produced mild inflammation but significant VEGF-B expression in the lung.

Five days after administration of Ad.VEGF-A in a dose of $10^{10}$ VP, VEGF-A expression was consistent, and only mild inflammation was present. Human VEGF-A protein was detectable in BAL fluid from 6/6 rats, in concentrations ranging from 2.02 to 2.33 ng/ml. In a previous study, this level of VEGF-A protein expression was sufficient to protect rats against the development of hypoxic PH. No human VEGF-A was detected in plasma with this dose. In rats pretreated with Ad.nul, little or no human VEGF-A protein was detectable in BAL fluid (the highest concentration detected did not exceed 0.05 ng/ml). Mild inflammatory infiltrates and alveolitis (macrophages and polymonuclear cells) were found.

Evaluation of pulmonary edema after Ad.VEGF-B or Ad.VEGF-A administration. Five days after administration of $10^{10}$ VP in rats exposed to normoxia or hypoxia, there was a significant effect of adenoviral treatment ($P < 0.001$) and oxygenation condition ($P < 0.001$) on the wet-to-dry lung weight ratio, with no interaction. The hearts of Ad.VEGF-A-treated rats appeared normal, but the lungs were enlarged and edematous. Small pleural effusions were noted in some cases. With all oxygenation conditions, the wet-to-dry lung weight ratio was increased with Ad.VEGF-A compared with Ad.nul, Ad.VEGF-B167, or Ad.VEGF-B186 rats ($P < 0.001$, Fisher test; Fig. 4, A and B); the ratios were similar in these last three conditions and did not differ from those of sham rats not administrated with an adenovirus. The wet-to-dry lung weight ratio was also slightly increased after exposure to hypoxia compared with normoxia ($P < 0.001$).

Seventeen days after administration of $10^{10}$ VP, the wet-to-dry lung weight ratio was the same in all groups of rats exposed to normoxia (data not shown).

Evaluation of eNOS expression after Ad.VEGF-B or Ad.VEGF-A administration. Five days after adenovirus administration ($10^{10}$ VP), there were significant effects of adenoviral treatment ($P < 0.001$) and oxygenation condition on lung eNOS protein ($P < 0.01$), with no interaction. Lung eNOS protein was increased in the lungs of rats given Ad.VEGF-A, compared with Ad.VEGF-B or Ad.nul, with all oxygenation conditions ($P < 0.001$, Fig. 5, A and B). Expression of eNOS was also higher after hypoxia than after normoxia ($P < 0.01$).

Evaluation of NO production after Ad.VEGF-B or Ad.VEGF-A administration. Five days after adenovirus administration ($10^{10}$ VP) there were significant effects of adenoviral treatment ($P < 0.05$) and oxygenation condition on levels of NO-derived products in BAL fluid ($P < 0.05$), with no interaction. NOX levels were increased in the lungs of rats given Ad.VEGF-A, compared with Ad.VEGF-B or Ad.nul, with all oxygenation conditions ($P < 0.001$ Fig. 6, A and B). Levels of NOX were also higher after hypoxia than after normoxia ($P < 0.05$).

Effects of Ad.VEGF-B Pretreatment on Chronic Hypoxic PH, Comparison with Effects of Ad.VEGF-A

Administration of the different adenoviruses ($10^{10}$ VP) was well tolerated. No deaths or symptoms of respiratory failure were observed in normoxic rats or in rats subsequently exposed to chronic hypoxia.
Hemodynamic measurements and assessment of right ventricular hypertrophy in hypoxic rats pretreated with Ad.VEGF-B167 or Ad.VEGF-B186. After 15 days of exposure to hypoxia, final body weight, SAP, heart rate, and hematocrit were similar in rats pretreated with Ad.VEGF-B167, Ad.VEGF-B186, or Ad.nul (Table 3). However, PAP was lower in rats pretreated with Ad.VEGF-B186 than in rats pretreated with Ad.nul (P < 0.05 by one-way ANOVA). Right ventricular hypertrophy as assessed by the Fulton index was also significantly less marked in rats pretreated with Ad.VEGF-B167 or Ad.VEGF-B186 (P < 0.01) than in controls, whereas left ventricular weight was similar in the three groups. No difference in PAP or Fulton index was observed between Ad.VEGF-B167 and Ad.VEGF-B186 pretreatment.

Structural remodeling of distal pulmonary vessels in hypoxic rats pretreated with Ad.VEGF-B167 or Ad.VEGF-B186. As shown in Fig. 7, muscularization was also less marked in distal pulmonary vessels from rats pretreated with Ad.VEGF-B167 and Ad.VEGF-B186

Table 2. Lung histopathology 5 days after administration of various doses of Ad.nul, Ad.VEGF-B167, or Ad.VEGF-B186 in normoxic rats

<table>
<thead>
<tr>
<th>Inflammation (0–3)</th>
<th>Ad.nul</th>
<th>Ad.VEGF-B167</th>
<th>Ad.VEGF-B186</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity</td>
<td>10^9 VP</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Cell type</td>
<td>10^10 VP</td>
<td>1</td>
<td>2/3</td>
</tr>
<tr>
<td>Location</td>
<td>10^11 VP</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Ad.nul, control adenovirus vector; Ad.VEGF-B, VEGF-B adenovirus vector; VP, viral particles.

Fig. 4. Wet-to-dry lung weight ratio 5 days after administration of 10^10 viral particles (VP) of Ad.VEGF-B167, Ad.VEGF-B186, Ad.VEGF-A165, Ad.nul, or sham rats not administered with an adenovirus. A: normoxic rat lungs; B: hypoxic rat lungs. There was a significant effect of the oxygenation condition (P < 0.01) and adenoviral pretreatment (P < 0.001), with no interaction. *P < 0.001 compared with Ad.nul, Ad.VEGF-B167, or Ad.VEGF-B186-pretreated rats (Fisher test after 2-way ANOVA, n = 8 in each experiment).

Fig. 5. Lung endothelial nitric oxide synthase (eNOS) expression assessed by Western blot analysis 5 days after 10^10 VP adenoviral administration of Ad.VEGF-B167, Ad.VEGF-B186, Ad.VEGF-A165, or Ad.nul in normoxic rat lungs (A) and hypoxic rat lungs (B). Top of A and B: representative blots with each lane corresponding to 1 rat lung. Bottom of A and B: quantification of the density (means ± SE) of lungs from n = 8 rats for each group. Expression of eNOS was increased after pretreatment with Ad.VEGF-A compared with Ad.nul, Ad.VEGF-B167, and Ad.VEGF-B186 (P < 0.001) and was also higher after hypoxia than after normoxia (P < 0.01), with no interaction (Fisher test after 2-way ANOVA).
than in controls, the percentage of muscularized arteries being reduced at both the alveolar duct and the alveolar wall levels (P < 0.001, Kruskal-Wallis followed by Dunn’s test on ordinally classified vessels). There were no differences between Ad.VEGF-B167 and Ad.VEGF-B186 pretreatment.

**Table 3. Final BW, hemodynamic parameters, right ventricular hypertrophy, and hematocrit after 2-wk exposure to hypoxia of rats pretreated with Ad.nul, Ad.VEGF-B167, and Ad.VEGF-B186**

<table>
<thead>
<tr>
<th></th>
<th>Ad.nul (n = 6)</th>
<th>Ad.VEGF-B167 (n = 7)</th>
<th>Ad.VEGF-B186 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final BW, g</td>
<td>253 ± 5.3</td>
<td>242 ± 8.3</td>
<td>234 ± 8.2</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>35.3 ± 1.15</td>
<td>32.8 ± 0.96</td>
<td>30.6 ± 0.4*</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>120 ± 13</td>
<td>103 ± 9</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>278 ± 35</td>
<td>267 ± 22</td>
<td>280 ± 16</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.56 ± 0.01</td>
<td>0.48 ± 0.02</td>
<td>0.43 ± 0.02*</td>
</tr>
<tr>
<td>RV, g</td>
<td>0.28 ± 0.01</td>
<td>0.23 ± 0.01*</td>
<td>0.21 ± 0.01*</td>
</tr>
<tr>
<td>LV + S, g</td>
<td>0.49 ± 0.01</td>
<td>0.48 ± 0.03</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>60.0 ± 3</td>
<td>60.3 ± 1.5</td>
<td>57.4 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. PAP, pulmonary artery pressure; SAP, systemic arterial pressure; RV, right ventricle weight; LV + S, left ventricle plus septum weight. *P < 0.01 compared with control rats treated with Ad. nul.

Table 4. **Final BW, hemodynamic parameters, right ventricular hypertrophy, and hematocrit after 2-wk exposure to hypoxia of rats pretreated with Ad.nul, Ad.VEGF-B186, and Ad.VEGF-A165**

<table>
<thead>
<tr>
<th></th>
<th>Ad.nul (n = 8)</th>
<th>Ad.VEGF-B186 (n = 7)</th>
<th>Ad.VEGF-A165 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final BW, g</td>
<td>249 ± 6.6</td>
<td>231 ± 6.8</td>
<td>221 ± 8.3</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>30.7 ± 2.4</td>
<td>26.4 ± 1.1</td>
<td>27.3 ± 1.3</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>92 ± 6</td>
<td>98 ± 6</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>310 ± 19</td>
<td>277 ± 10</td>
<td>280 ± 15</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.43 ± 0.02</td>
<td>0.36 ± 0.02</td>
<td>0.35 ± 0.01*</td>
</tr>
<tr>
<td>RV, g</td>
<td>0.21 ± 0.09</td>
<td>0.17 ± 0.01*</td>
<td>0.18 ± 0.01*</td>
</tr>
<tr>
<td>LV + S, g</td>
<td>0.49 ± 0.01</td>
<td>0.48 ± 0.03</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>55.9 ± 1.15</td>
<td>55.3 ± 1.4</td>
<td>55.4 ± 2.15</td>
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</tbody>
</table>

Values are means ± SE. Only 7 of 8 Ad.nul, 6 of 7 VEGF-B186, and 7 of 8 Ad.VEGF-A165 pretreated rats had PAP and SAP measurements. *P < 0.05 compared with control rats treated with Ad.nul; †P < 0.01 compared with control rats treated with Ad.nul.
DISCUSSION

Our findings show that ablation of endogenous VEGF-B expression in mice does not aggravate the development of PH in response to 3 wk of hypoxia. Whatever the oxygenation condition, neither PAP nor right ventricular hypertrophy differed between VEGF-B−/− and wild-type mice. Moreover, VEGF-B gene inactivation had no effect on distal vessel muscularization or vascular density, which were similar in the two groups of hypoxic animals. In contrast, VEGF-B167 or VEGF-B186 overexpression in the lungs induced by adenoviral gene transfer had similar protective effects against the development of hypoxic PH as did VEGF-A. Right ventricular hypertrophy was less severe and distal vessel muscularization less marked in rats pretreated with Ad.VEGF-B186 or Ad.VEGF-A compared with those given Ad.nul. Moreover, vascular density was similarly increased in animals pretreated with Ad.VEGF-B186 or Ad.VEGF-A compared with the controls similarly exposed to 2 wk of hypoxia.

Although endogenous VEGF-B has been shown to be coexpressed with VEGF-A in the lung (33), our data suggest that, in contrast to other angiogenic factors, endogenous VEGF-B neither affects the pulmonary circulation in normoxia nor significantly counteracts the development of hypoxic PH. Indeed, various studies indicate that exposure to hypoxia is associated with activation of endogenous lung angiogenic processes (8, 18, 27, 28). Labeling studies in rats have demonstrated a burst of endothelial cell division in intra-acinar arteries at the end of the first week of exposure to hypoxia (29). Moreover, recent results obtained by our group by quantification of lung immunoreactivity for factor VIII suggest an increase in lung vascular density in mice exposed to hypoxia (36). Among angiogenic factors, lung VEGF-A, which is expressed at a high level in animals chronically exposed to hypoxia (8, 34, 42), has been shown to protect against the development of chronic hypoxic PH (37, 41). Inhibition of VEGF receptors through tyrosine kinase inhibitors causes mild PH and pulmonary vascular remodeling in normoxic rats and severe irreversible PH in chronically hypoxic rats (40). Moreover, counteracting lung angiogenesis by overexpression of angiotatin aggravates PH in chronically hypoxic mice (36). Previous data suggest that activation of VEGFR-1 by VEGF-B may specifically potentiate the response to VEGF-A and contribute to angiogenesis under abnormal conditions such as ischemia (7). Whereas both VEGF-A−/− and VEGF-A+/− mice are unable to survive to term due to a diffuse impairment of blood vessel formation in the early embryo (6), VEGF-B−/− mice appear healthy and fertile (2). However, their hearts are reduced in size and display impaired recovery from experimentally induced ischemia (2). Our present results obtained in normoxic mice as well as during development of hypoxic PH differ markedly from those reported with VEGF-B−/− gene deletion in the heart. The prenatal and postnatal heart is one of the organs with the highest level of VEGF-B expression (31, 33). This may

![Graph A](http://ajplung.physiology.org/)

![Graph B](http://ajplung.physiology.org/)

Fig. 8. Percentage of nonmuscular, partially muscular, and fully muscular vessels at the alveolar duct or alveolar wall levels in rats pretreated with Ad.VEGF-B186 (n = 7), Ad.VEGF-A (n = 8), or Ad.nul (n = 8) and exposed to hypoxia for 2 wk. Muscularization of intra-acinar vessels was similar with Ad.VEGF-B186 and Ad.VEGF-A pretreatment but was significantly reduced compared with Ad.nul pretreatment, at both the alveolar duct and alveolar wall levels (\(**P < 0.001\), Kruskal-Wallis test followed by Dunn’s test on ordinally classified vessels).

**Structural remodeling of distal pulmonary vessels in hypoxic rats pretreated with Ad.VEGF-B186 or Ad.VEGF-A.** As shown in Fig. 8, muscularization was also less marked in distal pulmonary vessels from rats pretreated with Ad.VEGF-B186 or Ad.VEGF-A, compared with controls, the percentage of muscularized arteries being reduced at both the alveolar duct and the alveolar wall levels (\(P < 0.001\), Kruskal-Wallis test followed by Dunn’s test on ordinally classified vessels).

**Vascular density in hypoxic rats pretreated with Ad.VEGF-B186 or Ad.VEGF-A.** Vascular density was assessed by quantifying the surface area per field that stained for *U. europaeus*. Because only areas without bronchioli or arteries 15 μm or more in diameter were considered, our count represents only distal vessel density (Fig. 9B). Staining of large arteries was used as the positive control (Fig. 9A). As shown in Fig. 9, C and D, vascular density was increased in the lungs from rats chronically exposed to hypoxia, compared with similarly pretreated normoxic animals (\(P < 0.001\)), and there was a significant interaction between the various adenovirus treatments and the oxygenation conditions (\(P < 0.05\)). Pretreatment with Ad.VEGF-B186 or Ad.VEGF-A significantly increased lung vascular density in chronically hypoxic rats (\(P < 0.01\) and \(P < 0.001\) respectively, Fig. 9D) but not in normoxic rats (Fig. 9C), compared with Ad.nul controls.
explain why VEGF-B can influence the development and function of the coronary circulation in response to ischemia. Compared with VEGF-B, VEGF-A is predominantly expressed in the lung (30, 33). This difference in expression between the two proteins, which is further amplified by hypoxia (31, 41), may make VEGF-B unable to potentiate the effect of VEGF-A in the lung. We also cannot rule out that, in the lung, upregulation of VEGF-A in VEGF-B−/− mice may compensate for the lack of VEGF-B. Our present results are in slight discordance with those of Wanstall et al. (43), who recently reported blunting of PH and vascular remodeling in VEGF-B−/− mice exposed to chronic hypoxia. We have no explanation for this discordance except that they studied female mice instead of male in our present study.

In the present study, using adenoviral gene transfer, we examined the effect of lung VEGF-B overexpression in the development of PH. As previously demonstrated by our group for VEGF-A (35), intratracheal administration of Ad.VEGF-B167, Ad.VEGF-B186, or Ad.VEGF-A ensured efficient local gene transfer, with secretion of the protein by transduced cells. In accordance with our previous study (35), protein secretion was demonstrated by a dose-dependent increase in protein levels in BAL fluid. Moreover, as assessed by the level of VEGF-A in BAL fluid 4 days after intratracheal administration of 10^10 VP of Ad.VEGF-A, the level of VEGF-A overexpression was similar to that previously shown by our group to be associated with significant attenuation of PH (35). VEGF-B levels in BAL fluid peaked on day 4 after adenoviral administration, but the proteins were not detected after day 10. No human VEGF-B167, VEGF-B186, or VEGF-A was detected in plasma with the dose used in our study, suggesting a minimal risk of diffusion and expression in other organs. As indicated by our histological data, efficient gene transfer was obtained at the expense of mild inflammation, in both normoxic and hypoxic rats. Thus administration of 10^10 VP of Ad.VEGF-B167, Ad.VEGF-B186, or Ad.VEGF-A was associated with only small patchy macrophagic alveolitis.
With this dose of adenovirus, overexpression of VEGF-B_{167} or VEGF-B_{186} in lung tissue was associated with significant attenuation of PH. PAP was significantly lower with Ad.VEGF-B_{186} than with Ad.nul pretreatment in rats exposed to similar hypoxic conditions. Moreover, right ventricular hypertrophy and the percentage of muscularized arteries at both the alveolar duct and wall levels were less severe in rats pre-treated with either Ad.VEGF-B_{167} or Ad.VEGF-B_{186} than in those given Ad.nul, whereas SAP, left ventricular weight, and hematocrit were similar.

The protective effects of VEGF-B overexpression against hypoxic PH were comparable to those obtained with VEGF-A, as shown by the similar right ventricular hypertrophy and percentage of muscularized arteries at both the alveolar duct and wall levels in all three groups of animals overexpressing a VEGF protein.

In the present study, exposure to hypoxia for 5 days was associated with a twofold increase in eNOS expression in lung tissue as well as in levels of NO-derived products in BAL fluid from Ad.nul-pretreated control rats. This is in accordance with the increases in eNOS protein and activity previously found in lung tissue from hypoxic rats (12, 14, 24–26, 44). Administration of Ad.VEGF-A caused a further increase in eNOS expression in lungs and NO-derived products in BAL fluid from both normoxic and hypoxic rats. In our previous study, we also observed that lung VEGF-A overexpression was associated with an increase in eNOS activity (35). This is consistent with studies of systemic vessels, in which VEGF-A overexpression within the vascular wall restored endothelium-dependant relaxation of these vessels and protected against vasoconstriction (1). Therefore, the attenuation of hypoxia-induced pulmonary vascular remodeling by VEGF-A overexpression in our study can be ascribed in part to protection of endothelial function and enhanced release of endothelial NO (23). This effect of VEGF-A has also been reported in cultured endothelial cells, where it was related to activation of the KDR receptor tyrosine kinase and of a downstream protein kinase C signaling pathway (21, 22, 38). In contrast, overexpression of VEGF-B, which binds to VEGFR-1 but not to VEGFR-2, did not affect eNOS expression in the lungs. However, it has been suggested that VEGFR-1 may function as an inert decoy by binding to VEGF-A, which has far greater affinity for VEGFR-1 than for VEGFR-2, thus regulating the amount of VEGF-A available for activating VEGFR-2 (16). It has also been suggested that VEGF-B may enhance the angiogenic response to VEGF-A by forming VEGF-A/VEGF-B heterodimers that activate VEGFR-2 (11). It is unlikely that such mechanisms account for the protective effect of VEGF-B overexpression against hypoxic PH. Although VEGF-B_{167} or VEGF-B_{186} overexpression attenuated PH to the same extent as VEGF-A did, there was no effect on the eNOS protein level and NO production. Mechanisms other than a transfer of VEGF-A from receptor VEGFR-1 to VEGFR-2 have been suggested to explain the VEGF-B-induced amplification of the angiogenic response to VEGF-A (5, 15, 19, 20). Previous studies have shown that activation of VEGFR-1 specifically potentiates the angiogenic response to VEGF but not to basic fibroblast growth factor (7). Another ligand of VEGFR-1, PlGF, has no effect on eNOS expression (38) but has recently been shown to amplify the migration, proliferation, and survival of capillary endothelial cells in response to VEGF-A. This effect was observed only in cells without endogenous PlGF production (7). This may be the case in the lung, where production of endogenous VEGF-B or PlGF, the two specific ligands of VEGFR-1, is probably very low under usual conditions and are not up-regulated as VEGFR-1 is by hypoxia. If the effects on eNOS result from VEGFR-2 activation, our results would indicate that VEGFR-1-transmitted intracellular signals mediate the ability of VEGF-B overexpression to attenuate hypoxic PH without increasing eNOS expression. A selective Src-kinase inhibitor was recently shown to completely block PlGF-dependent amplification of the VEGF-A response in PlGF−/− endothelial cells, whereas it did not affect the response to VEGF-A in these same cells (7). In our study, 5 days after Ad.VEGF-A administration, there was some evidence of lung edema as shown by an increase in wet-to-dry lung weight, whereas no such an effect was observed after treatment with Ad.VEGF-B. This finding further supports the hypothesis that the two proteins protect against PH through different mechanisms.

Together, our present results obtained in mice deleted for the VEGF-B gene provide evidence that, in the lung, endogenous VEGF-B does not significantly counteract the development of chronic hypoxic PH. This redundant role of VEGF-B in the pulmonary circulation is probably due to a low level of expression compared with VEGF-A, since we demonstrated that VEGF-B, when overexpressed in the lung by means of adenoviral gene transfer, was as potent as VEGF-A in attenuating the development of PH and vascular remodeling. Our data obtained by quantification of distal vessel endothelial marking also strongly suggest that both VEGF-B and VEGF-A can stimulate angiogenesis in the pulmonary circulation. However, our finding that VEGF-B does not share with VEGF-A the ability to stimulate eNOS expression and NO production strongly suggests that different molecular mechanisms underlie the effects of VEGF-A and VEGF-B in this rat model of PH. The effects of VEGF-B may depend specifically on intracellular signals mediated by VEGFR-1 activation. Moreover, the fact that, when overexpressed, VEGF-B was as potent in attenuating PH as was VEGF-A but did not significantly increase vascular permeability may have implications for the treatment of PH.

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