

Genetic Delivery of Bevacizumab to Suppress Vascular Endothelial Growth Factor-Induced High-Permeability Pulmonary Edema

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Abstract

High-permeability pulmonary edema causing acute respiratory distress syndrome is associated with high mortality. Using a model of intratracheal adenovirus (Ad)-mediated overexpression of human vascular endothelial growth factor (VEGF)-A₁₆₅ in mouse lung to induce alveolar permeability and consequent pulmonary edema, we hypothesized that systemic administration of a second adenoviral vector expressing an anti-VEGF antibody (Ad α VEGFAb) would protect the lung from pulmonary edema. Pulmonary edema was induced in mice by intratracheal administration of AdVEGFA165. To evaluate anti-VEGF antibody therapy, the mice were treated intravenously with Ad α VEGFAb, an adenoviral vector encoding the light and heavy chains of an anti-human VEGF antibody with the bevacizumab (Avastin) antigen-binding site. Lung VEGF-A₁₆₅ and phosphorylated VEGF receptor (VEGFR)-2 levels, histology, lung wet-to-dry weight ratios, and bronchoalveolar lavage fluid (BALF) levels of total protein were assessed. Administration of Ad α VEGFAb to mice decreased AdVEGFA165-induced levels of human VEGF-A₁₆₅ and phosphorylated VEGFR-2 in the lung. Histological analysis of Ad α VEGFAb-treated mice demonstrated a reduction of edema fluid in the lung tissue that correlated with a reduction of lung wet-to-dry ratios and BALF total protein levels. Importantly, administration of Ad α VEGFAb 48 hr after induction of pulmonary edema with AdVEGFA165 was effective in suppressing pulmonary edema. Administration of an adenoviral vector encoding an anti-VEGF antibody that is the equivalent of bevacizumab effectively suppresses VEGF-A₁₆₅-induced high-permeability pulmonary edema, suggesting that anti-VEGF antibody therapy may represent a novel therapy for high-permeability pulmonary edema.

Introduction

PULMONARY EDEMA, a significant cause of morbidity and mortality in a critical care setting, is characterized by excessive extravascular fluid in the lungs (Staub, 1974; Fraser *et al.*, 1999; Matthay *et al.*, 2002; Matthay and Martin, 2005). The alteration of fluid balance in the lung that results in pulmonary edema can be caused by increased hydrostatic pressure or increased permeability of the pulmonary capillary bed (Guyton and Lindsey, 1959; Gorin and Stewart, 1979; Montaner *et al.*, 1986; Matthay *et al.*, 2002). High-permeability pulmonary edema results from an increase in the vascular permeability of the lung, causing an accumulation of fluid and protein in the lung interstitium and air spaces (Gorin and Stewart, 1979; Montaner *et al.*, 1986; Matthay *et al.*, 2002). The increase in permeability can be initiated by a variety of factors, and is directly related to reversible physical modifications of the pulmonary endothelium (Fraser *et al.*, 1999; Matthay and Martin, 2005; Leaver and Evans, 2007; Wheeler and Bernard, 2007).

Various experimental and human studies support the concept of vascular endothelial growth factor (VEGF) playing an important role in the pathogenesis of high-permeability pulmonary edema (Becker *et al.*, 2000; Kaner *et al.*, 2000; Thickett *et al.*, 2001; Karpaliotis *et al.*, 2002; Carpenter *et al.*, 2003, 2005; Choi *et al.*, 2003; Gurkan *et al.*, 2003; Lee *et al.*, 2004; Bhandari *et al.*, 2006, 2008; Godzich *et al.*, 2006; Kunig *et al.*, 2006). VEGF was originally described as a “vascular permeability factor” related to its ability to induce permeability in guinea pig skin (Senger *et al.*, 1983; Dvorak *et al.*, 1995). It is now recognized that VEGF is a critical regulator of vascular permeability in several organs (Roberts and Palade, 1995; Dvorak, 2006; Breen, 2007). The lung is particularly vulnerable, as there are 500-fold higher levels of VEGF in normal human lung epithelial lining fluid (ELF) than in human plasma (Kaner and Crystal, 2001); if the tight junctions of the respiratory epithelium are disturbed, the high concentrations of VEGF in lung ELF can reach the alveolar capillary endothelium, interact with the endothelial cell VEGF receptors, and induce vascular permeability (Kaner and Crystal, 2004;

Mura *et al.*, 2004; Voelkel *et al.*, 2006; Gropper and Wiener-Kronish, 2008; Kosmidou *et al.*, 2008). Overexpression of human VEGF-A₁₆₅ by intratracheal administration of an adenovirus (Ad)-based gene transfer vector (AdVEGFA165) to mice causes pulmonary edema and increased vascular permeability, suggesting a significant role for VEGF in the development of pulmonary edema (Kaner *et al.*, 2000).

Bevacizumab (Avastin), a humanized IgG1 monoclonal antibody specific for human VEGF-A, is effective at inhibiting VEGF-dependent processes (Ferrara *et al.*, 2004, 2005). Bevacizumab binds to all VEGF-A isoforms, and prevents VEGF-A from activating the two major VEGF receptors, VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR). In this study, we hypothesized that genetic delivery of an anti-VEGF antibody should be effective in suppressing VEGF-A₁₆₅-induced high-permeability pulmonary edema. To assess this, we used an adenoviral gene transfer vector (Ad α VEGFAb) expressing the heavy and light chains of a monoclonal antibody with a VEGF-A₁₆₅ antigen recognition site identical to bevacizumab to suppress human VEGF-A₁₆₅-induced pulmonary edema. The data demonstrate that a single intravenous administration of Ad α VEGFAb is effective in reducing AdVEGFA165-induced pulmonary edema, including high-permeability pulmonary edema initiated 48 hr before Ad α VEGFAb administration.

Materials and Methods

Adenoviral vectors

All adenoviral vectors were replication-deficient recombinant adenovirus type 5-based vectors with E1 and E3 deletions and under the control of the cytomegalovirus promoter/enhancer (Hackett and Crystal, 2008). Ad α VEGFAb expresses a full-length antibody that has specificity for human VEGF-A (Watanabe *et al.*, 2008). The expression cassette in Ad α VEGFAb encodes the anti-human VEGF light chain and heavy chain sequence separated by a poliovirus internal ribosome entry site (IRES) to facilitate expression of both protein subunits from a single promoter (Watanabe *et al.*, 2008). From 5' to 3', the expression cassette in the Ad α VEGFAb vector contains the cytomegalovirus promoter/enhancer, the anti-human VEGF light chain-coding sequence, the poliovirus IRES, the anti-human VEGF heavy chain-coding sequence, and the simian virus 40 polyadenylation signal. Synthetic antibody heavy and light chain variable domains selected for the study were derived from the protein sequence for antibody A.4.6.1, the murine antibody that was humanized to generate bevacizumab (Kim *et al.*, 1992). The coding sequences for the VEGF-binding site were identical to that of bevacizumab (Baca and Wells, 1997). The variable domains were incorporated into full-length heavy and light chains by adding murine IgG1 constant domain and the murine κ constant domain onto the variable regions by overlap polymerase chain reaction (PCR). AdVEGFA165 expresses the human VEGF-A₁₆₅ cDNA (Muhlhauser *et al.*, 1995; Kaner *et al.*, 2000). Concerning the negative control vectors, Ad α PAAb encodes an unrelated antibody against anthrax protective antigen (De *et al.*, 2008), AdLacZ encodes β -galactosidase (Hersh *et al.*, 1995), and AdNull encodes no transgene (Hersh *et al.*, 1995). Propagation, purification, and titration of the adenoviral vectors was as previously described (Rosenfeld *et al.*, 1991, 1992; Mittereder *et al.*, 1996).

Anti-VEGF antibody levels after Ad α VEGFAb administration

Male C57BL/6 mice, 8 to 10 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY) and were housed under pathogen-free conditions. To determine which administration route of the Ad α VEGFAb vector would deliver the highest levels of anti-VEGF antibodies to the lung, Ad α VEGFAb (10^{11} particle units, PU) in 100 μ l of phosphate-buffered saline, pH 7.4 (PBS) was administered by the intravenous, intratracheal, or intrapleural route to C57BL/6 mice. After 1 to 140 days, bronchoalveolar lavage fluid (BALF) and serum were collected. BALF was collected by cannulating the trachea with a 24-gauge angiocatheter and flushing and aspirating three times with 500 μ l of PBS, and then centrifuged at 3500 rpm for 5 min. Blood was collected via the tail vein, allowed to clot for 60 min, and centrifuged at 13,000 rpm for 10 min. Anti-human VEGF antibody levels in mouse BALF and serum were assessed by a human VEGF-specific enzyme-linked immunosorbent assay (ELISA) using flat-bottomed 96-well EIA/RIA plates (Corning Life Sciences, Lowell, MA) coated with 0.1 μ g of human VEGF-A₁₆₅ per well in a total volume of 100 μ l of 0.05 M carbonate buffer containing 0.01% thimerosal overnight at 4°C. The plates were washed three times with PBS and blocked with 5% dry milk in PBS for 30 min. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Serial serum dilutions in PBS containing 1% dry milk were added to each well and incubated for 60 min. The plates were washed three times with PBS-Tween and 100 μ l/well of 1:10,000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 1% dry milk was added and incubated for 60 min. The plates were washed four times with PBS-Tween and once with PBS. Peroxidase substrate (100 μ l/well; Bio-Rad, Hercules, CA) was added; after 10 min, the reaction was stopped by addition of 2% oxalic acid (100 μ l/well). Absorbance at 415 nm was measured. Antibody titers were calculated with a log (optical density)–log (dilution) interpolation model and a cutoff value equal to 2-fold the absorbance of background (Plikaytis *et al.*, 1991) and normalized to total protein using a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL).

Organ distribution of anti-VEGF antibody mRNA expression levels after intravenous Ad α VEGFAb administration

Quantitative TaqMan real-time PCR analysis was used to evaluate organ distribution of anti-VEGF antibody mRNA expression levels after intravenous Ad α VEGFAb (10^{11} PU) administration. Primers and probe for the anti-VEGF antibody gene were 5'-GGTCTTAAGTGGATGGGATGGA-3', 5'-TGTGAACCTGCGCTTGAAATC-3', and FAM-TAATAC TTATACTGGAGAACCCTACCTACGCTG-TAMRA, respectively. Five days after vector administration, liver, lung, heart, spleen, and kidney were collected and total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) followed by DNA digestion with deoxyribonuclease I, amplification grade (Invitrogen). First-strand cDNA was synthesized from 1 μ g of total RNA in a 50- μ l reaction volume, using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) with random hexamers as primers for RT-PCR. Quantitative

TaqMan real-time PCR relative to an 18S ribosomal RNA (rRNA) primer and probe set as the internal control (Applied Biosystems) was performed to assess the expression levels of anti-VEGF antibody mRNA in the sample, using the $\Delta\Delta C_t$ method.

Pulmonary edema therapy model

AdVEGFA165, 10^{11} PU in $100\ \mu\text{l}$ of PBS, was administered intratracheally to mice to induce edema as previously described (Kaner *et al.*, 2000). In this prior study, evaluation of the time course of edema formation demonstrated a peak of the lung wet-to-dry ratio between days 3 and 5, but there was a significant increase in the ratio relative to animals receiving a control vector throughout a 10-day time course. As a negative control, AdLacZ or AdNull, 10^{11} PU in $100\ \mu\text{l}$ of PBS, was administered intratracheally to mice. Ad α VEGFAb, 10^{11} PU in $100\ \mu\text{l}$ of PBS, was administered intravenously either at the same time as AdVEGFA165, or at various times after AdVEGFA165 administration. As a negative control, AdNull or Ad α PAAb, 10^{11} PU in $100\ \mu\text{l}$ of PBS, was administered intravenously at the same time points. PBS was an additional negative control.

Lung wet-to-dry weight ratio

Five days after AdVEGFA165 administration, lungs were excised *en bloc* and dissected away from the heart and thymus. The lungs were immediately weighed and then placed in a desiccating oven at 65°C for 48 hr, at which point dry weight was achieved. The ratio of lung wet-to-dry weight was used to quantify lung water content (Staub, 1974; Kaner *et al.*, 2000).

Assessment of lung VEGF-A₁₆₅ levels and VEGFR-2 phosphorylation

BALF was collected and centrifuged as described previously. Human VEGF-A₁₆₅ levels in BALF were measured by an ELISA (R&D Systems, Minneapolis, MN) with a human VEGF-specific primary antibody that has no cross-reactivity with mouse VEGF. Phosphorylation of VEGFR-2 was assessed by Western analysis. Frozen whole lung tissues were homogenized in $300\ \mu\text{l}$ of protein extraction reagent (Thermo Scientific) with protease and phosphatase inhibitors. The homogenates were centrifuged at 13,000 rpm for 20 min at 4°C , and the supernatants were collected. Pooled protein ($100\ \mu\text{g}$ from $n = 3$ mice per group) was evaluated for total VEGFR-2 levels with rabbit anti-VEGFR-2 (Cell Signaling Technology, Danvers, MA) as the primary antibody and a horseradish peroxidase-linked donkey anti-rabbit IgG antibody (GE Healthcare Life Sciences, Piscataway, NJ) as the secondary antibody. The same samples were analyzed for phosphorylation of VEGFR-2, using HRP-conjugated mouse anti-phosphotyrosine clone 4G10 (Millipore, Billerica, MA) as the primary antibody. Detection was with ECL reagent (GE Healthcare Life Sciences). The intensities of the bands were analyzed with MetaMorph image analysis software (Universal Imaging, Downingtown, PA). Immunoprecipitation with the anti-VEGFR-2 antibody was done to enrich for VEGFR-2 before phosphotyrosine immunoblotting; however, this was not an improvement over direct Western analysis, likely because of the insensitivity of the anti-VEGFR-2 antibody.

Lung histopathology

To assess lung histopathology, mice were exsanguinated, and 2 ml of PBS was injected via the jugular vein to perfuse the lungs. Lungs were inflated to total lung capacity with air injection via an angiocatheter placed in the trachea and tied with sutures. The air-inflated lungs were removed *en bloc*, placed in an uncovered container with 300 ml of PBS, and submerged by covering with saturated gauze. The PBS was heated to 60°C , 4 min in a microwave oven (Turner *et al.*, 1990). The lungs were then fixed in 4% paraformaldehyde followed by paraffin embedding.

Measurement of BALF total protein levels

BALF was collected as described previously at various time points and under conditions as detailed in text. Photos were taken of the lavage fluid before centrifugation. The BALF was then centrifuged to remove blood proteins and total protein levels were measured by the bicinchoninic method (Thermo Scientific) as specified by the manufacturer.

Statistical analysis

All data are shown as means \pm standard error. Statistical comparison was made by a two-tailed Student *t* test, and a value of $p < 0.05$ was accepted as indicating significance.

Results

Comparison of serum and BALF anti-VEGF antibody levels after administration of Ad α VEGFAb by different routes

To determine the administration route of Ad α VEGFAb required to achieve the highest lung anti-VEGF levels, BALF and serum anti-VEGF antibody levels were assessed after intravenous, intratracheal, or intrapleural administration of the same dose of the vector. Intravenous Ad α VEGFAb administration resulted in the highest antibody expression levels in both serum (Fig. 1A) and BALF (Fig. 1B) compared with intratracheal or intrapleural administration ($p < 0.0001$). No anti-VEGF antibody was detected in the BALF and serum from mice that received intravenous administration of the control Ad α PAAb or AdNull vector. Assessment of the expression profile of anti-VEGF antibody levels in serum and BALF over 20 weeks after intravenous Ad α VEGFAb administration showed that serum and BALF anti-VEGF antibody levels peaked on days 7 and 5, respectively, and then gradually decreased over time (Fig. 1C and D).

Organ distribution of anti-VEGF antibody mRNA expression levels after intravenous Ad α VEGFAb administration

To evaluate the organ distribution of anti-VEGF antibody mRNA expression levels after intravenous Ad α VEGFAb administration, anti-VEGF antibody mRNA expression levels relative to endogenous 18S rRNA in various tissues were assessed by quantitative TaqMan real-time PCR. The highest amount of anti-VEGF antibody mRNA expression level was found in the liver, followed by the spleen (Fig. 2).

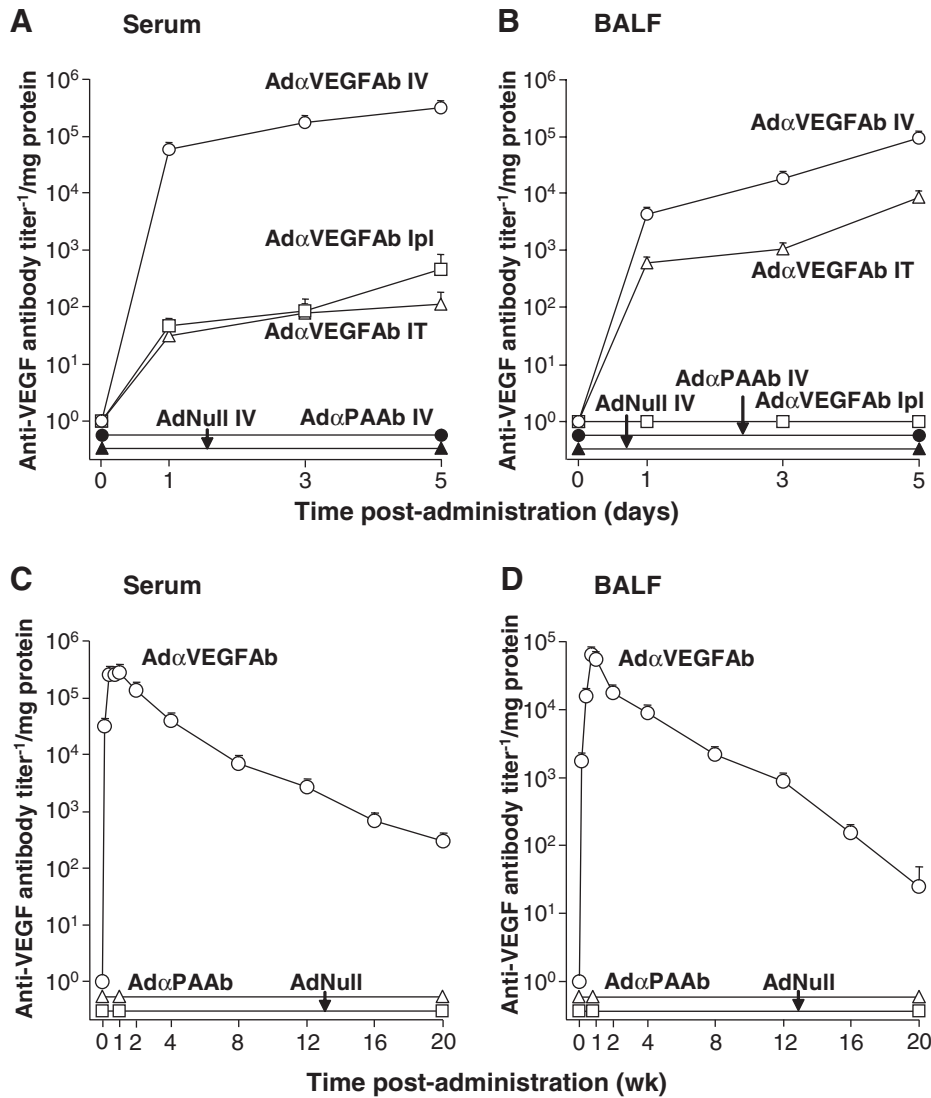


FIG. 1. Comparison of serum and bronchoalveolar lavage fluid (BALF) anti-vascular endothelial growth factor (VEGF) antibody levels after administration of the Ad α VEGFAb vector by different routes. (A and B) Time course of serum and BALF anti-VEGF antibody levels after vector administration by different routes. Ad α VEGFAb (10^{11} particle units [PU]) was administered to C57BL/6 mice by the intravenous (IV), intratracheal (IT), or intrapleural (Ipl) route. Ad α PAAb and AdNull were administered intravenously as controls. (A) Serum anti-VEGF antibody levels. (B) BALF anti-VEGF antibody levels. (C and D) Anti-VEGF antibody levels in serum and BALF over 20 weeks after intravenous administration of Ad α VEGFAb. (C) Serum anti-VEGF antibody levels. (D) BALF anti-VEGF antibody levels. Ad α PAAb and AdNull were administered intravenously as controls. For all panels, for direct comparison, data are presented relative to total protein levels in serum or BALF, respectively. Data for each panel were obtained from $n = 5$ animals per group.

Effects of intratracheal AdVEGFA165 administration on lung wet-to-dry weight ratio

The lung wet-to-dry weight ratio, a measure of extravascular lung water, was quantified as a function of AdVEGFA165 dose after intratracheal administration. In previous studies, we have demonstrated that after intratracheal adenoviral vector administration to the epithelial surface of the respiratory tract, the respiratory epithelium is the only category of cells that express the transgene (Mas-trangeli *et al.*, 1993). The lung wet-to-dry ratio showed a dose-dependent increase, with the highest dose (10^{11} PU) resulting in a significant increase over that observed with AdLacZ at the same dose or with PBS (Fig. 3; $p < 0.0005$ compared with

all other groups). Although AdLacZ administration resulted in a slightly elevated lung wet-to-dry ratio relative to PBS (9.8%), the lung wet-to-dry ratio induced by AdVEGFA165 is statistically significant relative to the same dose of AdLacZ. On the basis of these data, intratracheal AdVEGFA165, at a dose of 10^{11} PU, was used for all subsequent experiments to initiate high-permeability pulmonary edema.

Effects of Ad α VEGFAb on lung VEGF-A₁₆₅ levels and VEGFR-2 phosphorylation

To evaluate the effect of intravenous administration of Ad α VEGFAb on lung VEGF-A₁₆₅ and VEGFR-2 signaling *in vivo* induced by intratracheal administration of

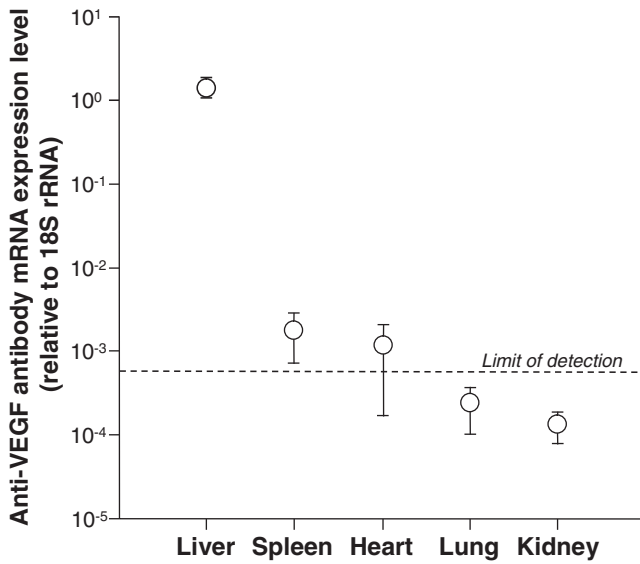


FIG. 2. Organ distribution of anti-VEGF antibody mRNA expression levels after intravenous administration of Ad α VEGFAb. Ad α VEGFAb (10^{11} PU) was administered intravenously. On day 5, various organs were collected and anti-VEGF antibody mRNA expression levels relative to endogenous 18S rRNA were assessed by quantitative TaqMan real-time PCR (limit of detection, 0.000567). Data were obtained from $n = 2$ animals per group.

AdVEGFA165, BALF levels of human VEGF-A₁₆₅ and lung tissue VEGFR-2 phosphorylation levels were assessed. Treatment with Ad α VEGFAb induced a significant reduction of BALF levels of human VEGF-A₁₆₅ (Fig. 4A; $p < 0.005$, Ad α VEGFAb compared with all control treated groups) and downregulation of VEGFR-2 phosphorylation (Fig. 4B) compared with Ad α PAAb-, AdNull-, or PBS-treated mice.

Effects of Ad α VEGFAb on VEGF-A₁₆₅-mediated high-permeability pulmonary edema

To determine the effects of Ad α VEGFAb on VEGF-A₁₆₅-mediated high-permeability pulmonary edema, AdVEGFA165 was administered intratracheally to mice concurrently treated by intravenous injection of Ad α VEGFAb, control vectors, Ad α PAAb or AdNull, or PBS. For these experiments, we did not include a direct comparison of the effect of Ad α VEGFAb with the effect of purified anti-VEGF antibody; the differences in half-lives between Ad α VEGFAb-expressed antibody and purified antibody, and the differences in antibody distribution after intravenous administration of Ad α VEGFAb versus purified antibody, would make data interpretation and a meaningful comparison difficult. Treatment with Ad α VEGFAb resulted in a marked reduction of intraalveolar edema as assessed by lung histopathology in Ad α VEGFAb-treated mice as compared with the Ad α PAAb-, AdNull-, or PBS-treated mice, whose alveoli were filled with bloody edema fluid (Fig. 5). The lung wet-to-dry weight ratio demonstrated a significant ratio reduction in Ad α VEGFAb-treated mice relative to animals that received Ad α PAAb, AdNull, or PBS (Fig. 6; $p < 0.0001$, Ad α VEGFAb

compared with all control treated groups and similar to naive animals, $p > 0.5$).

Assessment of BALF recovered from mice receiving intratracheal AdVEGFA165 and treated with Ad α PAAb, AdNull, or PBS showed that BALF was bloody in all cases. In contrast, BALF from Ad α VEGFAb-treated mice was similar to that of naive animals (Fig. 7A). To evaluate the effect of Ad α VEGFAb on alveolar capillary protein leak induced by AdVEGFA165, BALF total protein levels were quantified. Intravenous administration of Ad α VEGFAb resulted in a significant reduction of total protein levels in BALF relative to animals that received Ad α PAAb, AdNull, or PBS (Fig. 7B; $p < 0.005$, Ad α VEGFAb compared with all control groups).

To determine the effects of delayed administration of Ad α VEGFAb on VEGF-A₁₆₅-mediated high-permeability pulmonary edema, AdVEGFA165 was administered intratracheally to mice and then treated by intravenous injection of Ad α VEGFAb or the control vector Ad α PAAb 0, 6, 12, 24, or 48 hr later; animals were killed on day 5 after AdVEGFA165 administration for analysis of BALF total protein levels. Delayed administration of Ad α VEGFAb resulted in a marked reduction of BALF total protein levels relative to animals that received Ad α PAAb at all time points tested (Fig. 8; $p < 0.01$, Ad α VEGFAb compared with control groups), consistent with the concept that Ad α VEGFAb was an effective treatment after the initiation of pulmonary edema.

Discussion

Pulmonary edema is a common clinical problem with significant consequences for lung mechanical functions and gas

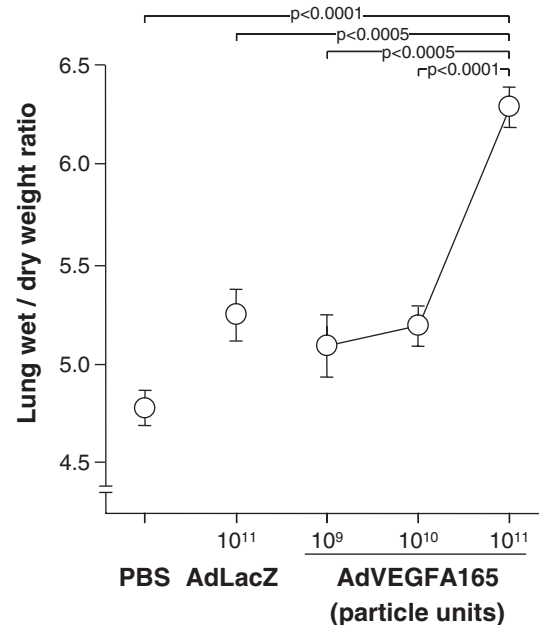


FIG. 3. Lung wet-to-dry weight ratio after intratracheal administration of AdVEGFA165. Increasing doses (10^9 , 10^{10} , or 10^{11} PU) of AdVEGFA165 or AdLacZ, or PBS, were administered intratracheally on day 0. On day 5, the lung wet-to-dry ratio was quantified. Data were obtained from $n = 5$ animals per group.

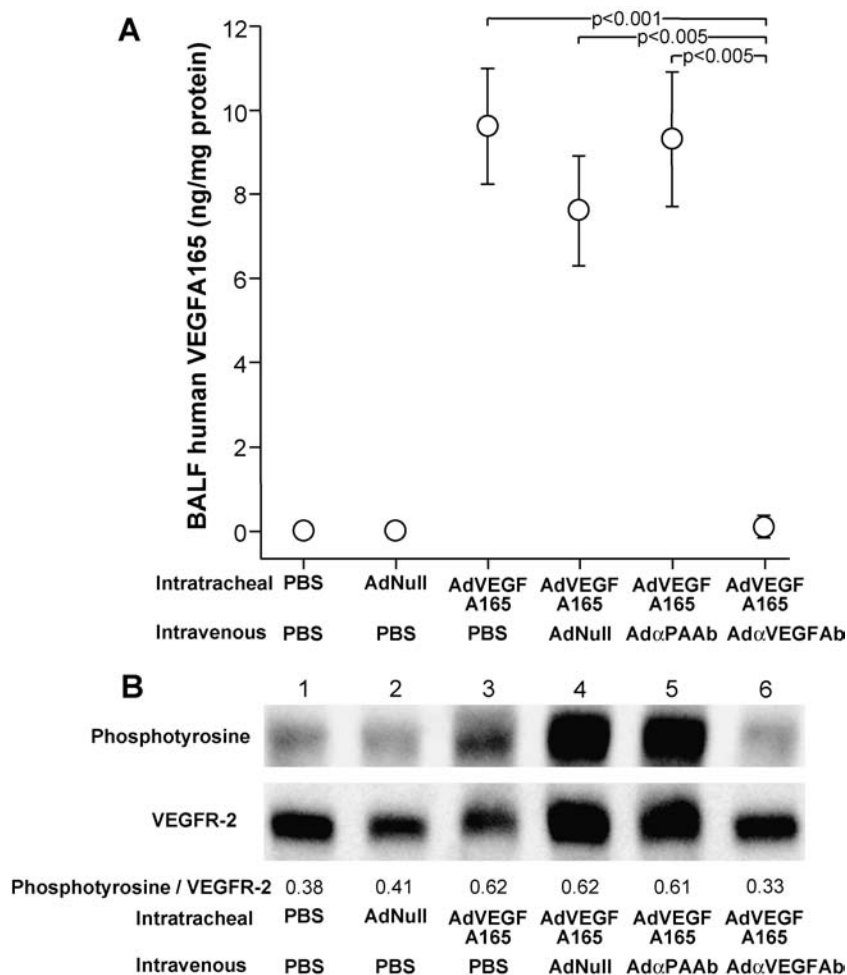


FIG. 4. Effects of intravenous administration of Ad α VEGFAb on lung VEGFR-2 signaling induced by intratracheal administration of AdVEGF A165. AdVEGF A165 (10^{11} PU) was administered intratracheally and at the same time Ad α VEGFAb or controls Ad α PAAb, AdNull, or PBS was administered intravenously (10^{11} PU). On day 5, BALF and lung tissues were collected. (A) BALF human VEGF-A₁₆₅ levels after intratracheal administration of AdVEGF A165 without and with parallel intravenous administration of Ad α VEGFAb. Human VEGF-A₁₆₅ levels are normalized to total protein. Data were obtained from $n = 4$ or 5 animals per group. (B) Lung tissue VEGFR-2 phosphorylation levels after intratracheal administration of AdVEGF A165 without and with parallel intravenous administration of Ad α VEGFAb. Lung homogenates were assessed for total VEGFR-2 expression and phosphorylated VEGFR-2 levels by Western analysis with rabbit anti-VEGFR-2 and horseradish peroxidase (HRP)-conjugated mouse anti-phosphotyrosine antibodies. The mean intensities of the bands (phosphotyrosine/VEGFR-2) were analyzed with image analysis software. Lane 1, PBS + PBS; lane 2, AdNull + PBS; lane 3, AdVEGF A165 + PBS; lane 4, AdVEGF A165 + AdNull; lane 5, AdVEGF A165 + Ad α PAAb; lane 6, AdVEGF A165 + Ad α VEGFAb. Data were obtained from pooled samples from $n = 3$ animals per group.

exchange (Fraser *et al.*, 1999; Matthay and Martin, 2005; Leaver and Evans, 2007; Wheeler and Bernard, 2007). VEGF has been implicated in a variety of conditions characterized by vascular leakage, including pulmonary edema (Kaner *et al.*, 2000; Ferrara, 2004; Mura *et al.*, 2004; Dvorak, 2006; Voelkel *et al.*, 2006; Breen, 2007; Kosmidou *et al.*, 2008). In the present study, we tested the hypothesis that an anti-VEGF antibody, the murine equivalent of bevacizumab with an anti-human VEGF-binding site, would inhibit VEGF-induced high-permeability pulmonary edema. The results indicate that delivery of an anti-VEGF antibody with an adenoviral gene transfer vector (Ad α VEGFAb) to mice is effective in suppressing AdVEGF A165-induced high-permeability pulmo-

nary edema as measured by human VEGF-A₁₆₅ levels in BALF, lung phosphorylated VEGFR-2 levels, histology, lung wet-to-dry weight ratios, and BALF total protein levels. Importantly, and relevant to the possible clinical application of this strategy to treat pulmonary edema, administration of Ad α VEGFAb 6 to 48 hr after induction of pulmonary edema with AdVEGF A165 was successful in preventing the pulmonary edema.

VEGF and high-permeability pulmonary edema

VEGF belongs to a gene family that includes VEGF isoforms A–E and placenta growth factor, proteins that have multiple

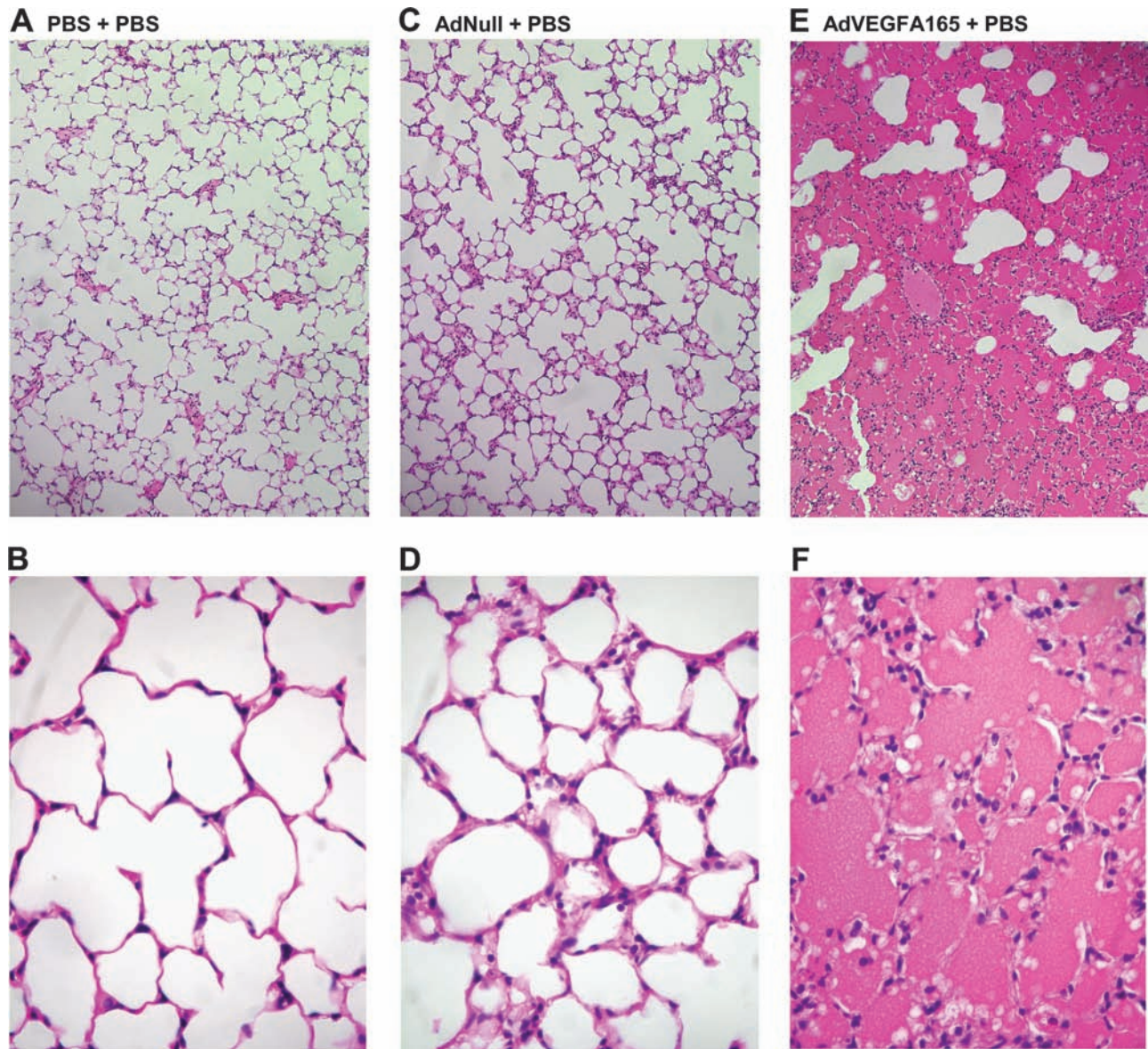


FIG. 5A-F (continued next page).

diverse roles and biological functions (Ferrara *et al.*, 2003; Ferrara, 2004). VEGF-A is the prototype member of the family that arises from alternative splicing of the eight-exon VEGF gene to yield four isoforms of 121, 165, 189, or 206 amino acids (Tischer *et al.*, 1991; Ferrara *et al.*, 2003; Ferrara, 2004). VEGF-A₁₆₅, the most common isoform, binds to VEGFR-1 and VEGFR-2 and induces receptor dimerization and phosphorylation of specific tyrosine residues for functional signal transduction (Matsumoto and Claesson-Welsh, 2001; Kowanzet and Ferrara, 2006; Olsson *et al.*, 2006).

VEGF is a potent stimulus of growth of vascular endothelial cells, and this activity is relevant to recruiting a vasculature to developing tumors (Folkman, 1971; Ferrara, 2002). VEGF is also a permeability factor, capable of inducing vascular leakage (Senger *et al.*, 1983; Dvorak *et al.*, 1995; Roberts and Palade, 1995; Ferrara, 2004; Dvorak, 2006; Breen, 2007). In this regard, VEGF induces increased vascular permeability in

multiple organs, including skin, muscle, gastrointestinal tract, CNS, and lung (Senger *et al.*, 1983; Roberts and Palade, 1995; Bates *et al.*, 1999; Proescholdt *et al.*, 1999; Kaner *et al.*, 2000; Schoch *et al.*, 2002; Rosenstein and Krum, 2004; Breen, 2007). VEGF-dependent increases in permeability and fenestration have been observed in large vessels (Hippenstiel *et al.*, 1998), microvascular vessels (Esser *et al.*, 1998), and cultured endothelial cells (Bates *et al.*, 1999). Many of the effects of VEGF on endothelial cells are related to the VEGF-induced production of endothelial cell nitric oxide (Fukumura *et al.*, 2001).

Several animal studies have demonstrated a role for VEGF in the development of high-permeability pulmonary edema (Mura *et al.*, 2004; Voelkel *et al.*, 2006; Kosmidou *et al.*, 2008). When administered intratracheally to the lung, an adenoviral gene transfer vector expressing VEGF promotes pulmonary edema and vascular leakage, and this effect is abrogated by pretreatment with an adenoviral vector expressing sFlt-1, the

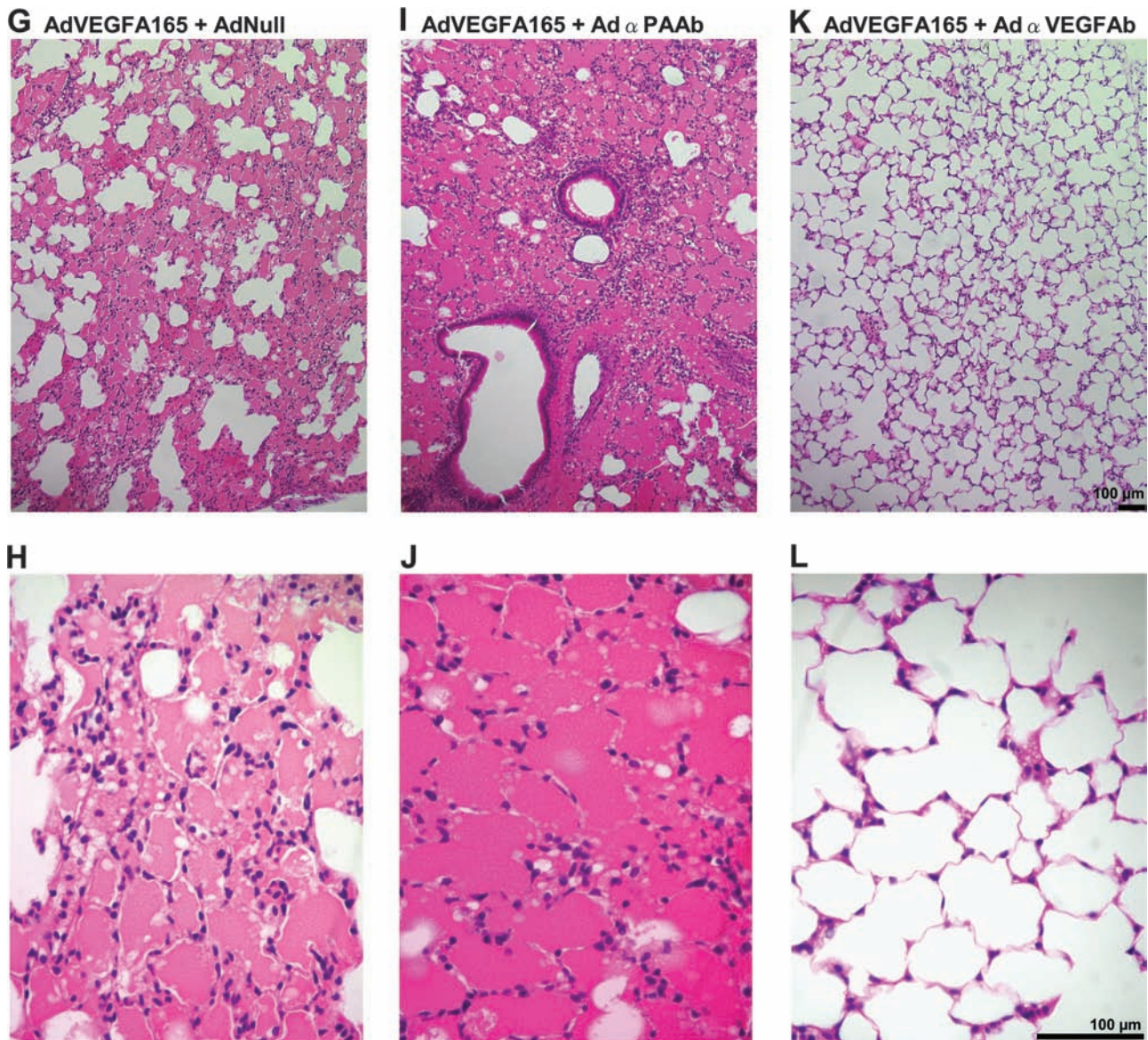


FIG. 5. Lung histopathology after intratracheal administration of AdVEGFA165 and treatment with Ad α VEGFAB. AdVEGFA165 (10^{11} PU) was administered intratracheally and at the same time Ad α VEGFAB or control Ad α PAAb, AdNull, or PBS was administered intravenously (10^{11} PU). On day 5, lungs were fixed by the microwave technique and paraffin sections were stained with hematoxylin and eosin. (A and B) PBS + PBS; (C and D) AdNull + PBS; (E and F) AdVEGFA165 + PBS; (G and H) AdVEGFA165 + AdNull; (I and J) AdVEGFA165 + Ad α PAAb; and (K and L) AdVEGFA165 + Ad α VEGFAB. Scale bars [shown in (K) and (L)]: 100 μ m.

soluble form of VEGFR-1 (Kaner *et al.*, 2000). In ferrets (Becker *et al.*, 2000) and rats (Choi *et al.*, 2003), ventilator-induced lung injury is associated with elevated lung or serum VEGF levels and vascular leak. Activation of the stress protein response prevents the development of pulmonary edema by inhibiting VEGF cell signaling in rats after ischemia-reperfusion injury (Godzich *et al.*, 2006). In a murine model of pulmonary edema induced by lipopolysaccharide inhalation, there are elevated lung levels of VEGF (Karpaliotis *et al.*, 2002). The VEGFR-2 levels in lung homogenate are upregulated in a murine model of pulmonary edema after intratracheal hydrochloric acid-induced lung injury, and these levels were further elevated under conditions of high tidal volume ventilation (Gurkan

et al., 2003). Inhibition of VEGF activity with a soluble VEGF-Trap decoy receptor markedly reduces pulmonary vascular protein extravasation in hypoxic endothelin-B receptor-deficient rats (Carpenter *et al.*, 2003), consistent with the concept that increased VEGF expression in the lung contributes to vascular permeability. Endothelin-driven increases in lung VEGF levels were associated with the development of pulmonary edema (Carpenter *et al.*, 2005). Administration of recombinant human VEGF during hyperoxia-induced acute lung injury transiently increases pulmonary edema (Kunig *et al.*, 2006). Finally, lung-targeted VEGF-A₁₆₅ transgenic mice develop pulmonary edema after induction of VEGF-A₁₆₅ expression (Lee *et al.*, 2004; Bhandari *et al.*, 2006, 2008).

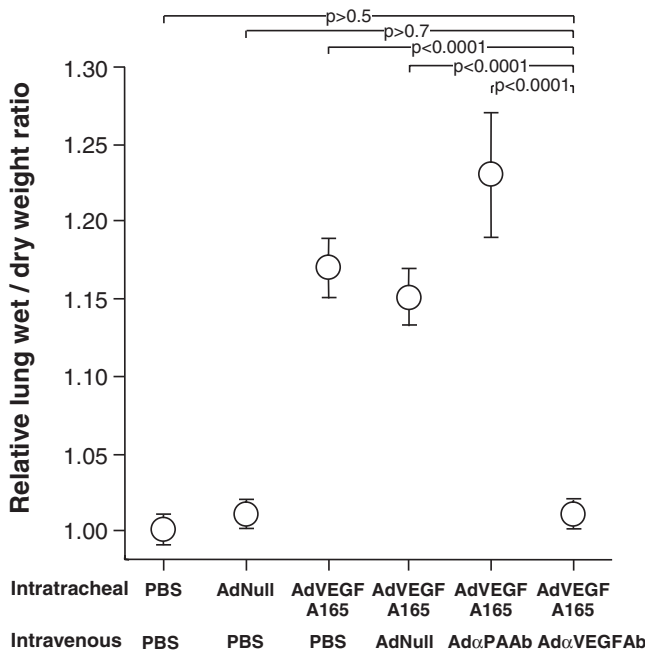


FIG. 6. Lung wet-to-dry weight ratio after intratracheal administration of AdVEGFA165 and treatment with AdαVEGFAB. AdVEGFA165 (10^{11} PU) was administered intratracheally and at the same time AdαVEGFAB or control AdαPAAb, AdNull, or PBS was administered intravenously (10^{11} PU). On day 5, the lung wet-to-dry ratio was quantified. Data were normalized according to the mean level of the PBS + PBS group. Data were obtained from $n = 15$ animals per group.

In humans, elevated plasma VEGF levels are present in patients with acute respiratory distress syndrome (ARDS) as well as patients at risk for developing ARDS (Thickett *et al.*, 2001). In this study, there was a correlation between VEGF levels and disease outcome, with higher VEGF levels in nonsurvivors.

Bevacizumab (Avastin)

Bevacizumab binds specifically to all human VEGF isoforms and bioactive proteolytic fragments, but not to mouse or rat VEGF, and prevents the binding of VEGF to its receptors (Ferrara *et al.*, 2004, 2005). In preclinical studies, inhibiting VEGF activity with bevacizumab effectively suppresses tumor growth in immunodeficient mice with human tumor xenografts (Presta *et al.*, 1997; Sweeney *et al.*, 2001; Fox *et al.*, 2002). In clinical studies, bevacizumab, in combination with standard chemotherapy regimens, significantly prolongs the survival of patients with metastatic cancers of the colon, breast, kidney, and lung (Yang *et al.*, 2003; Hurwitz *et al.*, 2004; Miller *et al.*, 2005; Sandler *et al.*, 2006). The efficacy of bevacizumab as a cancer therapy led to its approval by the U.S. Food and Drug Administration in 2004 as the first anti-angiogenic agent for the treatment of cancer (Ferrara *et al.*, 2004).

The murine anti-VEGF monoclonal antibody A.4.6.1 is the precursor to the humanized form of the antibody bev-

acizumab (Kim *et al.*, 1992; Fox *et al.*, 2002). Humanization of A.4.6.1 to reduce immunogenicity and to increase its half-life in humans resulted in an antibody molecule that is 93% human and 7% murine (Baca *et al.*, 1997; Fox *et al.*, 2002); both antibodies are specific for human VEGF and both are effective inhibitors of tumor growth *in vivo* (Gerber and Ferrara, 2005).

The activity of bevacizumab is comparable to that of other VEGF inhibitors, such as soluble VEGF receptors, that have higher binding affinity for VEGF (Kuo *et al.*, 2001; Holash *et al.*, 2002; Ferrara *et al.*, 2004). These effects may be related to the relatively longer half-life of the antibody, biodistribution, or stability of antibody-VEGF binding. However, all these therapeutic regimens require frequent administrations of large doses of the inhibitors (Holash *et al.*, 2002; Ferrara *et al.*, 2004). In a previous study, we demonstrated that genetic

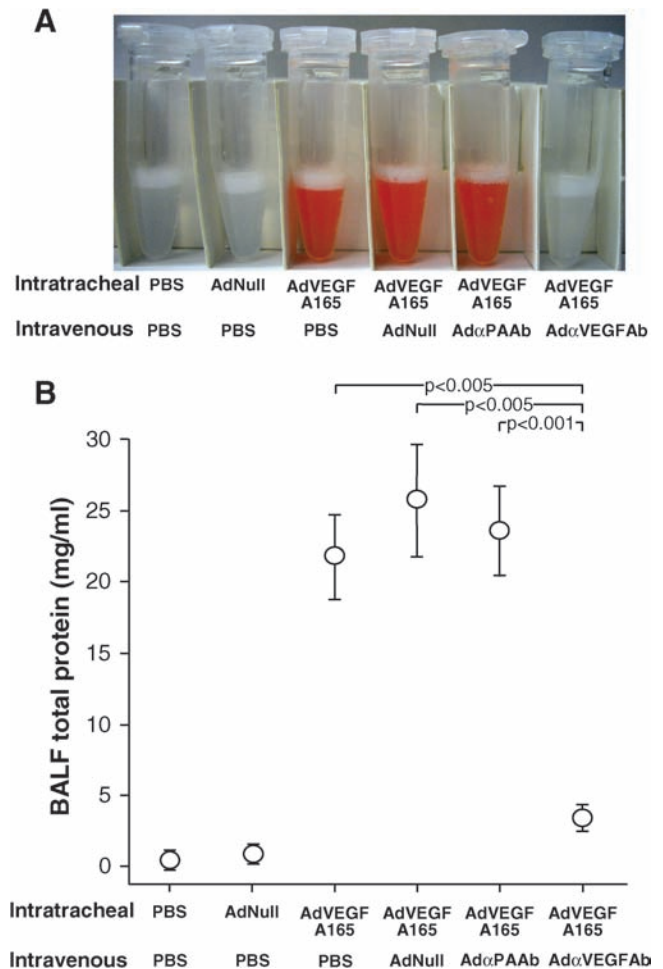


FIG. 7. BALF total protein levels after intratracheal administration of AdVEGFA165 and treatment with AdαVEGFAB. AdVEGFA165 (10^{11} PU) was administered intratracheally to mice and at the same time AdαVEGFAB or control AdαPAAb, AdNull, or PBS was administered intravenously (10^{11} PU). On day 5, BALF was collected and total protein levels were measured. (A) Photos of BALF under various conditions before centrifugation. (B) BALF total protein levels after centrifugation. Data were obtained from $n = 4$ or 5 animals per group.

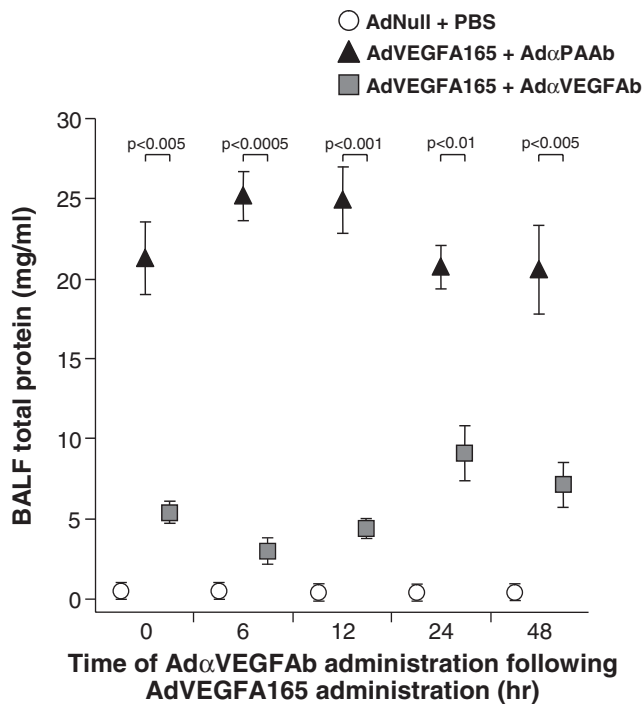


FIG. 8. Suppression of AdVEGFA165-induced pulmonary edema by administration of Ad α VEGFAB after the pulmonary edema is induced. Shown is the BALF total protein level after intratracheal administration of AdVEGFA165 and treatment with Ad α VEGFAB 0 to 48 hr afterward. AdVEGFA165 (10^{11} PU) was administered intratracheally to mice and, at various times ranging from 6 to 48 hr later, Ad α VEGFAB or a control vector, Ad α PAAb, was administered intravenously (10^{11} PU). On day 5, BALF was collected and centrifuged, and total protein levels were measured. Data were obtained from $n=3$ animals per group.

delivery of monoclonal antibody A.4.6.1 suppressed tumor growth in a human tumor xenograft model after a single administration, suggesting that genetic delivery of anti-VEGF antibodies may be a strategy to further increase antibody half-life and consequent bioavailability (Watanabe *et al.*, 2008).

Treatment of high-permeability pulmonary edema with bevacizumab

The effects of bevacizumab in inhibiting angiogenesis and tumor growth are striking and suggest that inhibition of other VEGF-dependent processes with bevacizumab would be similarly effective. Although there have been no published studies demonstrating the utility of bevacizumab as a therapy for high-permeability pulmonary edema in humans, there are anecdotal reports that bevacizumab is effective in treating pleural effusion (Badros *et al.*, 2005; Pichelmayer *et al.*, 2005; Hoyer *et al.*, 2007). The connection between VEGF and the establishment of high-permeability pulmonary edema suggests that anti-VEGF antibodies are a viable therapeutic strategy for this condition. Keeping in mind that VEGF has multiple functions, including a role in maintaining alveolar structure and function and in repair after injury (Mura *et al.*,

2004; Voelkel *et al.*, 2006; Kosmidou *et al.*, 2008), transient administration of anti-VEGF to treat high-permeability pulmonary edema will have to be assessed from a safety viewpoint. With this caveat, the data obtained from the current study demonstrate the efficacy of genetic delivery of an anti-VEGF antibody in suppressing VEGF-induced high-permeability pulmonary edema in a mouse model and suggest that bevacizumab, either directly administered or genetically delivered, is a novel strategy for the prevention of high-permeability pulmonary edema.

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