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Exposure to hypoxia leads to the development of pulmonary hypertension (PH) as a consequence of pulmonary smooth muscle hyperplasia. Hypoxia concomitantly stimulates lung expression of angiogenic factors. To investigate the role of angiogenesis processes in development of hypoxic PH, we examined the effects of lung overexpression of angiostatin, an angiogenesis inhibitor, on development of hypoxic PH and lung endothelial cell (EC) density. Angiostatin delivery was achieved by a defective adenovirus expressing a secretable angiostatin K3 molcule driven by the cytomegalovirus promoter (Ad.K3). Comparison was made with a control vector containing no gene in the expression cassette (Ad.CO1). Treatment with Ad.K3 (300 plaque-forming units [pfu]/cell) inhibited cultured human pulmonary artery EC migration by 100% and proliferation by 50%, but was without effects on human pulmonary artery smooth muscle cells. After intratracheal administration of Ad.K3 (10^9 pfu) to mice, angiostatin protein became detectable in bronchoalveolar lavage fluid. Mice pretreated with Ad.K3 1 d before a 2-wk exposure to hypoxia (10% O_2) showed more severe pulmonary hypertension than Ad.CO1-pretreated controls, as assessed by higher right ventricular systolic pressure (36.5 ± 2.4 versus 30.2 ± 1.4, respectively), aggravation of right ventricular hypertrophy ($P < 0.05$), and muscularization of distal vessels ($P < 0.01$). Lung factor VIII, CD31 immunostaining, as well as eNOS expression were significantly increased after exposure to hypoxia in Ad.CO1-pretreated controls, but decreased in both normoxic and hypoxic animals after treatment with Ad.K3. The results show that inhibition of hypoxia-induced stimulation of lung angiogenic processes aggravates development of hypoxic PH. This suggests that endogenous lung angiogenesis counteracts development of hypoxic PH.

Hypoxia is a well recognized stimulus for pulmonary blood vessel remodeling. One mechanism that may account for this effect is a direct action of hypoxia on the expression of specific genes involved in pulmonary artery smooth muscle cell proliferation, such as those encoding the serotonin transporter and endothelin (1, 2). Hypoxia is also a potent stimulus for the expression of angiogenic factors, which trigger endothelial cells (3, 4). Numerous angiogenic factors and cytokines, including vascular endothelial growth factor (VEGF) and VEGF receptors, platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factor (FGF), transforming growth factor, angiogenin, and prostaglandin E2, have also been shown to be increased in lungs from experimental hypoxic animals (5–7) as well as in tissues from patients with chronic hypoxemic lung disease (8). The physiologic or pathologic consequences of hypoxia-induced activation of lung angiogenic processes, however, are not completely understood. Pharmacologic blockade of VEGF receptors aggravates (9), whereas lung VEGF overexpression by adenovirus mediated gene transfer attenuates, development of hypoxic pulmonary hypertension (10). However, VEGF is only one among various angiogenic molecules that are upregulated during chronic hypoxia (5, 7). Moreover, VEGF receptors are present not only on endothelial cells but also on lung epithelial cells (11) and monocytes (12, 13). Finally, the regulation of angiogenesis is a complex process that depends upon the local balance between proangiogenic and antiangiogenic molecules. In the present study, we questioned whether changing the angiogenic set point by inducing lung overexpression of an endogenous angiogenesis inhibitor would alter development of hypoxic pulmonary hypertension.

Angiostatin, a potent naturally occurring inhibitor of angiogenesis generated by proteolysis of plasminogen (14), is considered as one of the most effective and specific inhibitors of angiogenesis. It inhibits proliferation and migration of endothelial cells (15, 16) without exerting toxic effects on the quiescent systemic vasculature. Produced by proteolytic cleavage, angiostatin has been shown to be a 38-kD internal fragment of plasminogen that consists of four kringle structures. Kringles 1–3 exert a potent inhibitory effect on endothelial cell proliferation, whereas kringle 4 is relatively ineffective (16).

In the present study, angiostatin delivery was achieved by a defective adenovirus expressing a secretable angiostatin K3 molcule driven by the cytomegalovirus promoter (Ad.K3). We first evaluated the efficiency of gene transfer in the lungs from mice with a single intratracheal instillation of the adenovirus by detecting the protein in bronchoalveolar lavage (BAL) fluid. We also evaluated, *in vitro*, the consequence of angiostatin expression on the migration and proliferation of microvascular endothelial cells and pulmonary artery smooth muscle cells. In the second part of the study, we evaluated the effect of lung angiostatin expression on pulmonary vascular density and development of

pulmonary hypertension in mice pretreated with intratracheal administration of Ad.K3 2 d before the start of a 2-wk exposure to either normoxia or hypoxia.

Materials and Methods

Recombinant Adenovirus Vectors

Ad.K3 is a defective recombinant E1E3-deleted adenovirus directing the expression and secretion of an angiostatin-like molecule (i.e., the N-terminal fragment of human plasminogen up to residue 333) from the cytomegalovirus immediate-early promoter (15). Ad.CO1 is a control virus that is identical to Ad.K3, but with no gene in the expression cassette. All adenovirus vectors were propagated in 293 cells and material purified by CsCl gradient centrifugation, dialyzed, and stored at $-80°C$. The titer of each viral stock was determined by plaque assay in 293 cells.

Effect of Angiostatin Expression on Vascular Cell Proliferation and Migration

Cell culture. Human pulmonary artery smooth muscle cells (PA-SMCs) were cultured from explants (17). In brief, pulmonary arteries (diameter: 5–10 mm) obtained from patients undergoing lung resection for lung carcinoma were kept in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen, Cergy Pontoise, France) at 4°C before their intimal cell layer and residual adventitial tissue were stripped off using forceps. The dissected media of the vessels was then cut into small pieces (3–5 mm), which were transferred into cell-culture flasks. To allow the PA-SMCs to grow out, the vessel tissues were incubated in DMEM supplemented with 10% fetal calf serum (FCS), 1 mM L-glutamine, and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin).Cultured PA-SMCs were used between passages 1 and 3.

Human microvascular endothelial cells (HMEC-1) obtained from E. W. Ades (Emory University Hospital, Atlanta, GA) were cultured in MCDB 131 (Gibco BRL) supplemented with 10% FCS, L-glutamine, antibiotics, 1 mg/ml hydrocortisone, and 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO) and grown at 37°C in 5% CO_2, in a humidified incubator.

Cell infection and Western blot analysis. Subconfluent cells were infected with Ad.K3 or Ad.CO1 at 10, 50, 100, 300, 500 plaque-forming units (pfu)/cell. Cell culture supernatants were collected 2, 4 and 6 d after infection.

The samples were run in a 10% sodium dodecyl sulfate–polyacrylamide gel before being transferred onto a nitrocellulose membrane. As a control, 100 ng of human plasminogen was run. After a 1-h incubation in a blocking buffer (0.15 M NaCl; 10 mM Tris-Hcl, pH 8.0; 0.005 Tween-20; and 5% BSA [TTBS], bovine serum albumin 5%), the membranes were incubated for 1 h with anti-human plasminogen, and for 1 h with a horseradish peroxidase–conjugated goat anti-mouse serum. After washing, the membranes were revealed by chemiluminescence using the ECL Plus kit (Amersham, Buckingham, U.K.).

Cell proliferation. The microculture tetrazolium (MTT) assay was used to evaluate the effect of the adenoviral constructs on PA-SMC and HMEC-1 proliferation. PA-SMCs and HMEC-1 were plated at a density of 5.10^3 cells/well in 96-well culture plates with DMEM or MCDB 131 supplemented with 20% FCS, and allowed to adhere. After 24 h, cells were subjected to growth arrest in medium containing 0.2% FCS and infected with Ad.K3 at 10 or 100 pfu/cell. Control cells were not infected, or infected with Ad.CO1 at the same dose. After 72 h, cells were supplemented with FCS (10%) and MTT assay performed 72 h later: MTT (0.2 mg/ml) was added to each well, which was incubated for 4 h at 37°C. Thereafter, the culture medium was removed and the MTT contained in cells was dissolved by adding 100 μl dimethylsulfoxide. The extent of reduction of tetrazolium salt to formazan within cells was then quantified spectrophotometrically at 520 nm and taken as an indicator of cell viability.

Cell migration assay. The cell migration assay was adapted from Planus and coworkers (18): HMEC-1 or PA-SMCs were subjected to growth arrest in medium containing 0.2% FCS and infected with Ad.K3. Control cells were not infected, or infected with Ad.CO1 at the same dose. After 72 h, cells were resuspended at 40.10^6 cells/ml in culture medium containing 15% FCS and 0.3% agarose, and were maintained at 37°C to prevent setting of the agarose. Three-microliter drops of the cellular suspension were plated in the center of each well of a 24-well tissue culture plate. Wells used for migration assay were precoated with type I collagen (0.5 mg/ml; Collaborative Biomedical Products, Becton Dickinson, France) for HMEC-1 and with poly-DL-ornithine (0.5 μg/ml; Sigma, St. Louis, MO) for PA-SMCs. The preparation was placed at 4°C for 20 min to allow the agarose to gel. Then, 0.5 ml of medium was added to cover the drops. The preparation was incubated at 37°C in 5% CO_2 for 24 h. Samples were fixed and stained with Diff Quik kit. Images were imported into an image analysis software (Perfect Image, Clara Vision, Orsay, France). This allowed calculation of the cell migration under each condition.

In Vivo Angiostatin Expression

Animals and delivery of adenovirus vectors to the lungs. Male C57BL/6J mice (20 - 25 g body weight) were used for all studies. All animal care and procedures were in accordance with institutional guidelines. Ad.K3 or Ad.CO1 as the control, was diluted before use with sterile saline, pH 7.4, in a final volume of 50 μl. Mice were anesthetized with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g). Intratracheal instillation of 50 μl/mouse of diluted Ad.K3 or Ad.CO1 was performed using a standard procedure, as previously described (10).

Human angiostatin protein detection in BAL fluid. To evaluate gene transfer efficiency, human angiostatin protein was assessed by Western blot analysis in BAL fluid from normoxic mice 5 d after administration with various doses of Ad.K3 (10^8 to 10^9 pfu) and 5, 10, and 15 d after administration of Ad.K3 or Ad.CO1 at 10^9 pfu.

After intraperitoneal administration of pentobarbital (6 mg/100 g), BAL was performed immediately after blood sampling. A total of 2 ml of warm phosphate-buffered saline (PBS) was used for each mouse. Then, the supernatants of BAL samples spun at 2,000 rpm, 4°C, for 15 min, were stored at $-20°C$.

Histologic evaluation of inflammation after gene transfer. To evaluate the inflammatory response after adenovirus administration, histologic examination was also performed in normoxic mice 4 d after administration with Ad.K3 or Ad.CO1 (10^8 and 10^9 pfu).

Immediately after BAL, the lungs were 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 using a previously described empiric semiquantitative scale based on inflammatory cell type and location (alveoli, bronchi, blood vessels) and on presence of edema and hemorrhage. Epithelial damage in bronchi, bronchioles, or alveoli was scored 0–4 (absent to severe). Extension of inflammation was also scored 0–4 as follows: 0, none; 1, small patchy areas involved; 2, < 10%; 3, 10–50%; 4, > 50% of section area.

Effect of angiostatin expression on development of hypoxic pulmonary hypertension. To examine the effect of angiostatin expression on the pulmonary circulation of normoxic mice and development of hypoxic pulmonary hypertension, Ad.K3 or Ad.CO1 (10^9 pfu) were administered intratracheally 2 d before the beginning of exposure to normoxia or hypoxia. Hemodynamic measurements

and assessment of right ventricular hypertrophy and pulmonary vascular remodeling were performed.

After 15 d of continuous exposure to normoxia or hypoxia (17 d after gene transfer), mice were anesthetized and ventilated with room air at a tidal volume of 0.2 ml and a rate of 90 breaths/min. After incision of the abdomen and diaphragm, a 26-gauge needle connected to a pressure transducer (Gould P23 ID, Gould Electornics, Ballainvilliers, France) was inserted into the right ventricle and right ventricular systolic pressure (RVSP) was immediately recorded. Next, blood was sampled for hematocrit determination. Finally, after an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and exsanguination, the thorax was opened and the lungs and heart were removed. The right ventricle (RV) was dissected from the left ventricle + septum (LV+S), and these dissected samples weighed.

The lungs were fixed by intratracheal infusion of 4% aqueous buffered formalin at a pressure of 23 cm H$_2$O. The entire specimen was placed in a bath of the same fixative for 1 wk. A midsagittal slice of the right lung including the apical, azygous, and diaphragmatic lobes was processed for paraffin embedding. Sections (5 μm thick) were cut for light microscopy and stained with hematoxylin-phloxin-saffron and orcein-picroindigo-carmine.

In each mouse, a total of 40 intra-acinar vessels accompanying either alveolar ducts or alveoli were analyzed by an observer blinded to the treatment. Each vessel was categorized as nonmuscular (no evidence of any vessel wall muscularization), partially muscular (SMCs identifiable in less than three-fourths of the vessel circumference), or fully muscular (SMCs in more than three-fourths of the vessel circumference). Muscularization was defined as the presence of typical SMCs stained red with phloxin, exhibiting an elongated shape and square-ended nucleus, and bound by two orcein-stained elastic laminae. The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number counted in the same experimental group.

Effect of angiostatin expression on pulmonary vascular density. To assess the consequence of angiostatin expression on pulmonary vascular density in various oxygenation, mice were pretreated with Ad.K3 or Ad.CO1. After 2 wk of continuous exposure to normoxia or hypoxia, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). After exsanguination, the lungs were removed and fixed in the distended state by infusion of alcohol formalin. A midsaggital slice of the right lung was processed for paraffin embedding, and 5-μm sections were prepared. After toluene treatment and rehydratation, the sections were pretreated for 10 min in a microwave oven in 10 mM citrate buffer (pH 6.0) and endogenous peroxidase activity quenched by incubation with 3% H$_2$O$_2$ for 5 min. Then, sections were washed in PBS and incubated for 60 min with a rabbit polyclonal serum raised against human Von Willebrand factor or CD31 (dilution 1:200, 1:100, respectively; Dako, Trappes, France). After three washes, the sections were incubated with biotinylated goat anti-rabbit IgG antibodies for 30 min. Then, the peroxidase staining of the slides incubated in streptavidin biotin horseradish peroxidase solution was performed using 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB; Sigma) and hydrogen peroxide. Finally, the sections were stained with hematoxylin. Vascular density was assessed in 10 selected fields and stored as digital fields image. Images were imported into an image-analysis Perfect Image. This allowed calculation of the total tissue area and the area of positive immunostaining. The lung immunoreactivity for Factor VIII and for CD31 expressed per area of lung parenchyma.

Effect of Angiostatin on eNOS Lung Expression

Immediately after removal, the lungs were quickly frozen in liquid nitrogen. After thawing at 0°C, the tissues were sonicated in 0.1 mM PBS containing antiproteases (1 μM leupeptin and 1 μM pepstatin A) and the detergent CHAPS (20 mmol/liter). The homogenate was centrifuged at 3,000 × g for 10 min at 5°C. The supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a nitrocellulose membrane by electroblotting in a transblot BioRad transfer apparatus, for 12 h at 4°C. Before the transfer, the gels, Whatman filter paper, and nitrocellulose membrane were soaked in electroblotting buffer (25 mM Tris-HCl; 193 mM glycine; 20% methanol, pH 8.0) for 15 min. After transfer, the membrane was blocked using 1× TTBS (0.15 M NaCl; 10 mM Tris-HCl, pH 8.0; 0.05% Tween-20; and 5% bovine serum albumin) for 1 h at room temperature. The eNOS protein was detected by incubating the membrane overnight at 4°C with mouse polyclonal anti-eNOS (Transduction Laboratory) diluted 1:1,000. The membrane was washed three times in 1× TBST. Specific protein was detected using a horseradish peroxidase–conjugated secondary antibody and ECL reagents (Amersham). The eNOS immunoreactivity was quantified using a semi-automated image analysis device (NIH image 1.52) that quantifies both the area and the intensity of immunoreactive bands using a ScanJet II scanner with DeskScan II (Hewlett Packard, France) software. Results are reported in arbitrary units.

Apoptosis assay. Apoptotic cells within the section from lung of mice exposed to normoxia or hypoxia after pretreatment with Ad.K3 or Ad.CO1 were detected by a kit using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling method (TUNEL; Boehringer Mannheim, Meylam, France). The apoptotic index was assessed on hematoxylin and eosin–stained histologic sections by counting nuclear and/or cytoplasmic features characterizing the apoptotic process. After image capture, apoptotic features were pointed on the screen and scored using an image analysis system (Perfect Image; Clara Vision, Orsay, France). The apoptotic index was defined as the ratio of this count over the total number of lung cells. Counts were determined in successive fields when fewer than 100 cells were present in a single field.

Statistical Analysis

All results are expressed as means ± SEM. A two-way ANOVA was performed to compare the effect of Ad.K3 versus Ad.CO1 pretreatment in normoxic and hypoxic mice, followed (when interaction was significant) by nonparametric Mann-Whitney test to compare Ad.K3 and Ad.CO1 pretreatment in each condition of oxygenation. To compare the degree of pulmonary vessel muscularization in two groups of mice, vessels were ordinally classified as nonmuscular, partially muscular, and muscular. Comparison of muscularization between two groups in each condition of oxygenation was performed using a nonparametric Mann-Whitney test.

Results

Evaluation of *In Vitro* Gene Transfer

As shown in Figure 1, transduction of HMEC-1 by Ad.K3 induced an accumulation of angiostatin protein in the culture media in a dose-dependent manner, whereas no signal was detected after infection with Ad.CO1. Angiostatin protein was detectable on Day 2, and was expressed over more than 6 d (Figure 1).

Effect of Angiostatin Expression on HMEC-1 and PA-SMC Migration

Four days after transfection with Ad.K3 (10–300 pfu/cell), there was a dose-dependent reduction of HMEC-1 migration, which was completely abolished at 300 pfu/cell. No
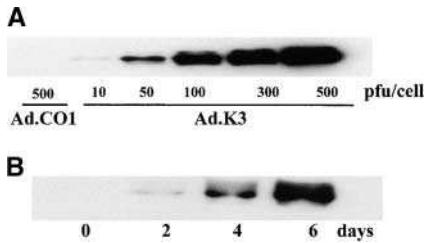
Figure 1. (*A*) Human angiostatin protein accumulation in supernatant of microvascular cells (HMEC-1) measured 4 d after cell transduction with Ad.K3 (10–500 pfu/cell) or Ad-CO1 (500 pfu/cell). (*B*) Time-course of human angiostatin protein accumulation in supernatant of HMEC-1 transduced with Ad.K3 (50 pfu/cell). Angiostatin protein was detected using Western blot with the specific antibody. Measurements were performed in five separate experiments.

alteration of HMEC-1 migration was observed after treatment with Ad.CO1 (Figure 2A).

However, incubation of PA-SMCs in the presence of Ad.K3 did not alter their migration. As shown in Figure 2B, cell migration out of the agarose drop explant was similar in PA-SMCs treated with Ad.K3, Ad.CO1 or untreated cells.

Effect of Angiostatin Expression on HMEC-1 and PA-SMC Proliferation

HMEC-1 infected with Ad.K3 (50–300 pfu/cell) showed a slight reduction in the growth response to 10% FCS, which

became significant only at the highest dose (Figure 3A). This effect was not observed in the presence of Ad.CO1, the control construction. However, in PA-SMCs infected with Ad.K3, the proliferation index was not altered and was maintained at levels similar to those measured in the presence of Ad.CO1 (Figure 3B).

Evaluation of *In Vivo* Gene Transfer in Normoxic Mice

Dose-dependent protein expression and inflammatory response after adenovirus administration. After intratracheal administration of Ad.K3 (10^9 pfu) in normoxic mice, detectable amounts of angiostatin protein were found at Day 5 (Figure 4), but not at Days 10 and 15 after treatment After treatment with Ad.CO1 (10^9 pfu), no human angiostatin was detected in BAL fluid. With 10^9 pfu of either Ad.K3 or Ad.CO1, inflammation was characterized by patchy infiltrates of mononuclear cells, most of which were macrophages. Cell damage, edema, or hemorrhage was infrequent (Table 1). On the basis of this experiment, 10^9 pfu was the dose selected for further experiments, because it caused minimal inflammatory response but significant human angiostatin protein production in lungs.

Effect of Angiostatin Expression on Chronic Hypoxic Pulmonary Hypertension

Hemodynamic measurements and assessment of right ventricular hypertrophy. Under normoxic conditions, body weight, RVSP, heart rate, and RV weight over either body weight or LV+S weight were similar in Ad.CO1- and

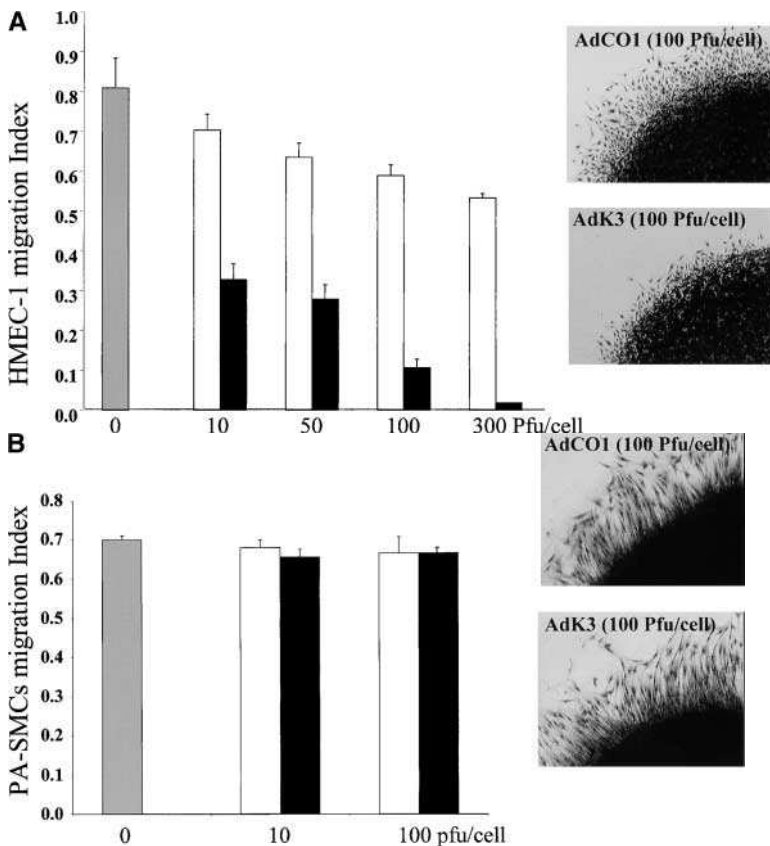*Figure 2.* Effects of Ad-K3 on HMEC-1 (*A*) or on PA-SMC (*B*) migration. The cells were starved of FCS for 72 h and transduced with Ad.CO1 (*open bars*) or Ad.K3 (*filled bars*) at increasing doses (10–300 pfu/cell). They were trypsinized and incorporated in agarose 0.3%. Cellular migration was measured 24 h after stimulation by 10% FCS. Values are means ± SEM of four independent experiments.
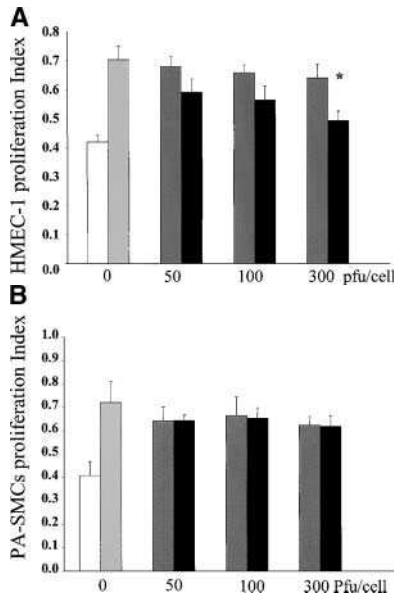
Figure 3. Effects of Ad.K3 on HMEC-1 (*A*) or PA-SMC (*B*) proliferation. The cells were starved of FCS for 72 h and transduced with Ad.CO1 or Ad.K3 at increasing doses. Cellular proliferation was measured by tetrazolium assay 72 h after stimulation by 10% FCS. Values are means ± SEM of four independent experiments. *Open bars*, FCS 0.2%; *lightly shaded bars*, FCS 10%; *darkly shaded bars*, Ad-CO1; *black bars*, Ad-K3.

Ad.K3-treated animals (Figure 5), and were similar to values previously reported in normoxic mice.

As compared with mice maintained under normoxic conditions, animals exposed to 10% O_2 during 15 d exhibited a significant increase in RVSP and development of right ventricular hypertrophy. However, under chronic hypoxic conditions, RVSP and right ventricular hypertrophy as assessed by RV/LV+S weight were higher in mice pretreated with Ad.K3 than in those given Ad.CO1 ($P < 0.05$ and $P < 0.01$, respectively), whereas left ventricular weight, hematocrit, and heart rate did not differ between the two hypoxic groups.

Structural remodeling of distal pulmonary vessels. In mice exposed to normoxia for 15 d after adenovirus administration, muscularization of distal vessels did not differ between Ad.K3- and Ad.CO1-treated animals, and were similar to values previously reported in normoxic mice (data not shown). After 15 d of exposure to hypoxia, muscularization of distal vessels, which was markedly increased as compared with normoxic animals, was also more marked in Ad.K3- than in Ad.CO1-pretreated mice exposed to similar hypoxic conditions (Figure 6; $P < 0.01$).
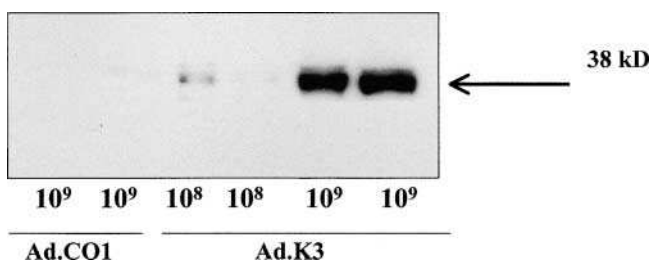

Figure 4. Human angiostatin protein in BAL fluid assessed by Western blot 5 d after intratracheal administration of normoxic mice with Ad.K3 or Ad.CO1 (10^8–10^9 pfu). A specific band (38 kD) was observed only in BAL from mice treated with Ad.K3 (10^9 pfu). Measurements were performed in four separate experiments.

Effect of hypoxia and angiostatin expression on vascular density. Vascular density was assessed by quantifying the surface area per field that scored immunoreactive for Von Willebrand factor and CD31. As shown in Figure 7 and in Figure 8A, there was an increase of vascular density in the lungs from mice chronically exposed to hypoxia as compared with similarly pretreated normoxic animals ($P < 0.001$). Pretreatment with Ad.K3 significantly reduced lung vascular density in both normoxic and chronically hypoxic mice as compared with control Ad.CO1 mice ($P < 0.01$), with no significant interaction between oxygenation condition and adenovirus pretreatment (two-way ANOVA).

Effect of hypoxia and angiostatin on eNOS expression. To evaluate the consequences of exposure to hypoxia and treatment with angiostatin on eNOS expression, the levels of eNOS protein were estimated by immunoblotting assay in lungs from mice treated with AdCO1 or with Ad.K3 and maintained under normoxic or hypoxic conditions. As shown in Figure 8B, eNOS protein levels was increased in the lungs from mice chronically exposed to hypoxia as compared with similarly pretreated normoxic animals ($P < 0.01$). Pretreatment with Ad.K3 significantly reduced lung eNOS protein levels in both normoxic and chronically hypoxic mice as compared with control Ad.CO1 mice ($P < 0.05$), with no significant interaction between oxygenation condition and adenovirus pretreatment (two-way ANOVA).

Effect of angiostatin expression on lung cell apoptosis. Lung sections from mice exposed to normoxia or hypoxia during 2 wk and pretreated with Ad.K3 or with Ad.CO1 (10^9 pfu) exhibited a few and identical number of apoptotic cells (data not shown). The apoptotic index did not differ between Ad.K3- and Ad.CO1-treated animals, the respective values being $3.1 \pm 0.7/1000$ versus $2.4 \pm 0.5/1000$ (NS) during normoxia, and $2.4 \pm 0.8/1000$ versus $1.9 \pm 0.6/1000$ (NS) during hypoxia

Discussion

The present results show that adenoviral-mediated lung overexpression of the angiogenesis inhibitor, angiostatin, aggravates development of hypoxic pulmonary hypertension in mice. Evidence for an activation of endogenous angiogenic processes in the lung during exposure to hypoxia was highly suggested by the observation that expression of endothelial markers like factor VIII, CD31, and eNOS was increased in chronically hypoxic mice. This change was suppressed by pretreatment with Ad-angiostatin, which also inhibited *in vitro* endothelial cell growth and migration. Because treatment with Ad-angiostatin did not affect PA-SMC function *in vitro*, but potentiated pulmonary hypertension and aggravated structural vascular remodeling in chronically hypoxic mice, the results indicate that counteracting lung angiogenic processes aggravates development of hypoxic pulmonary hypertension.

Hypoxic pulmonary hypertension is the most common form of pulmonary hypertension. Of variable severity, it affects patients of all ages and occurs in subjects who live at high altitude as well as in patients with chronic hypoxemic lung disease. Increased expression of the angiogenic factor VEGF and its receptors has been found in the lung from chronically hypoxic rats (5, 7). In addition to VEGFA, –C,

TABLE 1
Lung histopathology on day 5 after infection in normoxic mice

	Ad.CO1 10^8 pfu ($n = 5$)	Ad.K3 10^8 pfu ($n = 5$)	Ad.CO1 10^9 pfu ($n = 5$)	Ad.K3 10^9 pfu ($n = 5$)
Angiostatin in BAL fluid	No	No	No	Yes
Type of inflammatory cells	Macrophages	Macrophages	Macrophages	Macrophages
Inflammation extent (0–4)	1	1	1/2	1/2
Edema	0	0	0	1
Hemorrhage	0	0	0	0
Epithelial damage	0	0	0	0

Definition of abbreviations: BAL, bronchoalveolar lavage; pfu, plaque-forming units.
n indicates number of animals. The extent of inflammation was scored as 1 (small patchy area), 2 ($<$ 10%), 3 (10–50%), or 4 ($>$ 50% of section area). Edema, hemorrhage, and epithelial damage were scored from 0–4 (none to severe).

and -D, other growth factors or cytokines including PDGF, acidic and basic FGF, transforming growth factor, angiogenin, and prostaglandin E2 have also been shown to increase during exposure to hypoxia (6), suggesting that activation of several lung angiogenic processes contribute to the lung adaptation to chronic hypoxia. In systemic vessels, hypoxia or ischemia induced by occlusion of large systemic arteries results in development of newly formed collateral arteries (19). In the pulmonary circulation, no such an increase in new vessel formation has been angiographically documented. However, labeling studies in rat have suggested a burst of endothelial cell multiplication in intraacinar arteries at the end of the first week of exposure to hypoxia (20). In the present study, lung density of factor VIII and CD31 immunostaining was increased in mice exposed to chronic hypoxia. These results suggest that activation of lung angiogenic processes in response to hypoxia is associated with an increased number of endothelial cells in distal pulmonary vessels.

The physiologic or pathologic consequences of hypoxiainduced activation of lung angiogenic processes, however, are not completely understood. Until now, efforts have been devoted to specifically understand the role of VEGF. In previous studies, we have shown that adenoviral-mediated overexpression of VEGF in rat lungs attenuates develop-

ment of hypoxic pulmonary hypertension, and that this effect is concomitant to the improvement of endothelial function (10). In recent studies, inhibition of VEGF receptors through tyrosine kinase inhibitors was shown to cause severe pulmonary hypertension with selection and proliferation of apoptosis-resistant endothelial cells (9). Interestingly, inhibition of VEGF receptor tyrosine kinase–mediated activity was also shown to decrease alveolization in developing lung and to favor emphysema in the adults (21). Although these results point out important actions of VEGF in the lung, they did not specifically address the role of lung angiogenic processes during development of pulmonary hypertension. As mentioned earlier, angiogenic factors other than VEGF are stimulated during exposure to hypoxia. Moreover, evidence has now been provided that the VEGF action is not selective to endothelial cells and that functional VEGF receptors are present in many lung cell types including airway and alveolar epithelial cells, monocytes, and smooth muscle cells. Therefore, the role of endogenous angiogenic processes during exposure to hypoxia may not be fully investigated by the use of a specific inhibitor of only one among numerous angiogenic factors.

Physiologic angiogenesis is a highly regulated process under the control of both angiogenic and antiangiogenic factors. A number of endogenous angiogenesis inhibitors have been identified that antagonize the effects of VEGF, FGFs, and other angiogenic factors. Among those is angiostatin, a 38-kD fragment of plasminogen (14), which has been shown *in vitro* to inhibit endothelial cell proliferation and *in vivo* to exhibit potent antitumor and antiangiogenic
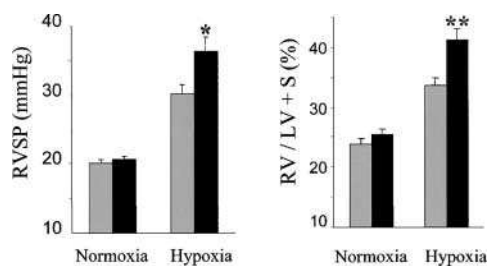

Figure 5. Right ventricular systolic pressure (RVSP) and ratio of right ventricle to left ventricle + septum weight (RV/LV+S) in mice pretreated with either Ad.K3 (*black bars*) or Ad.CO1 (*shaded bars*) and exposed to either normoxia or chronic hypoxia (2 wk). Pretreatment with Ad.K3 significantly increased RVSP and RV/LV + S in hypoxic rats but had no effect in normoxic mice. *$P <$ 0.05 and **$P <$ 0.01 for comparison with values in mice pretreated with Ad.CO1 and similarly exposed to hypoxia. Values are means $\pm$ SEM for $n = 10$ animals in each group.
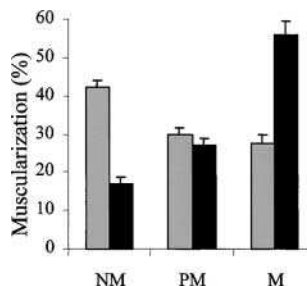

Figure 6. Percentage of nonmuscularized (NM), partially muscularized (PM), or fully muscularized (M) intra-acinar vessels in lungs from mice exposed to 2-wk hypoxia and pretreated with either Ad.K3 (*black bars*) or Ad.CO1 (*shaded bars*). Muscularization was significantly higher after Ad.K3 than Ad.CO1 pretreatment ($P <$ 0.01, nonparametric Mann-Whitney test on ordinally classified vessels). Values are means $\pm$ SEM for $n = 10$ animals in each group.
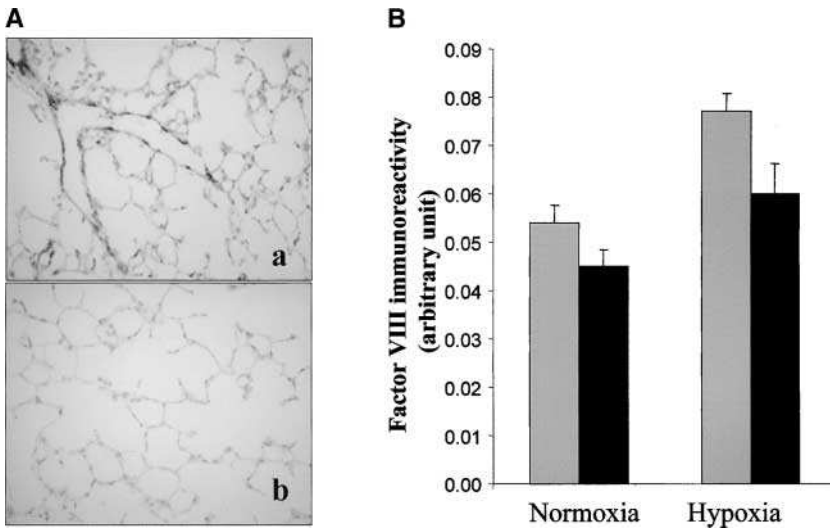
Figure 7. (*A*) Von Willebrand factor immunoreactivity in pulmonary arterial endothelial cells (a). No immunoreactivity is detected in section incubated with secondary antibody but no primary antibody (b). (*B*) Quantification of vascular density assessed by the ratio of Von Willebrand factor immunostaining density/total tissue area in lung sections from mice pretreated with Ad.CO1 (*shaded bars*) or Ad.K3 (*black bars*) (10^9 pfu) and exposed to normoxia or chronic hypoxia during 2 wk. Hypoxia significantly increased and pretreatment with Ad.K3 decreased vascular density with no interaction ($P < 0.01$ and $P < 0.05$, respectively, two-way ANOVA). Tissue section of $n = 9$ animals from each group were studied.

properties (15, 22, 23). Although its mechanism of action is not completely understood, numerous studies indicate that angiostatin is a highly specific angiogenesis inhibitor. In the present study, angiostatin delivery was achieved by a defective adenovirus expressing a secretable angiostatin K3 molcule from the cytomegalovirus promoter (Ad.K3). We found that angiostatin gene transfer in cultured endothelial cells inhibited cell migration and partially reduced cell proliferation. These results are consistent with those reported by studies using angiostatin protein. No cytotoxic or apoptotic effect was observed *in vitro*. More importantly, the effects of angiostatin appeared specific for endothelial cells, because human PA-SMCs treated with Ad.K3 remained functionally unaltered.

In vivo studies revealed that adenoviral transfer allowed efficient local overexpression of angiostatin in mice lungs.

Figure 8. (*A*) CD31 estimated by immunostaining density expressed by lung area of lung parenchyma. Tissue section of $n = 9$ animals from each group were studied. (*B*) eNOS protein levels assessed by Western blot in lungs from mice pretreated with Ad.CO1 (*shaded bars*) or Ad.K3 (*black bars*) (10^9 pfu) and exposed to normoxia or chronic hypoxia during 2 wk. $n = 5$ animals were studied in each group. Both lung CD31 and eNOS expression were significantly increased after hypoxia as compared with normoxia ($P < 0.001$ and $P < 0.01$, respectively) but decreased after Ad.K3 pretreatment as compared with Ad.CO1 ($P < 0.01$ and $P < 0.05$, respectively) with no interaction between oxygenation conditions and adenoviral pretreatment (two-way ANOVA).

After intratracheal administration of Ad.K3, the angiostatin protein was detected in BAL fluid for ~ 6 d, with a concentration peak on the fourth day. No angiostatin was detectable in the serum with this low dose of adenovirus, suggesting that angiostatin diffusion or expression in other organs was minimal.

As shown by our histologic study, administration of Ad.K3 or Ad.CO1 caused only mild inflammation in lungs from both normoxic and hypoxic mice. Only small patchy areas of macrophage infiltrates were observed, with no epithelial cell damage, edema, or hemorrhage.

The main finding from our study is that angiostatin overexpression in lung tissue from mice exposed to chronic hypoxia aggravated the development of pulmonary hypertension and right ventricular hypertrophy. The development of hypoxic pulmonary hypertension is associated with smooth muscle cell hypertrophy and hyperplasia in normally muscularized arteries, and with the appearance of new smooth muscle cells in nonmuscularized segments of the intra-acinar circulation (24). Angiostatin increased muscularization of the pulmonary resistance vessels, as shown by an increase in the percentage of muscularized arteries in hypoxic mice. These results are consistent with those of Taraseviciene-Stewart and colleagues (9), who recently reported that inhibition of the VEGF receptor 2 causes an increase in pulmonary artery medial thickening and led to severe pulmonary hypertension in chronically hypoxic rats. In line with these findings, we previously have shown that lung VEGF overexpression protects against pulmonary vascular remodeling in chronically hypoxic rats. Lung gene transfer of VEGF was associated with a lesser degree of medial thickening and muscularization in resistance vessels. Taken together, these observations strongly indicate that endogenous angiogenic processes counteract development of hypoxic pulmonary hypertension and negatively influence pulmonary vascular remodeling and smooth muscle cell growth.

Angiostatin might have affected the pulmonary vascular bed both functionally and structurally. Functional conse-
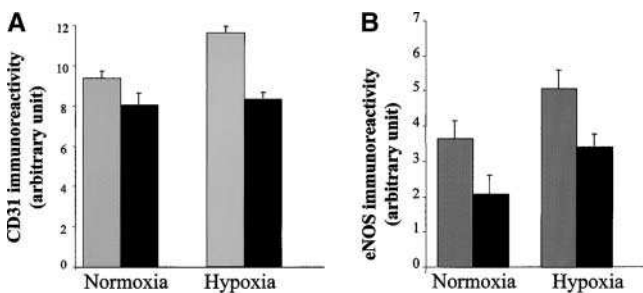
quences are those expected from an aggravation of endothelial dysfunction, which favors pulmonary vasoconstriction and/or enhance platelet–endothelial cell interaction, thereby increasing the release of platelet-derived constricting and growth factors such as serotonin or PDGF. Structural consequences are those expected from a reduced pulmonary vascular bed. Our observations that endothelial cell Factor WIII and CD31 immunostaining, as well as eNOS immunoreactivity, were decreased in lungs from both normoxic and hypoxic mice after lung angiostatin gene delivery is consistent with a decreased number of peripheral vessels. These data also suggest that even in normoxic conditions, pulmonary endothelial cells are sensitive to a change in the balance between angiogenic and antiangiogenic factors. The mechanism by which angiostatin leads to a reduction in endothelial cell labeling remains unclear. Angiostatin has been shown to induce endothelial cell apoptosis *in vitro* (25, 26). Although the apoptotic index did not differ between Ad.K3- and AdCO1-treated animals after exposure to hypoxia for 2 wk, we cannot exclude that angiostatin caused a reduction in peripheral vessels by inducing apoptosis of pulmonary vascular endothelial cells, either during normoxia or during hypoxia. The fact that angiostatin did not affect pulmonary hemodynamics and did not cause structural remodeling in normoxic mice may be related to the large number of endothelial cells in the lung, which contains approximately a third of the body endothelial cells. However, a decreased number of functional pulmonary vessels might have contributed to the increase in resistance and therefore to the increase in pulmonary artery pressure observed after exposure to hypoxia. According to the classical understanding of chronic hypoxic pulmonary hypertension, which is based on the well-established concept that vascular remodeling is a response to sustained pulmonary vasoconstriction and increased pulmonary artery pressure, angiostatin delivery might have increased physical forces exerted on the vascular wall, thereby triggering hypertrophy and proliferation of the vascular smooth muscle cells.

These results differ to some extent from those obtained by Taraseviciene-Stewart and coworkers (9). In this study, treatment of rats with SU5416, a selective VEGF receptor tyrosine kinase inhibitor caused mild pulmonary hypertension and pulmonary vascular remodeling in normoxic rats and severe, irreversible pulmonary hypertension associated with some endothelial cell proliferation in chronically hypoxic rats. It is not clear why pulmonary endothelial cells proliferate in response to SU5416. Because, in the study by Taraseviciene-Stewart and colleagues, endothelial cell death occurred before or concomitantly to endothelial cell proliferation, the authors proposed that endothelial cell death, together with chronic hypoxia, resulted in the selection of an apoptosis-resistant, proliferating endothelial cell phenotype. No such an effect of angiostatin was observed in our study where immunostaining to factor VIII or CD31-related antigen was markedly decreased, with no evidence of a significant increase in apoptosis of lung cells. Findings obtained in the present study might therefore be interpreted as reflecting the consequence of angiogenesis inhibition. The use of VEGFR2 inhibitors likely caused additional effects, potentially related to suppression of VEGF-dependent survival of endothelial cells or to other associated effects.

In conclusion, exposure to chronic hypoxia is associated with the activation of endogenous lung angiogenic processes, which attenuate the severity of pulmonary hypertension. This counteracting mechanism may not be found in other models of pulmonary hypertension, and may explain why hypoxic pulmonary hypertension is reversible and usually less severe than other forms of the disease. Moreover, they provide further support to the concept that stimulation of angiogenesis in the lung may be viewed as a therapeutic approach of pulmonary hypertension.

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