Adenoviral Vector Expressing Murine Angiostatin Inhibits a Model of Breast Cancer Metastatic Growth in the Lungs of Mice

Steve Gyorffy, Kay Palmer, and Jack Gaudie

From the Department of Pathology and Molecular Medicine,
Centre for Gene Therapeutics, McMaster University, Hamilton,
Ontario, Canada

Angiostatin, an internal fragment of plasminogen, has been shown to inhibit the process of angiogenesis or neovascularization. In this study, we have expressed the cDNA for murine angiostatin under the control of the human cytomegalovirus promoter from a human type-5 adenovirus and shown that this vector produces a protein which retains biological activity. Angiostatin expression was determined by Northern blot analysis and Western immunoblotting. Ad-angiostatin, but not a control vector Ad-dl70, significantly reduced the viability of infected human umbilical cord vein endothelial cells (HUVEC) *in vitro*. In an *in vivo* model of basic fibroblast growth factor-induced angiogenesis, Ad-angiostatin (1×10^9 pfu) could inhibit endothelial cell migration and the formation of capillaries within a Matrigel plug which had been implanted for one week subcutaneously into C57BL/6 mice. Endothelial cells in these plugs had an altered, rounded, phenotype with dark picnotic nuclei indicative of apoptosis, which was confirmed using transmission electron microscopy. In contrast, endothelial cells from bFGF alone or in combination with the control vector-treated plugs retained the long spindle shape characteristic of endothelial cells. Intranasal delivery of Ad-angiostatin into the lungs of FVB/n mice demonstrated comparable cellular infiltration in the recovered bronchoalveolar lavage fluid with no signs of abnormal pathology as compared to PBS or control vector-treated animals. In a pulmonary metastatic breast cancer model, the delivery of Ad-angiostatin (1×10^9 pfu) to the lung significantly delayed tumor growth as measured by the number of visible surface tumor nodules. This study has demonstrated that the specific targeting of tumors to inhibit angiogenesis using an adenovirus expressing angiostatin, may deliver localized concentrations of protein having a greater impact on inhibition of tumor growth. (*Am J Pathol* 2001, 159:1137-1147)

Tumors *in situ* that are smaller than 3 mm in diameter, exist in a pre-vascular state and are limited in their ability

to grow without perfusion from the blood supply.¹ Vascularization of the tumor increases the transport of oxygen and metabolites and removes waste products.¹ Change to the angiogenic state involves disruption of the balance between positive and negative regulatory factors controlling endothelial cell growth, proliferation and vascular formation. These angiogenic stimuli may be secreted by the tumor cells directly, by cells such as macrophages, or during the degradation of the extracellular matrix by metalloproteinase.¹ Positive mediators of angiogenesis include vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), which are thought to have short half-lives but can combine to have a synergistic effect.² Negative regulators of angiogenesis include thrombospondin and interferon- τ (IFN- τ).^{3,4} More recently, two powerful inhibitors of angiogenesis, angiostatin and endostatin have been reported.^{5,6,7} Angiostatin, a fragment of the plasma protein plasminogen was initially isolated from urine of mice with concomitant Lewis lung carcinoma.⁵

Previous studies have used repeated subcutaneous injections of angiostatin protein to demonstrate function,⁵ however the specific transient targeting of the angiostatin gene to a tumor site may be an attractive alternative using recombinant adenoviral vectors. This would allow for short term, concentrated expression of angiostatin in the tumor environment. This study demonstrates the construction and characterization of a recombinant, replication deficient type-5 adenovirus vector expressing the cDNA-encoding amino acids 98–458 of murine plasminogen. The biological activity is shown both *in vitro* and *in vivo* using human umbilical cord vein endothelial cells (HUVEC) and an *in vivo* Matrigel bioassay respectively. We also show that sustained expression of murine angiostatin by adenovirus delivered directly into the lung results in expression of angiostatin for up to seven days within the lung and significantly delayed the growth of metastatic breast tumor cells that had been embolized to the lung.

Supported by the Medical Research Council of Canada (MRC), The Hamilton Health Sciences Corporation, and St. Joseph's Hospital.

Accepted for publication May 31, 2001.

Address reprint requests to Jack Gaudie, PhD, FRSC, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5. E-mail: gaudie@mcmaster.ca.

Materials and Methods

Animals and Cell Culture

Six- to eight-week-old C57BL/6 and FVB/n female mice were obtained from Charles River (Troy, NY) and housed in a specific pathogen-free environment until use. All animal work was approved by the McMaster University animal ethics research board. Transgenic FVB mice expressing the polyomavirus (PyV) Middle T antigen under the transcriptional control of the mammary tumor virus (MMTV) long terminal repeat, spontaneously develop adenocarcinomas of the mammary epithelium in 8 to 10 weeks.⁸ The tumors were removed and processed to a single cell suspension with mechanical disruption in the presence of 25 mg collagenase (GIBCO, Burlington, ON) carried out in 100 ml of phosphate-buffered saline (PBS). The polyoma Middle T (PyMidT) cells were then placed in modified Eagle's medium (MEM) F-11 media containing 10% fetal bovine serum (FBS) (complete F-11) and cultured for 48 hours at 37°C and 5% CO₂ before intravenous delivery to syngeneic FVB mice.

Matrigel was purchased from Collaborative Biomedical Products, (Bedford, MA) and was stored at -20°C until use. All cell culture media was purchased from GIBCO (Burlington, ON).

Adenoviruses were propagated in 293 (Ad5 E1-transformed) human embryonic kidney cells maintained in complete F-11 containing 100 µg/ml penicillin and 100 U/ml streptomycin.⁹ Viruses were purified by double cesium chloride gradient centrifugation following growth in suspension 293 N3S cells using Joklik's medium and 10% FBS.⁹ A549 human lung carcinoma cells (ATCC CCL-185) were maintained in MEM media containing 10% FBS.

Human umbilical cord endothelial cells (HUVEC) were obtained from fresh umbilical cords by washing with 2% type 2 collagenase (GIBCO) in Hanks' buffered saline. HUVECs were maintained in monolayer on culture dishes pre-coated with 2% (w/v) type 2 collagen in PBS, M199 media supplemented with 10 ng/ml endothelial cell growth factor (ICN, Montreal, Quebec) and 10% FBS.¹⁰

Recombinant Adenovirus

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted from quick frozen livers of C57BL/6 mice using Trizol reagent (GIBCO). Reverse transcription reactions were carried out with 0.5, 1.0, 3.0, and 5.0 pg of total RNA in a 20 µl final volume using oligo dT primers supplied with the SuperScript RT reaction kit (GIBCO). Briefly, RNA and oligo dT primers were heated to 70°C for 10 minutes and then placed on ice. 10× RT buffer, MgCl₂ dNTPs and DTT were added to the reaction which was subsequently heated to 42°C for 5 minutes after which 1 µl of Superscript reverse transcriptase enzyme was added and the reaction incubated for another 50 minutes. The reaction was stopped by

heating to 70°C for 10 minutes and then treated with RNase for 20 minutes at 37°C.

Polymerase chain reactions were carried out in a Perkin Elmer 9600 thermocycler in a final volume of 100 µl using Vent polymerase (NEB, Mississauga, ON). The angiostatin cDNA is based on the corresponding amino acids 1–32 and 98–458 of the native murine plasminogen¹¹ and the protein sequence of angiostatin derived by enzyme proteolysis.^{2,12} Two polymerase chain reactions were carried out, one to form the sequence for amino acids 1–32 which also contained the native plasminogen signal sequence and a second reaction which added the bioactive four kringle region of angiostatin. The following primer pairs were used in the first reaction: forward primer, (A) 5'-TGGGGAATTCTTGTGGCCAGTCCCAA-CATGGACCATAAGGAAT3', reverse primer (B) 5'-ACC-ACTCGAGAAGAAGCCCCCTGTGTGCTTATGTAGCCATCCAT3', which contained an *Eco*RI and *Xho*I restriction enzyme site respectively. The second PCR reaction used the following primer pairs: (C) 5'-CTTCTCTCGAGTG-TATCTGTCTAGAATGTAAG3', reverse primer, (D) 5'-CC GAAGCTTTTATCATCCTGTCTCTGAGCTCCGCTTCAG3' which contained a stop codon. Primers C and D contained *Xho*I and *Hind*III restriction enzyme sites respectively. Cycling conditions for the first section were: denaturation at 94°C, 3 minutes; 94°C, 1 minute; annealing at 55°C, 1 minute; extension at 72°C, 1 minute for 35 cycles. The cycling conditions for the second segment were identical, except that the extension time was increased to 1 minute and 30 seconds. The PCR fragments were run on 1% agarose gels, excised, and isolated using QIAex II gel purification (Qiagen, Chatsworth, CA). The PCR fragments were restriction enzyme digested, re-purified, and ligated into the *Eco*RI and *Hind*III polylinker region of the adenovirus shuttle plasmid pAC-CMV forming a 1170 bp open reading frame which codes for murine angiostatin. The shuttle plasmid pACCMV-angiostatin was co-transfected into 293 cells with pJM17 using calcium phosphate co-precipitation.¹³ Ad-angiostatin was isolated from a single plaque, expanded in 293 cells and purified by cesium chloride centrifugation.⁹ The control vector Ad-dl70, without an expressed gene product, was previously described.¹⁴ All primers were synthesized at MOBIX (McMaster University, Hamilton, Ontario).

Northern Blots

Total RNA was isolated from A549 cells infected with Ad-dl70 or Ad-angiostatin at a multiplicity of infection (MOI) of 100 using Trizol (GIBCO). 20 µg of RNA was loaded per lane and electrophoresed through a 1% agarose formaldehyde denaturing gel at 20V. The RNA was transferred overnight by capillary action using 10× saline sodium citrate to Nytran membranes (ICN). Membranes were hybridized overnight at 55°C with α[³²P]-CTP random primed (Pharmacia, Uppsala, Sweden) mouse angiostatin cDNA polymerase chain reaction product as probe.

Western Blots

Supernatants from HUVEC or A549 cells infected 5 days earlier with Ad-angiostatin or Ad-dl70 at multiplicity of infection (MOI) 100, were incubated overnight at 4°C with 250 μ l of lysine-Sepharose (Pharmacia, Uppsala, Sweden) per ml of supernatant. The slurry was collected by centrifugation and washed with 50 mmol/L sodium phosphate buffer, pH 7.4. A 15- μ l aliquot was run on a 12% SDS-polyacrylamide gel under non-reducing conditions, and transferred to Immobulon-P membranes (Millipore, Mississauga, ON). The membranes were probed with a 1:500 dilution using a chicken anti-rabbit-plasminogen IgY antibody, followed by a secondary rabbit anti-chicken alkaline phosphatase conjugated antibody (Zymed, San Francisco, CA). A positive control, partially purified rabbit angiostatin derived from human urokinase digested rabbit plasminogen was used as a positive control. Prestained molecular weight markers were purchased from Novagen (Madison, WI).

In Vitro HUVEC Proliferation Assay (Direct Infection and Conditioned Media Assay)

HUVEC cells were plated in 6-well plates pre-coated with 2% gelatin in 3 ml of complete M199 media at a density of 120,000 cells per well. Cells were infected with Ad-dl70, or Ad-angiostatin at a MOI of 100 and were allowed to proliferate for seven days. Two ml of fresh complete M-199 media was added every other day to maintain cell viability. On day 7, the cells were removed with 0.5 $\times$ trypsin/EDTA (GIBCO), and total cells including those floating in the media were counted using trypan blue (GIBCO) exclusion staining to determine total numbers and viability.

To determine whether the angiostatin gene product was active on endothelial cell proliferation, HUVEC cells were plated in 96-well plates pre-coated with 2% gelatin in complete M199 media at a density of 5000 cells per well overnight. A549 cell culture supernatants infected for 48 hours with Ad-dl70 or Ad-Angiostatin at a MOI of 100 were filter sterilized and UV-irradiated for 30 minutes to inactivate the virus. Fresh M199 media containing 50, 25, or 12.5% conditioned A549 supernatants were added to the HUVEC cells in a final volume of 200 μ l. Two days later, the HUVEC cells were labeled with 1 μ Ci of [³H]thymidine, and incorporation was measured 18 hours later.

In Vivo Matrigel Bioassay for Angiostatin

Matrigel at 4°C was mixed with 100 ng/ml bFGF (GIBCO) alone or in combination with Ad-dl70 (1×10^9 pfu), and Ad-Angiostatin (1×10^9 pfu) in a final volume of 0.5 ml. The Matrigel mixture was then injected subcutaneously into the abdominal midline of C57BL/6 mice, where it polymerized to form a plug. The plug was removed on day 7, fixed in sodium phosphate (0.075 mol/L, pH 7.4) buffered, 10% formalin solution (Fisher Chemicals, Ottawa, ON) for 24 hours and then embedded in

paraffin. All tissues were sectioned (5 μ m thickness), mounted onto slides and stained with Masson's trichrome using standard techniques. Total cell invasion was determined by image analysis of the stained sections using a Sony CCD camera and Vidas (Kontron, UK) software. The amount of cell migration was determined by calculating the percent area occupied by the endothelial cells which stained red, versus the total area of the matrigel which stained blue/green.

Electron Microscopy

Portions of paraffin-embedded Matrigel plugs from the bFGF and bFGF plus Ad-angiostatin treatments were extricated. The samples chosen were based on regions of the Masson's trichrome stained sections that exhibited cellular morphological changes indicating endothelial infiltration, capillary development, or apoptosis.

The Matrigel was removed from the paraffin by heating and then dissolving the sample in xylene. This was followed by re-hydration using 50% ethanol/xylene, 70% ethanol/xylene, 100% ethanol, 90% ethanol, and finally 70% ethanol. The sample was then placed in 0.1 mol/L sodium cacodylate (NaCac) pH 7.4, followed by fixation in 2% glutaraldehyde buffered in 0.1 mol/L NaCac, pH 7.4. All procedures were carried out at room temperature. Treatment with 1% OsO₄ in 0.1 mol/L NaCac at 4°C for one hour ensued. The samples were dehydrated using a gradient of ethanol washes ranging from 50% to 100%, followed by propylene oxide treatment. The samples were then embedded in 100% Spurr's resin, heated overnight at 65°C and sectioned to a thickness of 1 μ m. Sections were placed on copper grids and examined using a JEOL 1200 EX Biosystem electron microscope (Tokyo, Japan) at 80 kV.

Lung Gene Expression and Bronchioalveolar Lavage

Ad-Angiostatin or control virus Ad-dl70 were introduced into the lungs of FVB/n mice by intranasal delivery (i.n.). Briefly, 1×10^9 pfu of vector, in a total volume of 30 μ l PBS was inserted into the nostrils of anesthetized mice in 15- μ l aliquots using a p10 Pipetteman and tip. Seven days post-infection, the lungs were removed, and bronchial alveolar lavage (BAL) fluid was obtained. The anesthetized mice were sacrificed by cutting the abdominal aorta and the entire lung with the attached trachea was removed from the chest cavity. A small diameter (2 mm) section of Tygon tubing attached to a 26-gauge needle and 1 ml syringe was inserted into the lungs through the trachea. BAL fluid was collected from the lungs by twice filling with 150 μ l of PBS, which was then aspirated. The lungs were fixed for 24 hours in 10% neutral buffered formalin and paraffin embedded. Sections (5 μ m) were hematoxylin and eosin (H&E) stained using standard techniques.

Pulmonary Metastatic Growth of Breast Cancer Cells

3.0×10^5 PyMid-T murine breast adenocarcinoma cells in a volume of 200 μ l of PBS were injected as a single cell suspension intravenously into the tail vein of FVB/n mice. This number of cells routinely shows significant metastatic lung nodules within 20 to 25 days after injection. Sixteen days post tumor injection, Ad-angiostatin, and Ad-dl70 (1×10^9 pfu) were delivered intranasally. Lungs were removed on day 24, weighed, and representative lungs from each treatment group were lavaged. Bronchoalveolar lavage fluid 0.5 ml was centrifuged to remove cells and the supernatant was concentrated using 6% trichloroacetic acid precipitation. The recovered protein was resolubilized in 50 μ l, 0.5 mol/L Tris buffer, pH 6.8 and run on a 12% SDS-PAGE gel under non-reducing conditions, transferred to Immobulon-P membranes, and Western immunoblotted as described above. The lungs were then formalin-fixed for 24 hours and the total number of surface lung metastases were counted under a stereomicroscope on 6X power. Following paraffin embedding, 5- μ m sections were cut and H&E stained to observe tumor vasculature and lung pathology. Statistical significance of differences was tested using Student's *t*-test.

Results

Homologous recombination between the shuttle plasmid pACCMV, carrying the open reading frame for murine angiostatin, and rescue plasmid pJM17 generated the adenovirus, Ad-angiostatin. Northern blot analysis of RNA from A549 cells infected with Ad-angiostatin revealed a positive single RNA species at approximately 1.2 kb, consistent with the expected mRNA message of murine angiostatin cDNA (Figure 1A). No angiostatin mRNA could be found in the Ad-dl70 infected A549 cells.

Angiostatin protein production from HUVEC cells infected with Ad-angiostatin at MOI 100 is shown in Figure 1B. Cell culture supernatants were harvested 5 days after infection and mixed with lysine-Sepharose to concentrate angiostatin from the media and subjected to gel electrophoresis. A single band is seen in lane 2 that has an apparent molecular weight of 50 kd. This single band may represent two angiostatin isoforms mixed together, as the unconcentrated culture supernatant (lane 3) gives rise to two distinct bands between 40 and 50 kd. This doublet may indicate differences in glycosylation. The chicken anti-rabbit plasminogen IgY antibody detected rabbit angiostatin (lane 4), derived from the proteolytic digestion of rabbit plasminogen, as well as bovine plasminogen from the culture media at 92 kd. However, no bands were detected in the 40 to 50 kd range in the Ad-dl70 control transfected sample (lane 1) indicating that the bands appearing in the Ad-Angiostatin lanes were not simply the result of degradation of serum-derived plasminogen.

Angiostatin Biological Activity in Vitro

Angiostatin biological activity was determined *in vitro* by two methods. HUVEC cells plated to a density of 1.2×10^5 cells per well were directly transduced at MOI of 100. Control wells were either uninfected, or infected with control vector Ad-dl70, or Ad-angiostatin. Seven days post-infection, the cells were gently removed and counted using trypan blue exclusion staining. Direct transduction of subconfluent HUVEC cells by Ad-angiostatin led to a 38% decrease in the number of viable cells seven days after infection. In contrast, uninfected and Ad-dl70 infected cells increased in cell numbers by 276% and 240%, respectively, during the same period (Figure 1C). HUVEC cells, whether uninfected or infected with control virus proliferated to a confluent state with the cells exhibiting the normal endothelial phenotype. In contrast, HUVEC cells infected with Ad-angiostatin were sparse, rounded, and detached.

To exclude the possibility of a direct viral toxic effect, conditioned media from A549 cells infected with Ad-angiostatin or Ad-dl70, was irradiated to inactivate any contaminating virus and was added to proliferating HUVEC cultures to measure [3 H]thymidine incorporation. Murine angiostatin was detected by Western immunoblotting in the Ad-angiostatin, infected A549 conditioned media (Figure 1A, lane 5). Addition of a range of dilutions of Ad-Angiostatin conditioned media induced a 54-to 106-fold inhibition of HUVEC cell proliferation compared to mock-transfected conditioned media (Figure 1D).

Angiostatin Biological Activity in Vivo

Matrigel, an extracellular matrix preparation from Englebreth-Holm-Swarm (EHS) murine sarcoma, is fluid at 4°C but solidifies at 37°C.¹⁵ This solid matrix, when combined with angiogenic factors such as bFGF, initiates endothelial cell migration and capillary tube formation into the matrix and is an excellent assay for angiogenesis studies *in vivo*.^{15,16}

To assess the biological activity of angiostatin derived from the adenoviral vector, mice were injected subcutaneously with Matrigel alone, Matrigel mixed with bFGF (100 ng/ml), or Matrigel plus bFGF plus control virus Ad-dl70 or Ad-angiostatin (1×10^9 pfu).

Histological sections of the Matrigel plugs stained with Masson's trichrome are shown in Figure 2. Seven days post-infection, the Matrigel alone revealed only a few endothelial cells invading from the tissue into the edge of the Matrigel in the absence of any angiogenic factor (Figure 2A). In contrast, the addition of bFGF to the matrigel caused a large increase in endothelial cell infiltration, and the development of capillary tubes containing red blood cells is evident (Figure 2B). Morphologically, the endothelial cells appear long and tapered with no distinct nuclear staining. Addition of the control adenovirus does not affect the process of endothelial cell invasion or capillary tube formation within the bFGF impregnated Matrigel as seen in panel C. In sharp contrast, the Ad-Angiostatin vector inhibited endothelial cell migration

Figure 1. *In vitro* expression and biological activity of murine angiostatin by Ad-angiostatin. **A:** Northern analysis. Northern blot of A549 cells infected at MOI 100 with Ad-dl70 or Ad-angiostatin. Total RNA, 20 μ g per lane, was loaded onto a 1% agarose denaturing gel (top, 18S and 28S ribosomal RNA present), transferred to Nytran membranes and hybridized to 32 P-labeled murine angiostatin cDNA. No angiostatin expression was observed in the Ad-dl70 infected A549 cells (lane 1). Infection of cells with Ad-angiostatin resulted in a message of approximately 1.2 kb consistent with the expected size of angiostatin mRNA (lane 2). **B:** Protein expression. Western blot of HUVEC and A549 culture supernatants infected at MOI 100 with Ad-angiostatin control virus Ad-dl70. Culture supernatants were harvested five days post infection. A 15 μ l aliquot of supernatant was run per lane on a 12% SDS polyacrylamide denaturing gel under non-reducing conditions and transferred to Immobulon-P membranes. The membrane was developed using a chicken (IgY) anti-rabbit plasminogen antibody followed by a goat anti-chicken alkaline phosphatase conjugated secondary antibody. Lysine-Sepharose concentrated supernatant from control Ad-dl70 infected HUVEC (lane 1). Culture supernatant from Ad-angiostatin infected HUVEC concentrated with lysine-Sepharose. Angiostatin band appeared at approximately 50 kD (lane 2); unconcentrated supernatant from Ad-angiostatin infected HUVEC, arrows indicate two angiostatin bands at approximately 40 to 50 kD thought to be different glycoforms of angiostatin (lane 3). Rabbit angiostatin derived from human urokinase digested rabbit plasminogen showing two angiostatin bands (lane 4). Radiation inactivated culture supernatant from Ad-angiostatin-infected A-549 cells used in HUVEC proliferation assay (lane 5) of **D**. Prestained molecular weight markers are in kD and are representative (Novagen). **C:** Biological activity. Ad-angiostatin reduces HUVEC cell viability in an *in vitro* bioassay. 120,000 HUVEC cells (line) were plated onto collagen coated plates and were uninfected or infected with Ad-dl70, or Ad-angiostatin at MOI 100. Columns represent the mean number of viable cells per well ($n = 6$ wells) seven days after infection. ($P < 0.005$ as compared to control uninfected HUVEC). **D:** Proliferation assay. Inhibition of HUVEC proliferation by murine angiostatin conditioned A549 culture supernatants. Fresh M199 media containing 50, 25, or 12.5% radiation-inactivated conditioned A549 culture media infected with Ad-dl70 or Ad-angiostatin was added to HUVEC cells in a final volume of 200 μ l. Two days later, HUVEC cells were labeled with 1 μ Ci of [3 H]thymidine, and incorporation was measured 18 hours later.

into the center of the matrigel. The cellular invasion was limited to a sharp line of demarcation near the outer edge of the plug and no vessel formation is evident (Figure 2, D and E). Moreover, the endothelial cell morphology is distinct, changing from a long tapered phenotype to a rounded cell with a pyknotic nucleus that exhibits intense nuclear staining suggesting apoptosis.

Image analysis of the Masson's trichrome-stained sections revealed that infiltration by endothelial cells ac-

counted for 22% of the Matrigel section in all treatment groups with the exception of Matrigel alone, which was not angiogenic. Electron microscopy of the bFGF plus control vector and bFGF plus Ad-angiostatin Matrigel sections confirmed the observations made with light microscopy alone. The most striking feature of both treatment groups is that the majority of the cells infiltrating into the Matrigel are endothelial cells (Figure 3, A and B). Moreover the formation of functional capillaries contain-

ing red blood cells is clearly evident. In addition, Weibel Palade bodies, the storage granule for von Willebrand factor (vWf), which are unique to endothelial cells, are also present. Fibroblasts are not prominent within the matrigel sections, even though 100 ng/ml of bFGF was added to the Matrigel. A small number of neutrophils and eosinophils were observed in both vector treatment groups but there was no sign of an inflammatory process.

The electron micrographs demonstrate apoptosis of the endothelial cells within the Ad-angiostatin-treated Matrigel, including cell shrinkage and rounding, condensation of the chromatin, fragmentation of the nucleus, and membrane bound blebbing of the cytoplasmic contents (Figure 3, C and D).^{17,18} Granulocytes were not affected by angiostatin and appeared in a normal, unstimulated state with their enzyme storage granules intact (not shown).

Lung Gene Expression and Bronchoalveolar Lavage

The expression of Ad-angiostatin in the lungs of mice did not induce any apparent abnormal pathology. Inflammation was limited to the extent of the control vector and no abnormal pathology was evident at seven days. Examination of the BAL fluid contents revealed similar amounts of infiltrating lymphocytes (Table 1) in both angiostatin and control vector treated lungs in FVB/n mice. Endothelial cells lining the veins and arteries appear intact and were not thrombosed. No necrosis or apoptosis of the lung tissue as a possible result of insufficient blood perfusion was observed.

Pulmonary Metastatic Growth of Breast Cancer

A model of breast cancer metastasis to the lung was established in FVB/n mice. Mice were injected in the tail vein with 3.0×10^5 PyMidT primary tumor cells and preliminary evidence of nodules on the lung surface appear at 14 to 15 days post injection. On day 16, 1×10^9 pfu control Ad-dl70 or Ad-angiostatin was administered by intranasal delivery. The mice were subsequently sacrificed one week later at which time the lungs were weighed, BAL fluid was collected, and the lung surface metastases counted.

Figure 2. *In vivo* biological activity of Ad-angiostatin. Matrigel plug bioassay for neovascularization in C57BL/6 mice. Sections stained with Masson's trichrome. **A:** Matrigel alone. No endothelial cell invasion is evident. **Arrow** marks depth of cellular infiltration. Magnification, $\times 400$. **B:** bFGF positive control. Addition of 100 ng/ml bFGF induces a large infiltration of mainly endothelial cells. Capillary formation is evident (**arrow**). Magnification, $\times 400$. **C:** Addition of control vector Ad-dl70 (1×10^9 pfu) does not diminish endothelial cell invasion nor capillary tube formation induced by bFGF. Magnification, $\times 400$. **D:** Ad-angiostatin (1×10^9 pfu/ml) inhibits bFGF induced endothelial cell invasion into the Matrigel. **Arrow** illustrates migration is limited to a narrow region bordering the edge of the Matrigel and abdominal tissue. Endothelial cell morphology is changed from the long spindle shaped phenotype to a rounded cell with a pronounced nucleus. Magnification, $\times 100$. **E:** Ad-angiostatin (1×10^9 pfu/ml) induces apoptosis. Cell death occurs along a sharp line of demarcation near the outer edge of the matrigel. Cell nuclei appear dark and dense (**arrow**) suggesting apoptosis. Magnification, $\times 400$.

Figure 3. *In vivo* Matrigel bioassay for neovascularization. Electron micrographs of bFGF (100 ng/ml)-treated Matrigel plugs removed from C57BL/6 mice seven days after injection of control or angiostatin vectors. **A:** Control. Migrating endothelial cell. Weibel Palade bodies (WP), markers of endothelial cells, are visible. Ribosome rich endoplasmic reticulum (ER) is predominant. Nucleus is intact and chromatin appears normal. Magnification, $\times 7,000$. **B:** Control. Cross-section through a capillary lined by three endothelial cells (E). Granulocyte (G), at the 11 o'clock position. Magnification, $\times 5,000$. **C:** Ad-Angiostatin. Endothelial cell undergoing apoptosis. Cytoplasm is fragmented and contains translucent cytoplasmic vacuoles (V). Cytoplasmic blebbing is evident. Magnification, $\times 5,000$. **D:** Ad-Angiostatin. Cell nuclei show dark chromatin and are fragmented, suggesting apoptosis. Magnification, $\times 11,000$.

Table 1. Total Cellular Components in BAL of Control and Ad-Angiostatin Vector-Treated FVB/n Mice at Day 7

Treatment	Cells/ml in BAL
PBS	530,000 $\pm$ 50,000 (<i>n</i> = 3)
Ad-dl70 1×10^9 pfu	580,000 $\pm$ 18,700 (<i>n</i> = 4)
Ad-angiostatin 1×10^9 pfu	581,000 $\pm$ 67,600 (<i>n</i> = 9)

Adenoviral-mediated gene transfer of angiostatin to the lung inhibited the growth of metastatic tumors as quantified by scoring of surface metastases. Gross examination of the lungs revealed hard yellowish tumor nodules on the surface of both treatment groups. However, the animals in the control treated group had significantly more surface nodules (98 ± 37 ; *n* = 7; Figure 4A) than those mice which had received Ad-Angiostatin (18 ± 13 ; *n* = 10; *P* < 0.0005). The average weight of lungs treated with control vector alone was 0.72 ± 0.24 g, whereas the angiostatin-treated animals had a mean lung weight of 0.44 ± 0.05 g (Figure 4B). This difference was also highly significant (*P* < 0.005). BAL fluid sampled from the lungs of control treated mice contained 2.3×10^6 ($\pm 2.1 \times 10^5$) cells/ml which were mainly macrophages, compared to only 6.4×10^5 ($\pm 4.2 \times 10^4$) cells/ml in the angiostatin-treated lungs. This value closely reflects the numbers obtained from normal non-tumor laden lungs and confirms our earlier findings that over-expression of angiostatin alone does not appear to contribute to any inflammation or lung pathology. Western immunoblotting of the concentrated recovered BAL fluids demonstrated two distinct bands corresponding to murine angiostatin between 40 and 50 kd in the Ad-angiostatin-treated lungs, but not in the BAL fluid recovered from control vector-treated lungs (Figure 4C). These bands are consistent with the *in vitro* culture production of angiostatin seen in infected HUVEC or A549 cell culture supernatants.

H&E stained sections of the lungs show metastatic nodules in all treatment groups which were not limited to the lung surface. Mice which received the control vector had extensive tumor growth throughout the entire lung. In contrast, the Ad-angiostatin-treated lungs contained lesions that were small and isolated but retained most of the normal alveolar architecture of a non-diseased lung.

Discussion

De novo development of endothelial cells into organized vessels occurs only in early embryonic development, and is known as vasculogenesis. Angiogenesis, the process of continued expansion of endothelial cells from pre-existing blood vessels in the embryo, is highly regulated in the mature animal and plays a role in many physiological functions.¹⁹ Angiogenesis is necessary for tissue growth, wound healing, and the menstrual cycle of females. However, uncontrolled angiogenesis is seen in many disease processes including retinal neovascularization, rheumatoid arthritis, hemangiomas, and psoria-

Figure 4. Inhibition of lung metastasis by Ad-angiostatin. **A:** Ad-angiostatin treated group (*n* = 10) had an average of 18 (± 13) nodules per lung as compared to 98 (± 37) nodules for the Ad-dl70-treated group (*n* = 7). This decrease in the number of surface nodules was significant compared to control-treated lungs (*P* < 0.0005). Each circle represents the number of nodules counted on the entire lung surface on day 23, seven days after administration of the vectors. **B:** Lungs from FVB/n mice bearing breast tumor metastases were removed and weighed seven days after intranasal delivery of Ad-angiostatin (*n* = 10) or Ad-dl70 (*n* = 7). Lung weight as a measure of tumor burden was decreased in the Ad-angiostatin-treated animals (*P* < 0.005). **C:** Expression of angiostatin in the recovered BAL fluid of Ad-angiostatin-treated lungs. The recovered BAL fluid from Ad-dl70 and Ad-angiostatin-treated mice was concentrated and Western immunoblotted to detect murine angiostatin protein. Angiostatin was detected in only the Ad-angiostatin-treated mice and could be seen as two bands (arrows) at approximately 40 to 50 kd, representing the two glycoforms of murine angiostatin (lane 1). No angiostatin bands could be detected in the BAL fluid of Ad-dl70 control-treated lungs (lane 2).

sis.¹ The growth of new blood vessels is especially significant to the support of tumor growth and metastasis. Angiostatin is a recently described fragment of the plasma protein plasminogen and possesses powerful anti-angiogenic properties.⁵ Initially isolated from the urine of mice with concomitant Lewis lung carcinoma (LLC), this 40- to -50 kd protein retains four of the five lysine binding kringle domains of the native plasminogen. A mechanism for generating angiostatin within the tumor environment has been suggested from *in vitro* studies using cultured tumor cell lines, including PC-3 prostate, Chinese hamster ovary (CHO), and HT1080 fibrosarcoma cells.^{20,21,22} In this mechanism, plasminogen is converted to plasmin and is then reduced by an as yet unpurified plasmin reductase enzyme which reduces the disulfide bonds within kringle 5 in the presence of a cofactor believed to be glutathione.^{22,23} The reduced plasmin is then enzymatically cleaved by serine proteases, possibly involving plasmin itself, in generating bio-active angiostatin.^{20,21,23} While this is one possible mechanism, others have described the conversion of plasminogen to angiostatin by macrophage-derived metalloelastase within a LLC tumor environment²⁴ and also by matrix metalloproteinase-2 expression directly from the LLC tumors themselves.²⁵

Other reports have used angiostatin derived from enzymatic digestion, or recombinant protein from *Pichia pastoris*²⁶ and baculovirus.¹² Our study has undertaken an alternative approach through an adenoviral gene therapy vector to express the murine angiostatin cDNA and allows for the *in situ* expression of angiostatin. Recombinant angiostatin has been reported to be fragile and may lose biological activity.²⁷ In addition, recombinant protein may not be correctly glycosylated resulting in a shorter circulating half-life.⁷ The murine angiostatin cDNA was created by reverse transcription PCR on total RNA isolated from the livers of C57BL/6 mice. Oligonucleotide primers were based on the DNA sequence of murine plasminogen to generate the recombinant murine angiostatin.¹¹ We fused the DNA of the native plasminogen signal sequence, including amino acids 1–32, to the region coding for the four kringle domains at amino acids 98–458. This cDNA was subsequently cloned into the adenovirus shuttle plasmid pACCMV, under the control of the human cytomegalovirus promoter, and rescued with the rescue plasmid pJM-17 to generate Ad-angiostatin.

Angiostatin mRNA was measured in A549 cells 36 hours after infection with Ad-angiostatin. Angiostatin protein production from infected HUVEC and A549 cell culture supernatant was confirmed by Western immunoblotting, using a unique chicken cross-reacting antibody raised against rabbit plasminogen. Concentration of the infected culture supernatants with lysine-Sepharose revealed a band at approximately 50 kd in the Ad-angiostatin, but not in the Ad-dl70-concentrated culture supernatants. No bands were visible in the Ad-dl70 control supernatant lane indicating that protein bands were not an artifact of plasminogen degradation from the cell culture media. The ability of angiostatin to bind to lysine-Sepharose would suggest that the lysine binding sites of

the four kringle domains, each containing three intrakringle disulfide bonds, have formed correctly.^{7,11}

Murine angiostatin expressed from the adenovirus vector effectively inhibited the proliferation of HUVEC cells *in vitro*. This effect was specific to Ad-angiostatin demonstrating that the activity of murine angiostatin is not limited to mouse endothelial cells. HUVECs infected directly with the Ad-angiostatin vector had an overall decrease in viability of 38% as compared to the initial number of viable cells seeded before viral infection. To rule out the possibility of a direct viral-induced toxic effect on endothelial cells, radiation inactivated culture supernatants from Ad-angiostatin infected A549 cells were added to HUVEC cells to measure changes in proliferation. The addition of Ad-angiostatin-infected A549 culture supernatants to subconfluent HUVEC cells demonstrated a significant reduction in HUVEC proliferation as measured by [³H]thymidine incorporation indicating the gene product had a direct inhibitory action on endothelial cells and this inhibition was not due to a toxic effect of the virus on these cells.

We have taken a unique approach to studying the effects of Ad-angiostatin in a Matrigel model of bFGF-induced angiogenesis. Matrigel is an extracellular matrix preparation which is composed primarily of proteoglycan, laminin and collagen.¹⁵ We have injected Matrigel subcutaneously alone, or in combination with bFGF and various adenoviral constructs, including Ad-Angiostatin into C57BL/6 mice for one week to study angiogenesis *in vivo*. Histological staining of the Matrigel sections with Masson's trichrome revealed no cellular invasion into Matrigel alone. The addition of an angiogenic factor such as bFGF induced a large infiltration of endothelial cells, with the formation of functional capillaries. The inclusion of Ad-angiostatin, but not a control virus, totally eliminated capillary formation and restricted endothelial cell migration to a small region along the edge of the Matrigel. The endothelial cells in the bFGF and bFGF plus control virus-treated sections appear to have normal morphology, whereas the angiostatin-treated cells are rounded and have dark pyknotic nuclei indicative of apoptosis. Angiostatin has been shown to be a potent inducer of apoptosis in endothelial cells.²⁸ Image analysis of the Masson's trichrome-stained Matrigel sections revealed that the migrating endothelial cells occupied 22% of the Matrigel section for all treatments which included the addition of bFGF, as compared to the Matrigel alone which was not angiogenic. Such an observation may indicate that migrating endothelial cells first respond to the angiogenic stimuli of the recombinant bFGF protein irrespective of the presence of any adenoviral vectors. However, further endothelial cell migration in the Ad-angiostatin-impregnated Matrigel is limited to the interface between the Matrigel and mouse tissues as the angiostatin is synthesized in this region. The angiostatin protein requires additional time to accumulate due to the initial requirement by the cells to take up the vector and synthesize the transgene. Therefore, following the initial angiogenic stimulus by recombinant bFGF, which does not have to be synthesized on site, endothelial cells migrate into the Matrigel. However, with time, increasing concen-

trations of angiostatin limits the migration and induces the apoptosis of the migrating endothelial cells in the Matrigel. Electron microscopy of the Matrigel sections confirmed that the majority of cells within the Matrigel were endothelial cells as noted by the presence of Weibel Palade bodies and the formation of developing capillaries containing red blood cells. Moreover, only the endothelial cells had undergone apoptosis in the angiostatin treated group and no apoptosis was found in the control vector treatment.

Angiostatin-based therapy could be applied to all forms of solid tumors to inhibit the growth of both the primary lesion and any metastatic disease. Anti-angiogenesis therapy may represent a universal treatment for cancer because all solid tumors require neovascularization to grow.

The reduction of a breast cancer metastasis in the lung is an example of how an adenovirus expressing angiostatin could be used to reduce the tumor burden. In the metastatic breast cancer model described here, intranasal delivery reduced the number of tumor nodules in the lungs of treated mice, but did not appear to induce any apparent abnormal pathology to the lung tissue. However, Ad-angiostatin treatment did not totally inhibit the growth of the metastatic nodules, and therefore, angiostatin therapy may need to be combined with traditional therapies like radiation or with gene therapy induced immunotherapy to achieve optimal results.

This report has demonstrated the construction and characterization of an adenoviral vector expressing the angiogenesis inhibitor angiostatin. The biological activity of this gene therapy vector in a Matrigel model not only highlights the biological activity of angiostatin *in vivo*, but also allows for an unobstructed view of the neovascularization process. The use of such a vector has many applications in treating disease, the foremost being cancer. The specific targeting of tumors as opposed to a systemic administration of recombinant protein would allow for sustained, high concentrations of angiostatin to remain in the tumor environment for maximum efficiency in inhibiting tumor growth.

Acknowledgments

We thank Dr. Mark Hatton for providing the anti-rabbit plasminogen antibody and human urokinase-derived rabbit angiostatin protein used in this study. We also thank Dr. Mary Richardson for her assistance and interpretation of the electron microscopy. We are greatly appreciative of the technical expertise of Duncan Chong and Xueya Feng in propagating and purifying all adenoviral vectors used in this study.

References

1. Folkman J: Clinical applications of research on angiogenesis. *N Engl J Med* 1995, 333:1757-1763
2. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Cao Y,

- Moses M, Lane WS, Sage EH, Folkman J: Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. *Cold Spring Harbor Symp Quant Biol* 1994, 59:471-482
3. Dameron KM, Volpert OV, Tainsky MA, Bouck N: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994, 265:1582-1584
4. Coughlin CM, Salhany KE, Gee MS, LaTemple DC, Kutenko S, Ma X, Gri G, Wysocka M, Kim JE, Liu L, Liao F, Farber J, Pestka S, Trinchieri G, Lee WMF: Tumor cell responses to IFN- γ affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity* 1998, 9:25-34
5. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994, 79:315-328
6. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997, 88:277-285
7. Sim BK, O'Reilly MS, Liang H, Fortier AH, He W, Madsen JW, Lapcevic R, Nacy CA: A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res* 1997, 57:1329-1334
8. Guy CT, Cardiff RD, Muller WJ: Induction of mammary tumors by expression of polyomavirus Middle T oncogene: a transgenic model for metastatic disease. *Mol Cell Biol* 1992, 12:954-961
9. Hitt M, Bett A, Prevec L, Graham FL: Construction and propagation of human adenovirus vectors. *Cell Biology: A Laboratory Handbook*. Edited by JE Celis. San Diego, Academic Press, 1998, pp 500-512
10. Battista P, Soderland C: Serum-free culture of human venous and arterial endothelial cells. *Cell Biology: A Laboratory Handbook*. Edited by JE Celis. San Diego, Academic Press, 1998, pp 137-141
11. Degen SJ, Bell SM, Schaeffer L, Elliott RW: Characterization of the cDNA coding for mouse plasminogen and localization of the gene to mouse chromosome 17. *Genomics* 1990, 8:49-61
12. Wu Z, O'Reilly MS, Folkman J, Shing Y: Suppression of tumor growth with recombinant murine angiostatin. *Biochem Biophys Res Commun* 1997, 236:651-654
13. Ghosh-Choudhury G, Haj-Ahmad Y, Brinkley P, Rudy J, Graham FL: Human adenovirus cloning vectors based on infectious bacterial plasmids. *Gene* 1986, 50:161-171
14. Bramson J, Hitt M, Gallichan WS, Rosenthal KL, Gaudie J, Graham FL: Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: *in vitro* and *in vivo* production of biologically active interleukin-12. *Hum Gene Ther* 1996, 7:333-342
15. Baatout S: Endothelial differentiation using Matrigel. *Anticancer Res* 1997, 17:451-456
16. Jain RK, Schlenger K, Hockel M, Yuan F: Quantitative angiogenesis assays: progress and problems. *Nat Med* 1997, 3:1203-1208
17. Wyllie AH, Kerr JF, Currie AR: Cell death: the significance of apoptosis. *Int Rev Cytol* 1980, 68:251-305
18. Gerritsen ME: Physiological functions of normal endothelial cells. *Vascular Medicine*. Edited by J Loscalzo, MA Creager, VJ Drar. New York, Little Brown, 1996, pp 3-38
19. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM: Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 1998, 91:3527-3561
20. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, Castellino FJ, Enghild J, Kwaan HC, Lee F, Kramer RA, Volpert O, Bouck N, Soff GA: The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Proc Natl Acad Sci USA* 1997, 94:10868-10872
21. Gately S, Twardowski P, Stack MS, Patrick M, Boggio L, Cundiff DL, Schnaper HW, Madison L, Volpert O, Bouck N, Enghild J, Kwaan HC, Soff GA: Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. *Cancer Res* 1996, 56:4887-4890
22. Stathakis P, Fitzgerald M, Matthias LJ, Chesterman CN, Hogg PJ: Generation of angiostatin by reduction and proteolysis of plasmin. *J Biol Chem* 1997, 272:20641-20645
23. Stathakis P, Lay AJ, Fitzgerald M, Schlieker C, Matthias LJ, Hogg PJ:

- Angiostatin formation involves disulfide bond reduction and proteolysis in kringle 5 of plasmin. *J Biol Chem* 1999, 274:8910–8916
24. Dong Z, Kumar R, Yang X, Fidler IJ: Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 1997, 88:801–810
25. O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, Folkman J, Moses MA: Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. *J Biol Chem* 1999, 274:29568–29571
26. Lucas L, Holmgren L, Jimenez GB, Mandriota SJ, Borlat F, Sim BKL, Wu Z, Grau Y, Shing Y, Soff GA, Bouck N, Pepper MS: Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* 1998, 92:4730–4741
27. Barinaga M: Cancer research: a surprising partner for angiostatin. *Science* 1999, 283:1831
28. Claesson-Welsh L, Welsh M, Ito N, Anand-Apte B, Soker S, Zetter B, O'Reilly M, Folkman J: Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc Natl Acad Sci USA* 1998, 95:5579–5583