Lung Overexpression of the Vascular Endothelial Growth Factor Gene Induces Pulmonary Edema

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We hypothesized that the angiogenic mediator, vascular endothelial growth factor (VEGF), known to be expressed in the lung and to be capable of inducing local edema in skin, might evoke the development of lung edema if expressed in excess amounts. To test this hypothesis, we developed an *in vivo* model of VEGF overexpression in the lung on the basis of delivery to the respiratory epithelium of the VEGF165 complementary DNA by an E1$^-$ adenovirus vector (AdVEGF165). Administration of AdVEGF165 by the intratracheal route (10^9 plaque-forming units [pfu]) to C57Bl/6 mice showed increased expression of VEGF messenger RNA in lung tissue by Northern analysis. Overexpression of VEGF protein in the lung at Days 1 to 10 was confirmed by enzyme-linked immunosorbent assay. Intratracheal administration of AdVEGF165 resulted in a dose-dependent increase in lung wet/dry weight ratios over time, lung histology showed widespread intraalveolar edema, and pulmonary capillary permeability was significantly increased as quantified by the Evans blue dye assay and [^{131}I]albumin permeability. To confirm the specificity of these observations, mice were pretreated with intranasal administration of an adenovirus vector expressing a truncated soluble form of the VEGF receptor *flt-1* (Ads*flt*). Ads*flt* (10^9 pfu) pretreatment completely abrogated the increased lung wet/dry weight ratio caused by AdVEGF165 administration, whereas an identical adenovirus vector with an irrelevant transgene had no effect upon subsequent AdVEGF165-induced pulmonary edema. Together, these data suggest that overexpression of VEGF in the lung may be one mechanism of increased pulmonary vascular permeability in the early stages of acute lung injury.

Pulmonary edema, defined as an excessive amount of extravascular water in the lungs, is a common clinical problem that occurs when fluid is filtered into the lungs faster than it is removed (1–5). The major consequence of pulmonary edema is in the gas exchange units, where swelling of the alveolar interstitium and flooding of the alveoli cause disturbances in lung mechanical functions and gas exchange (6–8). Although there are many causes of pulmonary edema, the pathophysiology of pulmonary edema can be generally classified as that due to increased pressure (e.g., heart failure) or due to increased permeability (e.g., adult respiratory distress syndrome [ARDS]) (9, 10). In the "increased permeability" category, more than 100 initiating agents have been described, all of which are associated with an alteration in the integrity of the barrier to the flow of protein and fluid into the lungs (9). This barrier is provided by the 70-m^2 continuous, nonfenestrated capillary bed, in which the endothelium is held together by tight junctions forming a "gasket-like" seal (11, 12). The barrier to protein and fluid flow into the alveolar interstitium via paracellular pathways is provided by these tight junctions, along with the endothelial basement membrane (12–14). Although there are other mechanisms of bulk water flow across the pulmonary endothelium (e.g., plasmalemmal vesicles and aquaporin [water channels] [15, 16]), it is likely that the induction of increased permeability of the pulmonary capillary bed is directly linked to reversible physical modifications of the pulmonary endothelium (17).

The present study is based on the hypothesis that acute pulmonary edema can be induced by acute, excess expression of the vascular endothelial growth factor (VEGF) gene in the lung. VEGF is a 34- to 46-kD dimeric glycoprotein produced by alternative splicing of the 7 exon VEGF gene located at p12-21 on chromosome 6 (18–20). The VEGF gene is widely expressed, but the highest level of expression in normal tissues is in the lung (21, 22). In normal human tissues, the VEGF gene is expressed as proteins of 206, 189, 165, and 121 amino acids (19). Although the major interest in VEGF has been as an initiator of angiogenesis or target for excess angiogenesis in association with tumors (18), VEGF was initially described as "vascular permeability factor," on the basis of its ability to induce permeability in guinea-pig skin (23). Little attention has been focused on this aspect of the function of VEGF, although it is known that excess VEGF can cause accumulation of fluid in the peritoneal space (23). At the cellular level, VEGF interacts with endothelial cells through specific kinase domain receptors (*flt-1*, *flk-1*/KDR, neuropilin) on the endothelial surface (19, 24, 25), initiating proliferation of the endothelium and induction of a complex series of events culminating in angiogenesis (19, 24, 26). Relevant to edema, *in vitro* studies have demonstrated that VEGF affects endothelial morphology within 10 min, inducing the formation of fenestrae in the capillary sheet (27–29). VEGF also stimulates actin stress fiber formation and new focal adhesions in endothelial cells, consistent with a regulatory role in endothelial morphology.

To evaluate the hypothesis that acute overexpression of VEGF can induce pulmonary edema, we have used an adenovirus (Ad) gene transfer vector to overexpress the hu-

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Abbreviations: adenovirus, Ad; analysis of variance, ANOVA; β-galactosidase, βgal; complementary DNA, cDNA; Evans blue dye, EBD; enzyme-linked immunosorbent assay, ELISA; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; interleukin, IL; messenger RNA, mRNA; phosphate-buffered saline, pH 7.4, PBS; plaque-forming units, pfu; vascular endothelial growth factor, VEGF.

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man VEGF165 complementary DNA (cDNA) in the lower respiratory tract of mice (30–32). The data demonstrate that increased expression of VEGF165 is associated with an increase in lung water, histologic evidence of edema, and increased lung permeability. Interestingly, the VEGF-induced pulmonary edema can be blocked by prior Ad-mediated transfer to the lung of a cDNA coding for the extracellular portion of *flt-1*, one of the major VEGF receptors.

Materials and Methods

Vectors

All Ad vectors were E1a$^-$, partial E1b$^-$, partial E3$^-$ based on the Ad5 backbone, containing a transgene (AdVEGF165, Ad*sflt*, Adβ-gal) or no transgene (AdNull) under control of the cytomegalovirus immediate-early promoter/enhancer in the E1a position (31, 32). AdVEGF165 expresses the human VEGF165 cDNA (33), Ad*sflt* expresses the truncated soluble form of the human *flt-1* cDNA (34), and Adβgal expresses the *Escherichia coli* β-galactosidase (βgal) gene (35). All of the Ad vectors were purified by cesium chloride density gradient ultracentrifugation, titered by plaque-forming assay on 293 cells, and demonstrated to be free of replication-competent Ad (31, 32).

Animal Protocol

C57Bl/6 mice, 8 to 10 wk old, weighing 18 to 20 g, were used for this study (Charles River, Wilmington, MA; and Taconic, Germantown, NY). Animals were anesthetized with 250 to 300 μl of 1:10 dilution of ketamine (Fort Dodge Animal Health, Ford Dodge, IA) and xylazine (Bayer, Shawnee Mission, KS) by intraperitoneal injection. A cutdown was performed to expose the trachea, and a 25-gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) was inserted under direct vision through the tracheal wall into the lumen at the level of the midportion of the trachea. Ad vectors were resuspended in phosphate-buffered saline, pH 7.4 (PBS), and diluted to a final volume of 50 μl, which was slowly instilled through the angiocath over a period of 1 to 2 min. The catheter was withdrawn and the incision was closed with sutures. At the appropriate time points, animals were anesthetized and killed with a lethal dose of ketamine/xylazine.

Northern Blot Analysis

At time of death, animals were exsanguinated, and 2 ml of PBS was injected into the right ventricle to perfuse the lungs. Lungs were removed *en bloc*, snap-frozen in liquid N_2, and homogenized in 2 ml of Trizol (GIBCO BRL, Grand Island, NY). After extraction, total cellular RNA (10 μg/lane) was transferred to nylon membranes after electrophoretic separation through a 1% agarose gel. Probes, including human VEGF165 cDNA (33), mouse VEGF164 cDNA, and the control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (36) were gel-purified and labeled with [^{32}P]–deoxycytidine triphosphate (dCTP) using a random-primer labeling kit (Stratagene, La Jolla, CA). Human *sflt* cDNA was labeled with [^{32}P]dCTP using Klenow DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Hybridizations were performed in QUICKHYB (Stratagene) for 2 h at 65°C using standard methods.

VEGF Protein Determination

After dissection as previously described, lungs were snap-frozen in liquid N_2 and homogenized in 2 ml PBS containing protease inhibitors. After centrifugation in a microfuge (4°C, 30 min), the supernatants were stored at −80°C until assayed. VEGF concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

Quantification of Lung Wet/Dry Weight Ratio

Lungs were excised *en bloc* and dissected away from the heart and thymus. The lungs were immediately weighed and then placed in a dessicating oven at 65°C for 48 h, at which point dry weight was achieved. The ratio of wet/dry weight was used to quantify lung water content (4).

Lung Histopathology

At the time of death, animals were exsanguinated, and 2 ml of PBS was injected into the right ventricle to perfuse the lungs. Lungs were removed *en bloc* and inflated to total lung capacity with air injected via an angiocath placed in the trachea and tied with sutures. The air-inflated lungs were placed in an uncovered container with 300 ml PBS and submerged by covering with saturated gauze. The PBS was heated to 60°C for 4 min in a microwave oven (37). The lungs were then transferred to 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 18 h and then transferred to PBS. The lungs were paraffin-embedded and butterfly-shaped sections of 5-μm thickness were cut and placed on glass microscope slides stained with hematoxylin and eosin (H&E) (American Histo Labs, Gaithersburg, MD). The H&E sections were imaged at both macroscopic and microscopic levels. Macroscopic imaging of lungs was accomplished by mounting histologic slides of lungs in a PathScan Enabler (Meyer Instruments, Inc., Houston, TX) and digitizing the slide in a Polaroid SprintScan slide scanner (Polaroid Corp, Cambridge, MA). Microscopic images were created on Kodak T160 slide film using a Nikon UFX-DX 35-mm automated photography system on a Nikon Microphot SA microscope equipped with a ×10 plan objective, ×2.5 projection lens, and neutral color balance filter.

Evans Blue Dye Assay

To further assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/ml. Animals were anesthetized, weighed, and injected with 20 mg/kg EBD in the left jugular vein. After 30 min, the animals were killed and the lungs perfused with 1 ml PBS containing 5 mM ethylenediaminetetraacetic acid. The lungs were excised *en bloc* and snap-frozen in liquid N_2. The frozen lungs were homogenized in 2 ml PBS. The homogenate was diluted with 2 vol of formamide and incubated at 60°C for 2 h, followed by centrifugation at 5,000 × *g* for 30 min. The supernatant was collected and absorbance was measured at 620 and 740 nm in a dual-wave spectrophotometer (38, 39). The EBD concentration was determined from standard absorbance curves evaluated in parallel. Correction for contaminating heme pigments was calculated by the formula $E_{620}(EBD) = E_{620} - (1.426 \times E_{740} + 0.030)$. The EBD concentration was expressed as a percentage of the total dose of EBD administered (40).

Extravascular Lung Water and Lung Vascular Protein Permeability

A vascular tracer, [^{131}I]albumin, was injected intraperitoneally 2 h before death. The lungs were removed *en bloc* and the blood was obtained at the end of the experiment. The lung and plasma radioactivity were determined by γ counting. Wet and dry lung weights were measured by the gravimetric method (41). Hemoglobin concentration was measured in ultracentrifuged (14,000 × *g* for 10 min) lung homogenate and in peripheral blood (41). Extravascular lung water content was determined by standard methods (41, 42). Lung vascular protein permeability was determined by subtracting lung intravascular (plasma) [^{131}I] radioactivity from total lung [^{131}I] radioactivity. Lung intravascular [^{131}I] radioactivity was calculated from the plasma [^{131}I] count multiplied by the blood volume in the lung (41).

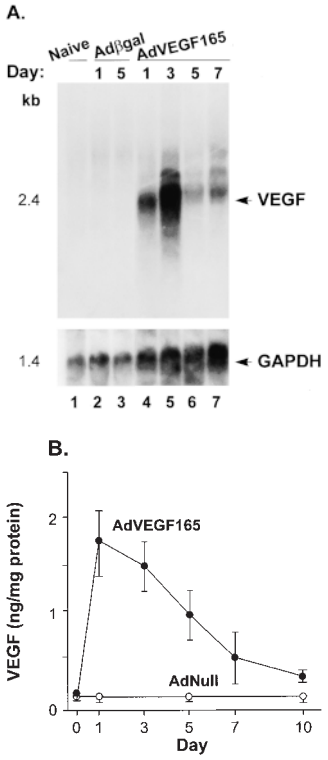

Figure 1. Expression of VEGF in mouse lungs after administration of AdVEGF165. (*A*) Northern blot analysis of VEGF mRNA expression. Total RNA (10 μg/lane) from mouse lungs harvested at various time points after intratracheal administration (10^9 pfu) of AdVEGF165, Adβgal, or naive mice were hybridized with a [^{32}P]-labeled human VEGF or human GAPDH cDNA probe. *Lane 1*, naive control; *lanes 2* and *3*, Adβgal Days 1 and 5; *lanes 4–7*, AdVEGF165 Days 1, 3, 5, and 7. (*B*) Quantification of VEGF protein from mouse lung extracts harvested at various time points after intratracheal administration of AdVEGF165. VEGF concentrations were determined by ELISA and normalized to total protein. The AdNull vector is used as a control.

Administration of Ad*sflt*

To determine whether pretreatment with an Ad vector expressing a soluble form of the *flt-1* VEGF receptor would prevent AdVEGF165-induced pulmonary edema, mice were anesthetized, restrained, and placed on an operating surface at an angle of 60 degrees. The Ad*sflt* vector (10^9 plaque-forming units [pfu]) resuspended in PBS (50 μl) was administered dropwise into the nasal passages with an insulin syringe and 27-gauge needle over a period of 20 to 30 min per animal. For confirmation of *sflt* expression, lungs were collected at various time points for Northern blot analysis as described earlier. In separate experiments, mice treated with intranasal administration of Ad*sflt* subsequently received intratracheal administration of 10^7 to 10^9 pfu AdVEGF165 or 10^9 pfu Adβgal on Day 3. After an additional 5 d, lungs were collected and wet/dry weight ratios were determined as previously described.

Statistical Analysis

Quantitative data are expressed as means ± standard error. Multiple groups were compared with unweighted means analysis of variance (ANOVA). $P \leq 0.05$ was considered significant. The significance of differences between individual groups was assessed with Newman–Keuls *post hoc* test using $\alpha = 0.05$. When only two groups were compared, the unweighted Student's *t* test was performed. Correlation was assessed with Pearson's linear regression. Statistical calculations were computed with the Number Cruncher Statistical System software program (NCSS, Kaysville, UT).

Results

Expression of VEGF *In Vivo*

Intratracheal administration of 10^9 pfu of AdVEGF165 resulted in enhanced expression of human VEGF messenger RNA (mRNA) in the mouse lungs (Figure 1A). The mRNA level (relative to GAPDH) peaked at Day 3 (Figure 1A, *lane 6*) and was readily detectable throughout the 7 d after administration (Figure 1A, *lanes 4–7*). No human VEGF mRNA was detected in the naive animals (Figure 1, *lane 1*), or animals receiving Adβgal (Figure 1A, *lanes 2* and *3*). Basal expression of mouse VEGF RNA was detectable when lung mRNA was hybridized with a mouse VEGF cDNA probe (not shown). Levels of GAPDH were used to control for loading of RNA. In a parallel fashion, the ratio of VEGF protein detected by ELISA normalized to total protein of lung extracts increased after AdVEGF165 administration, peaking at Day 1 (Figure 1B) and remaining at elevated levels at Day 10. These results indicate that intratracheal administration of AdVEGF165 results in overexpression of VEGF in the lung.

Physiologic Analysis of Effects of VEGF165 Overexpression in the Lung

The lung wet/dry weight ratio, a measure of extravascular lung water (4), was quantified as a function of AdVEGF165 dose after intratracheal administration (Figure 2A). The wet-to-dry weight ratio showed a dose-dependent increase, with the highest dose (10^9 pfu) resulting in a significant increase over that observed with the AdNull at the same dose ($P < 0.05$, ANOVA). These mice had a slightly increased respiratory rate but had no other obvious abnormalities. When the animals receiving the highest dose of AdVEGF165 were killed, the hearts appeared grossly normal but the lungs were visibly enlarged and edematous.

In a parallel study, evaluation of the time course of edema formation showed that the wet-to-dry weight ratio was significantly increased throughout Days 1 to 10 ($P < 0.05$, ANOVA) (Figure 2B) relative to naive controls ($P < 0.05$, ANOVA, not shown). The absolute magnitude of the

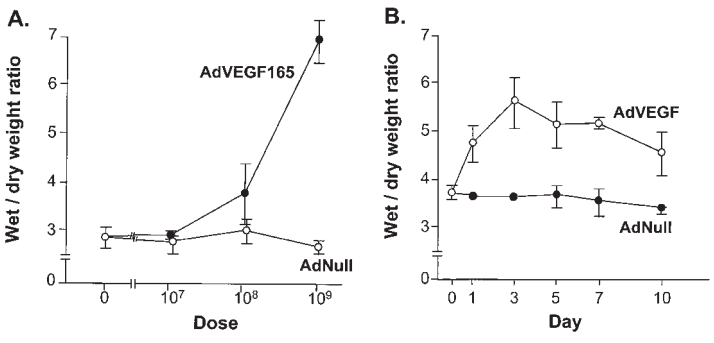

Figure 2. Effect of intratracheal administration of AdVEGF165 on lung wet/dry weight ratio. For each condition, $n = 3$ animals per group. (*A*) Lung wet/dry weight ratios as a function of AdVEGF165 dose 5 d after vector administration. The AdNull vector is used as a control. (*B*) Lung wet/dry weight ratios as a function of time after administration of 10^9 pfu AdVEGF165 compared with 10^9 pfu of AdNull.

increase at Day 5 was less than in the experiment shown in Figure 2A due to the variability between different groups of animals. The results with AdNull indicate the effect is specific for the transgene and not due to the Ad vector itself.

To rule out a possible contribution of neovascularization and hence increased pulmonary blood volume as an explanation for increased wet/dry weight ratios after administration of 10^9 pfu AdVEGF, lung hemoglobin content (39) was assessed in ultracentrifuged lung homogenates; no increases were found at any time point (Day 5 lung hemoglobin supernatant concentrations [means ± standard deviation]: naive [7.5 ± 1.0] × 10^{-2} g/dL; Adβgal [8.0 ± 0.6] × 10^{-2} g/dL; AdVEGF [7.7 ± 0.8] × 10^{-2} g/dL), indicating that increased pulmonary lung blood volume was not an explanation.

Histology of lung sections fixed by a microwave technique to maximize preservation of pulmonary edema (37) revealed dramatic interstitial and intra-alveolar edema throughout the lungs of mice treated with intratracheal AdVEGF165 (10^9 pfu) 5 d after administration (Figures 3C and 3F). The edema was patchy and slightly more prominent in the lower lung zones, as would be expected after intratracheal administration of vector. Mice treated with the same dose of AdNull showed mild interstitial inflammation (Figures 3B and 3E), but no other significant differences from mice treated with saline only as a control (Figures 3A and 3D).

To evaluate permeability of the mouse lung to macromolecules after administration of AdVEGF165, EBD, which binds rapidly and irreversibly to albumin in the circulation, was injected intravenously through a central vein to mice receiving 10^9 pfu AdVEGF165 intratracheally 5 d previously, and the mice were killed 30 min later. Compared with both the AdNull and naive controls, there was a 3-fold increase in the lung/total dose ratio of EBD after intratracheal AdVEGF165 administration ($P < 0.05$ ANOVA; Figure 4). These data are consistent with the lung wet-to-dry weight ratios and the histology demonstrating that lung permeability to macromolecules was significantly increased by VEGF overexpression.

To confirm these results, we quantified extravascular lung water and permeability to macromolecules using [^{131}I]albumin as a tracer. Animals were compared 5 d after intratracheal administration of 10^9 pfu of AdVEGF or Adβ-gal. There was a significant increase in wet/dry weight ratio corrected for vascular volume (Figure 5A, $P < 0.005$). The amount of [^{131}I]albumin accumulating in the lung increased 3-fold in AdVEGF-treated mice (Figure

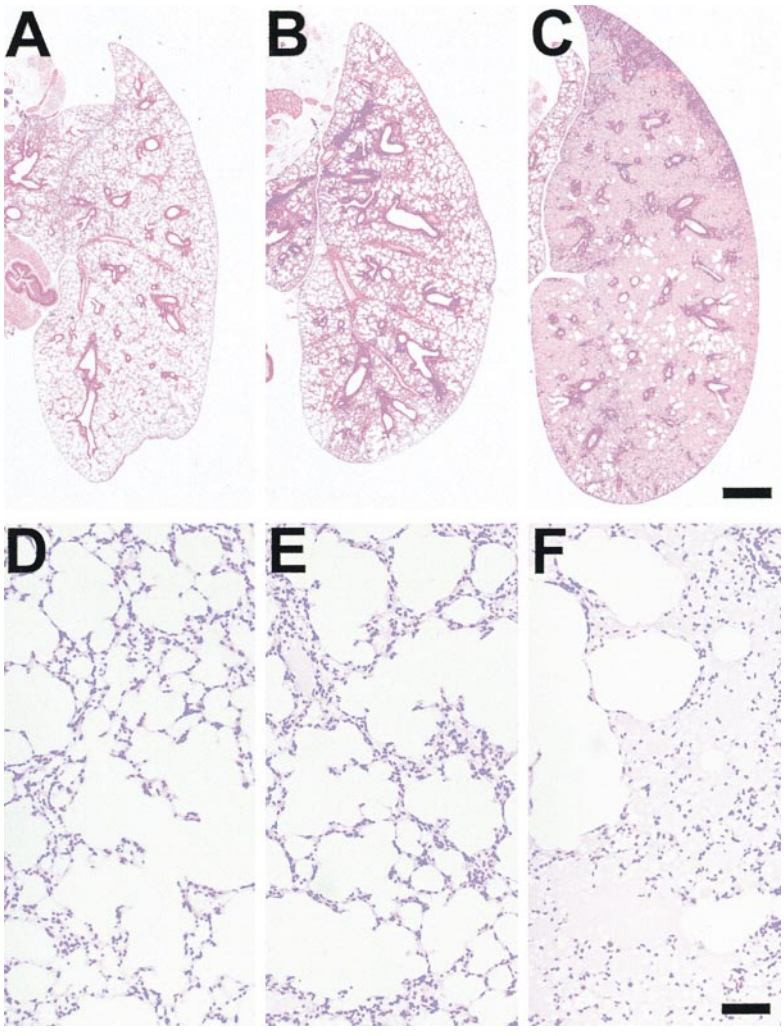

Figure 3. Lung histology (H&E stain) demonstrating pulmonary edema at Day 5 after intratracheal administration of AdVEGF165 (10^9 pfu). (*A–C*) Low-power views of mouse lungs. *Bar* = 1 mm. (*A*) Naive; (*B*) AdNull; (*C*) AdVEGF165. (*D–F*) High-power views of mouse lungs. *Bar* = 100 μm. (*D*) Naive; (*E*) AdNull; (*F*) AdVEGF165.

5B, $P < 0.005$). The increase in vascular leakage correlated positively with the increase in extravascular lung water (Figure 5C, $r = 0.761$, $P < 0.05$), indicating a linkage in magnitude of effect of increased permeability to water and macromolecules in individual animals. This experiment confirms the increase in extravascular lung water and permeability to macromolecules after intratracheal administration of AdVEGF.

Effect of Ads*flt*

The expression of Ads*flt* yields a truncated form of the human VEGF receptor gene *flt-1* (34, 43). The resulting protein is soluble, yet retains its ability to bind VEGF, and to VEGF receptors, with high affinity (43). Under conditions of excess soluble *flt-1* protein, VEGF is prevented from functioning via its naturally occurring receptor, either because it is bound in a multimeric complex and is thereby prevented from binding to its cognate cell surface receptors, or by "dominant-negative" binding of the *sflt* to the VEGF receptors (24). To use the ability of *sflt* to inhibit the biologic effects of VEGF, mice were treated with an Ad vector expressing the human *sflt-1* cDNA. After intranasal administration of Ads*flt*, the transgene was expressed in the lung parenchyma as demonstrated by mRNA expression by the mouse lung (Figure 6, *lanes 2–4*). No expression was detected in the naive mouse lung (Figure 6, *lane 1*).

Mice pretreated with 10^9 pfu Ads*flt* 3 d before intratracheal administration of AdVEGF165 were protected from pulmonary edema induced by the AdVEGF165 (Figure 7; $P < 0.05$, ANOVA; significant difference at $\alpha = 0.05$ for post-test comparing 10^9 pfu Ads*flt* treatment and no pretreatment; no significant difference between 10^9 pfu Ads*flt* pretreatment and naive). This effect was transgene-specific inasmuch as pretreatment with the same dose of Adβgal, an Ad containing an irrelevant transgene, had no protective effect on edema formation (post-test not significantly different at $\alpha = 0.05$ or 0.4, for comparison of Adβgal and no pretreatment). These data confirm that VEGF overexpression specifically causes pulmonary edema in the lung.

Discussion

Pulmonary edema, a condition characterized by excessive extravascular water in the lungs, can result from either increased pressure or permeability of the pulmonary capillary bed. The present study demonstrates that excess expression of VEGF within the murine lung is associated with increased-permeability pulmonary edema. Using an Ad gene transfer vector to overexpress the human VEGF165 cDNA in the epithelium of the murine lung over a 10-d period, lungs demonstrated an enhanced wet-to-dy ratio, histologic evidence of interstitial and intra-alveolar edema, and increased permeability to albumin, a macromolecule that normally has limited access to the extravascular compartment of the lung. Interestingly, the pulmonary edema mediated by the excess expression of VEGF165 was completely obviated by pretreatment of the lung with an Ad gene transfer vector expressing a truncated extracellular soluble form of sflt-1, one of the VEGF receptors, demonstrating the specificity of the VEGF effect.

Mechanisms of Pulmonary Edema

Permeabiity (i.e., nonhydrostatic) pulmonary edema is most commonly linked to local or systemic inflammatory processes, such as those associated with acute infection of the lung or systemic conditions such as sepsis, the post-

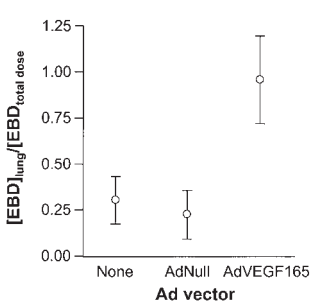

Figure 4. Physiologic assessment of lung permeability to albumin by EBD assay after intratracheal administration of AdVEGF165. EBD was administered intravenously 5 d after intratracheal administration of AdVEGF165 or AdNull (both 10^9 pfu) and the lungs were analyzed after 30 min. For each condition, $n = 3$ animals per group. Specific pulmonary permeability is expressed as the ratio of lung EBD concentration to total administered dose.

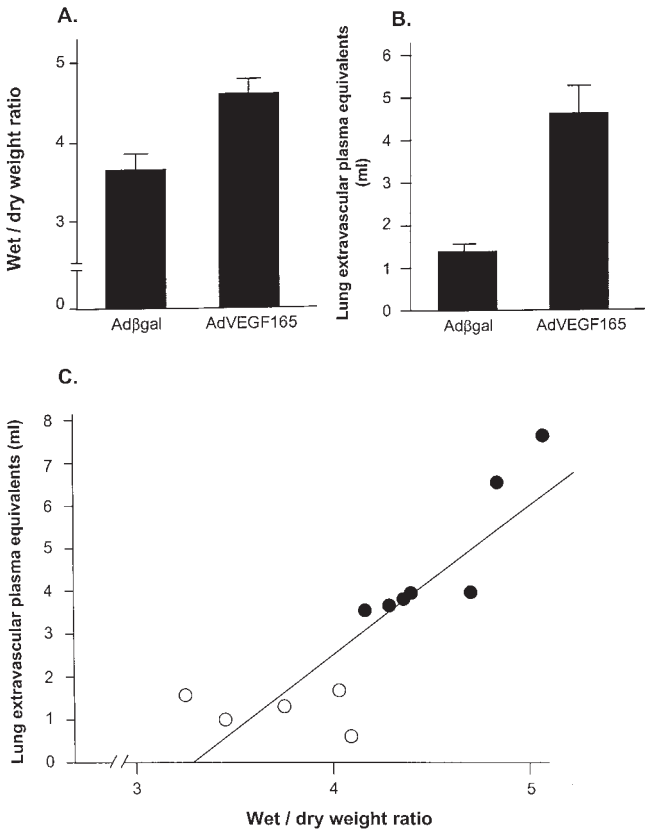

Figure 5. Effect of AdVEGF165 on extravascular lung water and albumin permeability. (*A*) Lung wet/dry weight ratios corrected for intravascular volume 5 d after intratracheal administration of 10^9 pfu AdVEGF165 or Adβgal. (*B*) Lung vascular permeability expressed as plasma equivalents (ml) in the extravascular space of the lung (*see* MATERIALS AND METHODS for calculation based on accumulation of [^{131}I]albumin in the extravascular space of the lung). (*C*) Correlation between index of lung vascular permeability (plasma equivalents, ml) and extravascular lung water in the same animals as in *A*.

operative state, pancreatitis, burns, and trauma (9). Although some instances of high-permeability pulmonary edema are linked to direct endotoxin effects on the pulmonary endothelium, there is increasing evidence that cytokines play a key role. In this regard, animal models and clinical studies have documented elevated systemic and lung levels of cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 in association with pulmonary edema (44). These cytokines have various effects on activating endothelium, inducing endothelial expression of adhesion molecules and leukocyte chemotaxis, leading to a local inflammatory response in the lung (45, 46). Although the effects of endotoxin and various cytokines on the pulmonary endothelium are clearly linked to neutrophil activation and adherence to endothelium, the link between these cytokines and edema formation per se is not clear.

The observations in the present study suggest the hypothesis that VEGF may be one of the mediators of high-permeability pulmonary edema. In this context, VEGF is normally expressed in the lungs (21), and excess amounts of VEGF will induce capillary permeability in the skin (23). Regarding a possible link between cytokines and VEGF, one of the cytokines elevated in the serum of patients with ARDS is IL-6, a potent inducer of VEGF by a transcriptional mechanism (47). In addition, other prominent cytokines in the inflammatory cascade, such as TNF-α and IL-1β, also induce VEGF synthesis (48, 49). VEGF has been shown to induce production of platelet-activating factor (50), a mediator of pulmonary edema in a model of acute lung injury (51).

We observed histologic evidence of mild inflammation in the lungs of mice after intratracheal administration of Ad vectors. The inflammation per se was not sufficient to induce edema by any of the parameters measured. Whether inflammation is necessary for the development of edema in the setting of VEGF overexpression remains to be tested.

In addition, VEGF directly induces a unique pattern of fenestration in venular and capillary endothelium in the skin within 10 min of administration (28), providing a morphologic correlate of edema formation. This concept is consistent with ultrastructural studies of pulmonary capillaries in the first few hours after acute lung injury where vascular tracers label sites of gap formation between endothelial cells (17). Whether the VEGF effect is directly on endothelial shape and, by implication, intercellular pore size, or whether there are other effects on caveolae, aquaporin, basement membranes or other endothelial-related structures such as vesiculo-vacuolar organelles, remains to be determined. There is no evidence that VEGF exerts an effect on alveolar epithelium, although experimental models support a primary role for the epithelium in maintaining the integrity of the alveolar capillary barrier (52).

It is interesting to note that VEGF has been detected in neutrophil granules, which are released upon neutrophil activation (53). Because neutrophils play a prominent role in acute lung injury (54), we hypothesize VEGF may be added to the list of mediators, such as reactive oxygen species and elastase and serine proteases released by activated neutrophils upon binding to the pulmonary capillary endothelium that lead to alteration of endothelial function (44).

In addition to its permeability-inducing properties, VEGF is also a potent angiogenic factor. This raises the consideration of whether our results could be explained by neovascularization in the lung. It takes several days to develop neovascularization, because there must be both endothelial mitosis and migration. After administration of vector, the transgene protein product may begin to appear within 6 h. However, we observed significant increases in wet/dry ratios at 24 h, which would be too soon for new blood vessels to be forming (55, 56). Moreover, hemoglobin in lung extracts did not increase after AdVEGF administration, which would be expected if new blood vessels accounted for the increased wet/dry ratios observed.

The peak ratio of VEGF/total protein occurred at Day 1 (Figure 1B), whereas peak VEGF mRNA was at Day 3 (Figure 1A). The reason for this divergence is uncertain, but may be accounted for by an influx of protein-rich edema fluid into the lung, increasing the denominator, or by increased degradation of VEGF protein by induction of proteases by Day 3. The reason for the discrepancy between peak lung VEGF mRNA expression on Day 3 (Figure 1A) and peak *sflt-1* mRNA expression on Day 1 (Fig-

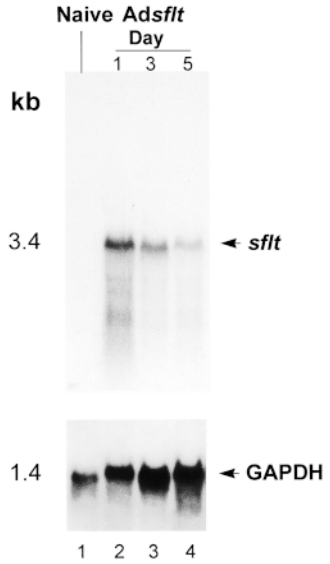

Figure 6. Expression of soluble *flt-1* by mouse lungs after intranasal administration of Ads*flt* by Northern analysis. Total RNA (10 μg/lane) from mouse lungs harvested at the indicated time points after intratracheal administration of Ads*flt* was hybridized with a [^{32}P]-labeled human *flt-1* cDNA probe. GAPDH was used as an RNA control. *Lane 1*, naive; *lanes 2–4*, Ads*flt*, Days 1, 3, and 5, respectively.

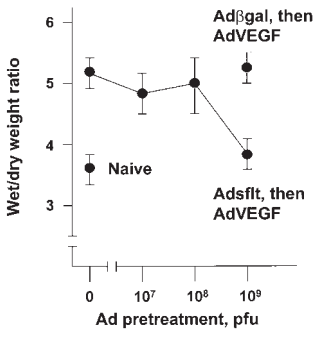

Figure 7. Effect of prior treatment with Ads*flt* on AdVEGF165-induced pulmonary edema. For each condition, $n = 3$ animals per group. Dose–response of intranasal Ads*flt* (10^9 pfu) administered 3 d before intratracheal administration of AdVEGF165 (10^9 pfu) by the intratracheal route. Intranasal administration of 10^9 pfu Adβgal followed by AdVEGF165 served as a control for the initial Ads*flt* administration. Naive mice were a control for the edema-inducing effect of AdVEGF165. Lung edema was quantified by wet/dry weight ratios 5 d after intratracheal AdVEGF165 administration.

ure 6) is also unclear but may be related to the relative stability or degradation rates of these mRNAs.

Gene Transfer Model of Pulmonary Edema

Underlying the use of an Ad vector to overexpress VEGF165 in the lung as a model of pulmonary edema is the concept that the development of a pathophysiologic condition such as increased-permeability pulmonary edema does not occur instantly with transient overexpression of a mediator such as VEGF. Although it was not the purpose of the present study to compare means of administration of VEGF to the lung as different strategies to evoke pulmonary edema, it is known that VEGF has a very short half-life, likely less than 1 h (57). A gene-transfer approach has certain advantages in the study of the pathophysiologic role of a specific protein in comparison with the direct administration of the purified protein in that protein production by the transferred gene is prolonged relative to direct protein administration. This is particularly useful in a model when the protein has a short half-life, is susceptible to proteolytic cleavage, or has systemic toxicities. Less obvious and more difficult to quantify is the spatial distribution of protein production, where a gene-therapy approach might lead to higher concentrations in a local milieu than would be achievable by direct protein administration. In this context, the strategy used in the present study may be adaptable to a broader approach of understanding the role of specific protein mediators in the pathogenesis of conditions such as pulmonary edema.

Implications for Treatment of Pulmonary Edema

The effect of AdVEGF in causing pulmonary edema can be abrogated by inactivation of VEGF by soluble flt-1, which binds to VEGF and/or prevents it from activating its cognate receptor(s). This observation suggests that activation of VEGF receptor(s) may be playing a role in edema formation. Questions that need to be addressed are the identity of the VEGF receptor(s) involved, whether they are upregulated in acute lung injury, and how the pattern of spatial and temporal expression influences the degree of edema formation. From the hypothesis of VEGF receptor involvement, it follows that VEGF receptor downregulation, antagonism, or inactivation might be candidate strategies for prevention or amelioration of pulmonary edema associated with systemic inflammation. Because gene therapy approaches now exist for overexpression of transgenes in lung epithelium (31, 32), alveolar macrophages (58, 59), and endothelium (60, 61), it may be possible to test these strategies in animal models.

In summary, we propose that VEGF may be one cytokine (among several) participating in the generation of pulmonary edema. Further studies will be necessary to determine whether VEGF receptor antagonism would be a useful approach to reducing edema formation.

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