

# Adeno-Associated Virus 2-Mediated Antiangiogenic Cancer Gene Therapy: Long-Term Efficacy of a Vector Encoding Angiostatin and Endostatin over Vectors Encoding a Single Factor

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## ABSTRACT

Angiogenesis is characteristic of solid tumor growth and a surrogate marker for metastasis in many human cancers. Inhibition of tumor angiogenesis using antiangiogenic drugs and gene transfer approaches has suggested the potential of this form of therapy in controlling tumor growth. However, for long-term tumor-free survival by antiangiogenic therapy, the factors controlling tumor neovasculature need to be systemically maintained at stable therapeutic levels. Here we show sustained expression of the antiangiogenic factors angiostatin and endostatin as secretory proteins by recombinant adeno-associated virus 2 (rAAV)-mediated gene transfer. Both vectors provided significant protective efficacy in a mouse tumor xenograft model. Stable transgene persistence and systemic levels of both angiostatin and endostatin were confirmed by *in situ* hybridization of the vector-injected tissues and by serum ELISA measurements, respectively. Whereas treatment with rAAV containing either endostatin or angiostatin alone resulted in moderate to significant protection, the combination of endostatin and angiostatin gene transfer from a single vector resulted in a complete protection. These data suggest that AAV-mediated long-term expression of both endostatin and angiostatin may have clinical utility against recurrence of cancers after primary therapies and may represent rational adjuvant therapies in combination with radiation or chemotherapy.

## INTRODUCTION

Increasing evidence demonstrates the importance of angiogenesis in solid tumor growth and metastasis (1–4). In the absence of neovasculature, tumors do not grow beyond a few millimeters and remain dormant (5, 6). Thus, novel antiangiogenic treatment strategies that can effectively control tumor growth are under intense investigation. Although many antiangiogenic factors have been implicated in the regulation of tumor growth and metastasis, the most potent have been angiostatin, endostatin, thrombospondin-1, tissue inhibitor of metalloproteases, and soluble vascular endothelial growth factor (VEGF) receptors (7–10).

Preclinical studies using purified antiangiogenic factors indicated therapeutic effects of antiangiogenic compounds in minimizing the size of established tumors (11–15). However, clinical trials with some of these factors have not demonstrated expected antitumor effects (16–19). Administration of purified antiangiogenic factors, although capable of producing significant growth inhibition of tumor cells in animal models, may be limited by their short half-life. Hence, production of antiangiogenic factors after gene transfer may overcome these limitations.

The potential of antiangiogenic gene therapy in cancer is currently being evaluated using viral and nonviral vectors (20–23). In contrast to genetic therapies targeting tumor cells directly with genes encoding prodrug-converting enzymes or cytokines/chemokines for oncolysis, which requires high-efficiency transduction of recombinant vectors to cancer cells directly, antiangiogenic gene therapy requires vectors capable of sustained, long-term expression without vector-associated toxicity or immunity. Additionally, systemic levels of antiangiogenic factors by gene transfer may be accomplished by targeting nontumor cells, using normal tissues to provide a stable platform for transgene expression as secretory proteins. Adeno-associated virus (AAV)-based vectors are nonpathogenic and less immunogenic compared with other gene therapy vectors. The AAV genome persists stably in transduced cells and affects long-term transgene expression. Thus, AAV meets the requirements for gene transfer vectors that may be used for antiangiogenic therapy.

The present study evaluated recombinant AAV (rAAV) encoding secretable forms of human angiostatin and endostatin. The results demonstrate a strong antiproliferative effect of rAAV-mediated angiostatin or endostatin gene transfer on primary human umbilical vein endothelial cells (HUVEC) *in vitro* and significant protective effect against the growth of a human angiogenesis-dependent tumor xenograft *in vivo*. Furthermore, the combination of both angiostatin and endostatin long-term gene therapy from a single vector resulted in a synergistic effect over therapy with vectors encoding a single factor alone.

## MATERIALS AND METHODS

**Cells and Reagents.** Human embryonic kidney cell line 293 was purchased from American Type Culture Collection and maintained in Iscove's modified essential medium supplemented with 10% newborn calf serum. Human ovarian cancer cell line SKOV3.ip1 was a kind gift of Dr. David Curiel (The University of Alabama at Birmingham, Birmingham, AL) and maintained as described previously (24). Primary HUVEC were a gift of Dr. Raj Singh (The University of Alabama at Birmingham, Birmingham, AL). Restriction endonucleases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corp. (Madison, WI). Mouse monoclonal (clone 79735) and goat polyclonal antibodies to human angiostatin were obtained from R&D Systems (Minneapolis, MN), and a mouse monoclonal antibody to human endostatin (clone EN2.1.99) was obtained from Leinco Technologies (St. Louis, MO). Secondary antibodies and colorimetric substrates were purchased from Amersham (Piscataway, NJ). Purified recombinant human angiostatin was purchased from R&D Systems.

**Construction of Recombinant Plasmids, Production, and Purification of rAAV.** All rAAV plasmids were constructed using pSub201 as the back bone (25). cDNA containing human angiostatin and endostatin sequences were isolated from a plasmid pBlast human Endo::Angio (Invivogen, San Diego, CA). For construction of the rAAV plasmid encoding endostatin, a region containing the human interleukin 2 secretory signal sequence was genetically fused to the endostatin coding region, amplified from the plasmid pBlast human Endo::Angio by PCR, and subcloned into an AAV plasmid containing cytomegalovirus (CMV) promoter, sequences of internal ribosome entry site (IRES), and a green fluorescent protein (GFP) gene followed by a synthetic polyadenylation signal sequence (polyA). Construction of rAAV encoding

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human angiostatin was done in two steps. Initially, the coding region of human angiostatin was amplified by PCR from the plasmid pBlast human Endo::Angio and subcloned in pBluescript vector (Stratagene, La Jolla, CA) with a double-stranded oligomer (5'-TCGAGATGGAACATAAGGAAGTGTTCTTCTACTTCTTTTATTCTGAAATCAGGTCAAG-3 and 5'-GATCCTTGACCTGATTTCAGAAATAAAGAAGTAGAAGAACCACTTCCTTATGTTCCATC-3') containing the plasminogen secretory signal sequence, in a three-way ligation. Subsequently, the region containing the secretory sequence and angiostatin gene was excised and subcloned downstream of CMV promoter in a rAAV plasmid as described for rAAV-endostatin construct.

A bicistronic rAAV plasmid containing both endostatin and angiostatin was constructed in three steps. First, the IRES and endostatin sequences were amplified by PCR and subcloned in pBluescript vector (Stratagene). Then, a region containing plasminogen secretory signal and angiostatin was isolated from the plasmid described above and subcloned upstream of IRES-endostatin cassette in pBluescript vector. Later, the portion containing plasminogen secretory sequence, angiostatin, IRES, and endostatin was isolated and subcloned in a rAAV vector containing CMV promoter and a synthetic polyA.

Packaging of all of the recombinant AAV plasmids was done in an adenovirus-free system as described previously (26). Purification of virions was done by discontinuous iodixanol gradient centrifugation followed by affinity purification on a heparin-agarose column (26). Particle titers of the purified virions were determined by quantitative slot blot analysis as described previously (27–29).

**Western Blot Analysis.** Western blot analysis was performed using conditioned media obtained from rAAV-transduced 293 cells. Briefly, conditioned media obtained after mock-transduction, transduction of AAV-endostatin, AAV-angiostatin, or AAV-endostatin plus AAV-angiostatin vectors was concentrated 5-fold, and 20  $\mu$ l from each was electrophoretically separated on 10% SDS polyacrylamide gels (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride membranes and immunodetection performed using mouse monoclonal antibodies to either human angiostatin (clone 79735) or endostatin (clone EN2.1.99) as primary antibody and goat antimouse secondary antibody conjugated to horseradish peroxidase as secondary antibody. Detection of bands was by enhanced chemiluminescent substrate as previously described (30).

**Endothelial Cell Proliferation Assay.** Early passage HUVECs were seeded into gelatin-coated 96-well tissue culture plates at a density of  $5 \times 10^3$  cells/well and grown in EGM-2 medium containing hydrocortisone, human fibroblast growth factor  $\beta$ , VEGF, ascorbic acid, heparin, human epidermal growth factor, and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). Twenty-four h later, 100  $\mu$ l of fresh medium containing 1, 10, or 25  $\mu$ l of conditioned medium from 293 cells transduced with 100 multiplicity of infection of rAAV encoding various trasgenes were added. As a positive control, purified recombinant human angiostatin was added at concentrations of 1, 10, or 25 ng/ml. Each condition was performed in triplicate. Seventy-two h later, cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay with a commercial kit (Promega), following the manufacturer's instructions. Proliferation index was expressed in percentage compared with HUVEC grown in the presence of complete medium without addition of any conditioned medium or recombinant angiostatin.

**In Vivo Studies.** Six-week old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD). Maintenance of the animals was done following the guidelines of the Institutional Animal Care and Use Committee, and all experimental procedures were approved by the Institutional Animal Care and Use Committee and the Occupational Health and Safety Department of the University of Alabama at Birmingham. Particles ( $3 \times 10^{11}$ ) of rAAV encoding GFP, endostatin, angiostatin, or endostatin plus angiostatin, in normal saline, were injected in a volume of 100  $\mu$ l into the quadriceps muscle of the hind limbs. Naïve animals did not receive any vector. Three weeks after vector administration, each mouse received implantation s.c. in two sites on bilateral flanks with  $10^7$  SKOV3.ip1 cells. Tumor size was measured at least twice every week with a digital caliper for two-dimensional longest axis ( $L$  in mm) and shortest axis ( $W$  in mm), and tumor volume calculated using the following formula: volume in  $\text{mm}^3 = (L \times W^2)/2$ . When tumor growth exceeded 1800  $\text{mm}^3$ , animals were humanely euthanized. Surviving mice were sacrificed 130 days after tumor cell implantation and experiments were terminated. Blood samples

were collected from all animals before vector administration, before tumor cell implantation, and at sacrifice, for ELISA measurements of serum angiostatin and endostatin levels. Regions of the quadriceps muscle of sham or vector injection were also isolated at the time of sacrifice for immunohistochemistry and *in situ* hybridization.

**ELISA for Serum Angiostatin and Endostatin Levels.** For the measurement of serum angiostatin, a sandwich ELISA was developed. Ninety-six-well ELISA plates were coated overnight at 4°C with a mouse antihuman angiostatin monoclonal antibody (clone 79735) at a concentration of 10  $\mu$ g/ml in borate saline buffer (BS; pH 8.6). Next day, the antibody was discarded and wells blocked with 150  $\mu$ l of BSA in BS (BS-BSA) for 45 min at room temperature. Serum samples, diluted 1:3 in BS-BSA were added to the wells and incubated overnight at 4°C. All of the samples were analyzed in triplicate. After washing five times with PBS containing 0.5% Tween 20, a polyclonal antihuman angiostatin antibody, biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin reagent (Pierce), was added at a concentration of 1  $\mu$ g/ml in BS-BSA and incubated at room temperature for 5 h. The contents were then discarded and plates washed five times with PBS containing 0.5% Tween 20 after which streptavidin-conjugated alkaline phosphatase was added and incubated for 30 min at room temperature. Color development was done with the addition of pNPP chromogenic substrate (Sigma) and incubated at room temperature for 20 min. Absorbance at 405 nm was measured in an ELISA plate reader. As a reference standard, known concentrations of human recombinant angiostatin from 0 to 1000 ng/ml were included in triplicate. Serum endostatin levels were determined using a commercial ELISA kit (Cytimmune Sciences Inc, College Park, MD) following the manufacturer's protocol.

**In Situ Hybridization.** A digoxigenin (DIG)-labeled DNA probe containing sequence of CMV promoter was generated by PCR using PCR-DIG labeling mix<sup>Plus</sup> (Roche Molecular Diagnostics, Indianapolis, IN) following manufacturer's protocol. Formalin-fixed tissues were sectioned at 5- $\mu$ m thickness, deparaffinized in xylene, and rehydrated through a series of graded-ethanol and PBS. Slides were then treated with 0.01 M citrate buffer (pH 6.0) at 42°C for 3 h. Prehybridization was performed at 65°C for 2 h in hybridization solution (ULTRAhyb, Ambion, TX). The hybridization reaction was carried out with approximately 400 ng/ml of DIG-labeled DNA probe at 65°C overnight. After thoroughly washing the excess probe, immunohistochemical detection of hybridization signals was performed using the DIG nucleic acid detection kit (Roche Molecular Diagnostics). Counterstaining of sections was done with diluted eosin solution for 1–2 min and slides mounted in Crystal/Mount (Biomedex, Forest City, CA).

**Immunohistochemistry.** Quadriceps muscles of mice were harvested and fixed immediately in alcoholic-formalin (PenFix; Richard-Allan, Kalamazoo, MI) for 18 h at room temperature. Tissues were dehydrated in graded alcohol and embedded in paraffin. Five- $\mu$ m sections on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling for 10 min in 0.01 M citrate buffer (pH 6.0) in a microwave oven. All sections were pretreated with a 3% aqueous solution of  $\text{H}_2\text{O}_2$  for 5 min to quench endogenous peroxidase. Sections were then treated with 3% goat serum for 1 h at room temperature to reduce nonspecific staining followed by 1-h incubation with monoclonal antibodies to either endostatin (clone EN2.1.99, 10  $\mu$ g/ml) or angiostatin (clone 79735, 5  $\mu$ g/ml). The remainder of staining procedure was performed using a Universal Mouse Kit (Biogenex, Sam Ramon, CA), which contained biotinylated goat antibody to mouse immunoglobulin and a horseradish peroxidase-streptavidin complex. Diaminobenzidine tetrahydrochloride was used as a substrate for the visualization of antigen-antibody complex. Slides were minimally counterstained with hematoxylin.

**Statistical Analyses.** Nonparametric Kuskall Wallis tests or Wilcoxon's rank-sum test were used to test for differences in cell proliferation between the groups, depending on the number of groups included in the test. The association for the presence or absence of tumor with each treatment condition was tested for statistical significance using  $\chi^2$  test. Next, the distribution of tumor volumes was examined and log transformed to fit a normal distribution. A general linear mixed model was fit to the data. The dependent variable was the logged tumor volume and the predictors were treatment, day after treatment, and an interaction between treatment and day. All three were statistically significant predictors.  $P < 0.05$  was considered to indicate significant difference between data sets.

## RESULTS

**Generation of High-Titer rAAV Encoding Human Angiostatin and/or Endostatin.** On the basis of the long-term expression capabilities of rAAV in transduced muscle tissue without deterioration of transgene-positive cells by host immune cells, we chose muscle as a secretory organ for angiostatin and endostatin transgene expression. We included signal peptide sequences upstream of both angiostatin and endostatin genes. The human interleukin 2 secretory signal was included upstream of the endostatin gene, and the human plasminogen signal sequence was included upstream of the angiostatin gene (Fig. 1A). Because previous studies using purified recombinant angiostatin and endostatin indicated that a combination of both the factors resulted in significant therapeutic benefit, we also produced a vector capable of bicistronic expression of angiostatin and endostatin using an IRES sequence (Fig. 1A), to determine whether long-term expression of these two antiangiogenic factors from the same vector would provide greater advantage over vectors encoding a single factor. The rAAV were packaged in a helpervirus-free system and purified using discontinuous gradient centrifugation and affinity chromatography. The titer of the vectors ranged between  $1-5 \times 10^{12}$  particles/ml.

**Expression of Angiostatin and Endostatin as Secretory Proteins.** The purified rAAV were tested in 293 cells for expression of antiangiogenic factors as secretory proteins in the medium. Cells were either mock-transduced or transduced with rAAV encoding endostatin alone, angiostatin alone, or the bicistronic vector containing both angiostatin and endostatin. After transduction, the cells were cultured for 48 h. The conditioned medium was harvested, concentrated, and subjected to SDS-PAGE and Western blot analysis using monoclonal antibody specific for either human angiostatin or endostatin. Results, shown in Fig. 1, B and C, documented expression of both angiostatin and endostatin as secretory proteins after rAAV-mediated gene transfer *in vitro*. The endostatin and angiostatin antibodies recognizing 20

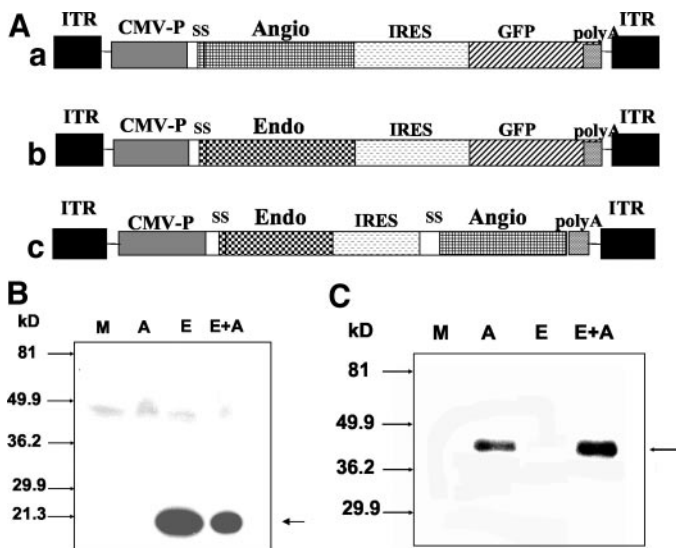


Fig. 1. Recombinant adeno-associated virus 2 (rAAV) encoding endostatin and/or angiostatin and Western blot analysis for transgene expression. A, rAAV containing human angiostatin (*Angio*) or endostatin (*Endo*) gene either in different vectors (a & b), or as bicistronically expressed proteins from a single vector (c) were subcloned under the control of the cytomegalovirus promoter (CMV-P). Secretory signal sequences (SS) of either interleukin 2 or human plasminogen gene were included upstream of the angiostatin and endostatin genes respectively for systemic secretion. ITR represents the inverted terminal repeat sequences of AAV. B and C, 293 cells were either mock-transduced (M) or transduced with rAAV encoding angiostatin (A), endostatin (E) or a bicistronic cassette encoding both endostatin and angiostatin (E+A). Forty-eight h after transduction, the culture supernatant was analyzed by Western blots using antibodies specific for human endostatin (B) or angiostatin (C). *polyA*, polyadenylation signal sequence.

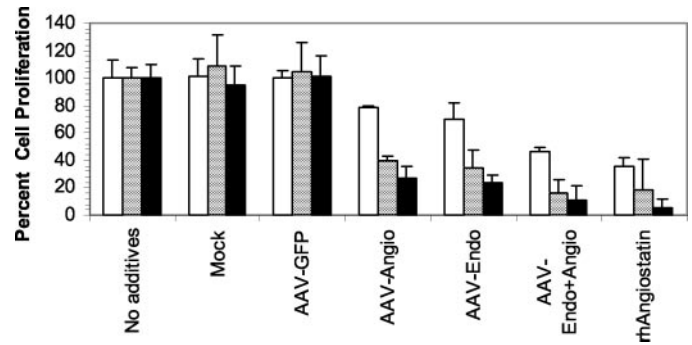


Fig. 2. Endothelial cell proliferation assay to determine the biological activity of endostatin (Endo) and angiostatin (Angio) produced after recombinant adeno-associated virus 2 (rAAV) transduction. rAAV encoding green fluorescent protein (GFP), Endo, Angio, or Endo + Angio was transduced into 293 cells at a multiplicity of 100. Forty-eight h later, 1 ( $\square$ ), 10 ( $\equiv$ ), or 25 ( $\blacksquare$ )  $\mu$ l of supernatant from the infected cells was tested on human umbilical vein endothelial cell (HUVEC) cultures. Cell proliferation was determined 72 h later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay and results expressed as percentage of control HUVEC with no treatment.

and 38 kDa proteins, respectively, also indicated the specificity of detection. Of interest, the detected amount of both factors was comparable when transduced as a single transgene or a bicistronic cassette. As expected, no signal was seen in conditioned medium from mock-transduced 293 cells.

**Characterization of Biological Activity of rAAV-Produced Angiostatin and Endostatin.** We next determined the biological activity of rAAV-expressed angiostatin and endostatin. Because our strategy for AAV-mediated gene therapy was to express the antiangiogenic factors as secreted proteins, the *in vitro* evaluation of biological activity was performed similar to *in vivo* strategy. rAAV encoding antiangiogenic factors were transduced into 293 cells, and the transgene products were obtained as secreted protein in the supernatant. Different concentrations of the supernatants were then added to early passage HUVEC cultures grown in the presence of 10 ng/ml VEGF. Differences in cell proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay. Results (Fig. 2) demonstrated a significant inhibition of HUVEC proliferation by conditioned media from both AAV-endostatin and AAV-angiostatin-transduced 293 cells as compared with medium from mock-transduced cells ( $P < 0.002$ ). The results also showed a dose-dependent growth-inhibitory effect with increasing amounts of conditioned medium. Interestingly, there was no statistically significant augmentation in the inhibitory effects on HUVEC proliferation in conditioned medium from cells transduced with angiostatin-endostatin bicistronic construct as compared with constructs expressing only a single factor ( $P > 0.05$ ). There was no inhibitory effect on HUVEC proliferation when conditioned medium obtained from rAAV-GFP-transduced cells was used, demonstrating specificity of the rAAV-expressed antiangiogenic proteins. Purified recombinant human angiostatin was used as a positive control. Although we did not quantitatively determine the amount of angiostatin or endostatin in the conditioned media in these experiments, results indicated inhibitory effects comparable with that of 25 ng/ml purified angiostatin protein when 10–25  $\mu$ l of conditioned medium was tested (Fig. 2).

***In Vivo* Vector Administration and Development of Xenograft Tumor Model.** As a model to evaluate the *in vivo* efficacy of AAV-mediated long-term expression of angiostatin and endostatin, we used athymic (nude) mouse s.c. xenografts of an angiogenesis-dependent human ovarian cancer cell line, SKOV3.ip1. Pilot studies indicated palpable SKOV3.ip1 tumors in nude mice 8–10 days after s.c. injection of  $10^7$  SKOV3.ip1 cells (data not shown). Because the present studies were primarily designed to evaluate the potential of AAV-

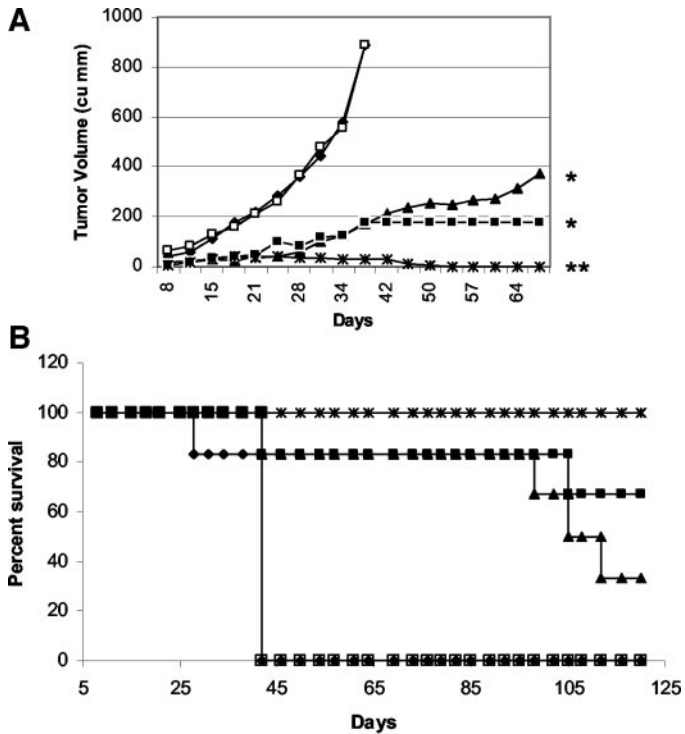


Fig. 3. Growth of SKOV3.ip1 tumor xenografts in athymic nude mice after injection of recombinant adeno-associated virus 2 (rAAV) encoding endostatin and angiostatin. Mice received i.m. injection with saline (◆), or with  $3 \times 10^{11}$  particles of AAV-GFP (□), AAV-endostatin (▲), AAV-angiostatin (■) or AAV-endostatin + angiostatin (\*), and 3 weeks later challenged by bilateral s.c. injection of  $10^7$  SKOV3.ip1 cells (six mice/group). A, average tumor volume in each group. The \* and \*\* denote  $P < 0.001$  and  $P < 0.0001$ , respectively, by comparison to naïve and AAV-GFP control groups. B, tumor-free survival. GFP, green fluorescent protein

mediated antiangiogenic gene therapy as a preventive therapy against tumor recurrence, animals first received injection with rAAV encoding angiostatin or endostatin. Because optimal expression of rAAV transgenes after i.m. injection is reached at about 3 weeks after administration, tumor challenge was done 3 weeks after vector. Each animal received two tumor implants on bilateral flanks. All naïve mice and control mice receiving injection with rAAV-GFP developed palpable tumors by day 8 after tumor cell implantation. Animals that developed tumors were monitored until tumor volume reached  $1800 \text{ mm}^3$ , and then animals were euthanized. Tumor-free animals were monitored up to 130 days before terminating the experiment.

**Inhibition of Tumor Growth after Injection of rAAV Expressing Endostatin, Angiostatin, or Endostatin Plus Angiostatin.** The results of the *in vivo* studies are shown in Fig. 3. Growth kinetics of SKOV3.ip1 tumors are shown in Fig. 3A, and tumor-free survival of mice is presented in Fig. 3B. There was a significant protective effect of AAV-mediated antiangiogenic gene expression with both endostatin and angiostatin compared with control or AAV-GFP-treated animals, as assessed by either tumor-free survival or tumor-growth kinetics. However, when both the factors were expressed from the bicistronic vector, the effect was more pronounced than therapy with vector expressing only a single factor ( $P < 0.0001$ ). Protection by AAV-endostatin alone was significantly less than by AAV-angiostatin alone (Fig. 3B). Interestingly, serum levels of endostatin or angiostatin were comparable in all mice within the same group irrespective of observed tumor growth. Mean tumor volumes were significantly less in animals that received angiostatin or endostatin gene therapy compared with either naïve animals or those given rAAV-GFP ( $P < 0.001$ ). Of note, in mice that received vector encoding both angiostatin and endostatin, only one animal developed a palpable

tumor, which regressed after day 30 (data not shown), after which all mice in that treatment group remained tumor free.

**Serum ELISA for Circulating Angiostatin and Endostatin Levels.** To determine the circulating levels of serum angiostatin, we developed a sandwich ELISA as described in the “Materials and Methods.” Using purified recombinant human angiostatin, the sensitivity of the ELISA was demonstrated to be  $12 \text{ ng/ml}$ . Serum samples were obtained from all animals (a) before tumor cell implantation and (b) before sacrifice of the animals because of either tumor burden or termination of the experiment. Results indicated that serum angiostatin levels remained stable up to 130 days after AAV-angiostatin injection, and levels were comparable with those in mice that received injection with the bicistronic AAV construct expressing both angiostatin and endostatin (Fig. 4A). Serum endostatin levels were determined using a commercial kit and demonstrated a pattern of endostatin expression that was similar to that for angiostatin (Fig. 4B). The serum angiostatin and endostatin levels appeared to have stabilized before the tumor cell implantation on day 21 after vector administration.

**Long-Term Retention and Expression of rAAV-Endostatin/Angiostatin.** An advantage of rAAV vectors in muscle-based gene therapy is the sustained presence of transgene(s) as either integrated or episomal copies for long-term expression. Because antiangiogenic gene therapy is directed toward inhibiting proliferation of tumor neovasculature and not tumor cells directly, a critical requirement for antiangiogenic therapy is stable expression of these factors at therapeutic levels. Studies have reported that administration of rAAV into muscle not only results in long-term expression of the transgene but also that vector administered this way does not elicit host immune response against the transgenic protein, which would otherwise eliminate transgene expressing cells (31, 32). Thus, in the present study,

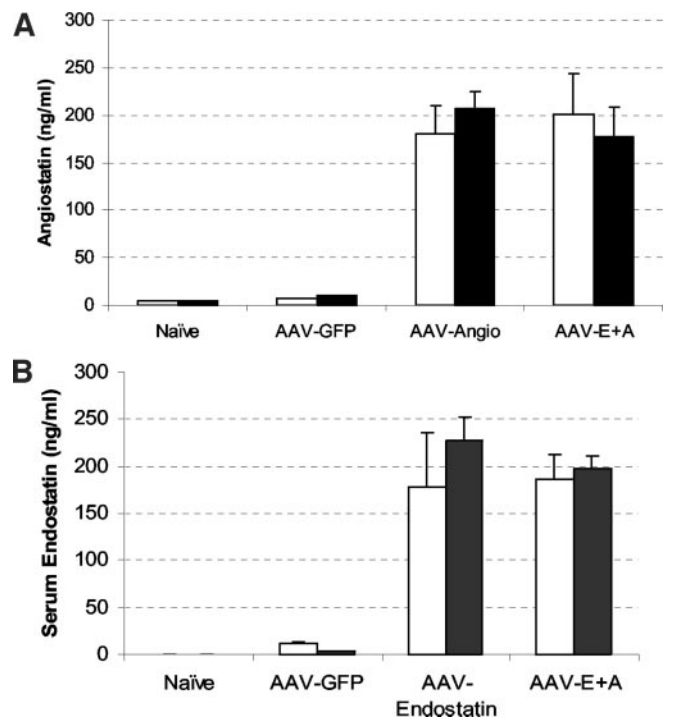
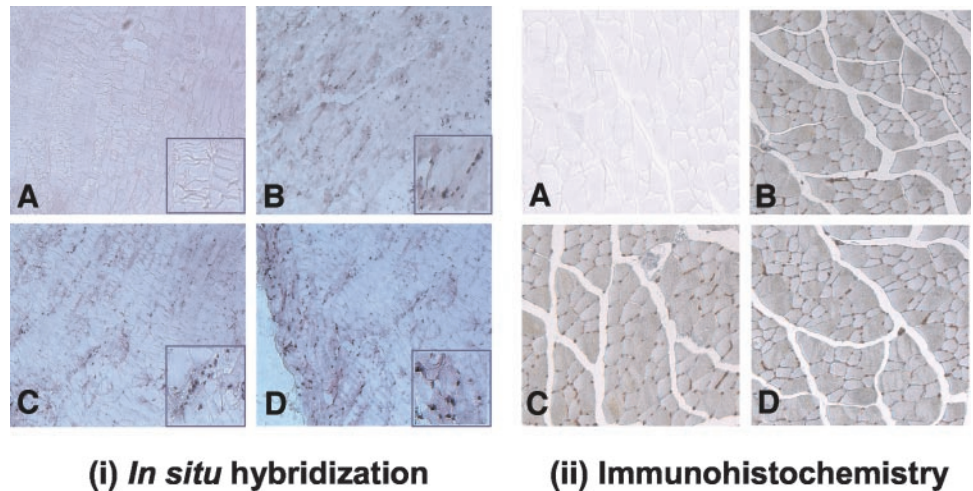


Fig. 4. Levels of human endostatin and angiostatin in mouse serum after injection of recombinant adeno-associated virus 2 (rAAV) encoding green fluorescent protein (GFP), endostatin (E), or angiostatin (Angio or A). Serum samples were obtained before tumor challenge (□) and at the time of sacrifice of mice because of either tumor burden or termination of the experiment (■). A, a sandwich ELISA was developed for measuring human angiostatin. B, ELISA for human endostatin used a commercial kit. Data show mean  $\pm$  SD of calculated concentrations of each factor (mean of triplicates).

Fig. 5. *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Five- $\mu$ m sections of injected muscles were used for *in situ* hybridization (left panels) and immunohistochemistry (right panels). A, uninjected. B, AAV-endostatin. C, AAV-angiostatin. D, AAV-endostatin + angiostatin bicistronic. For *in situ* hybridization, inserts show a larger magnification of representative areas. For immunohistochemistry, A, B, and D were stained with antibody specific for human endostatin, and C was stained with antibody specific for human angiostatin.



we used skeletal muscle as a target tissue for stable expression of rAAV and systemic secretion of endostatin and angiostatin using a signal peptide. To demonstrate persistence and expression of AAV transgene, we performed *in situ* hybridization and immunohistochemistry, respectively. Because the kringle 1–4 region of plasminogen (representing angiostatin) and the internal collagen XVIII domain (representing endostatin) show significant homology between human and mouse, we used a DIG-labeled probe to the transgene CMV promoter region for *in situ* hybridization analysis. Also, because all of the vector constructs used in the study contained the CMV promoter, it was possible to use the same probe for detection of vector genome in all treatment groups. Results (Fig. 5, left) demonstrate the persistence of AAV transgene in skeletal muscle of mice after 130 days. Although the *in situ* data cannot demonstrate stable integration of the vector into cell genomes, based on previous preclinical and clinical studies, it is clear that rAAV administered in skeletal muscle establishes long-term presence (33).

Immunohistochemistry was performed in paraffin sections of the transduced muscle to determine the expression of endostatin/angiostatin. Representative results (Fig. 5, right) indicate the presence of each antiangiogenic factor in the AAV-injected muscle tissue. On the basis of the immunohistochemistry results, it is most likely that the cells showing positive staining for angiostatin or endostatin are only those in which *in situ* hybridization identified the vector genome.

## DISCUSSION

Antiangiogenic therapy is a promising approach for the control of solid tumor growth and metastasis. Although several drugs have shown promise in controlling tumor neovasculature, a major problem in pharmacotherapy is side effects of constant drug administration and the limited half-life of antiangiogenic proteins (34). Gene therapy, on the other hand, offers advantages of maximizing cost effectiveness and maintaining sustained levels of antiangiogenic factors, which may enhance antitumor efficacy.

Although many factors are known to play important roles in new blood vessel formation, two major factors that play a key role are VEGF and fibroblast growth factor. Previous reports have presented contradictory results on the efficacy of antiangiogenic factors in controlling tumor growth in preclinical studies and clinical trials (19, 22, 35, 36). In the present study, we evaluated the potential of human angiostatin and endostatin using rAAV for stable transfer of genes encoding these factors. The major advantages of AAV vectors are

nonpathogenicity, less immunogenicity, and long-term stable expression of the antiangiogenic factors.

Because the initial discovery that biologically driven antiangiogenic agents are much safer and effective, studies have focused on testing their potential in preclinical and clinical applications (7–23). However, the possible mechanisms of action of these factors have only recently begun to be uncovered. Whereas angiostatin, a proteolytic fragment of plasminogen, acts by binding to  $\alpha\beta$ 3 integrin (37, 38), endostatin, an internal fragment of collagen XVIII, is believed to act by binding to tropomyosin, integrins, and matrix metalloproteases (39–41). Thus, it is clear that these two factors act on distinct pathways and targets. Hence, a treatment using these two factors should have an additive or even synergistic effect compared with therapy using only one factor. Results of our *in vivo* studies clearly demonstrate this. An apparently synergistic tumor protective effect was observed in mice that received the bicistronic vector encoding both angiostatin and endostatin as compared with mice that received vectors encoding only one of the factors.

The effect of a combination treatment of endostatin and angiostatin over that using a single factor therapy was reported previously using purified protein therapy (14). Interestingly, results of previous studies have suggested differences in the efficacy of protein as compared with gene therapy approaches. Whereas administration of 20 mg/kg endostatin as denatured purified protein was effective in controlling the growth of an angiogenesis-dependent Lewis lung carcinoma (11), gene therapy approaches using adenoviral or retroviral vectors produced only a modest therapeutic effect (42, 43). The reasons for this could be different pharmacokinetics and tissue distribution of the denatured purified protein compared with the *in vivo*-expressed factor or the nature of vectors used.

Despite encouraging results from preclinical studies using protein therapy, a major concern for clinical applications is the limited half-life of the purified proteins. Furthermore, the half-lives of endostatin and angiostatin are different, which may complicate drug scheduling. Stable gene therapy approaches such as described here can overcome these limitations by maintaining stable systemic levels of both angiostatin and endostatin. Although rAAV vectors have been used in many preclinical and a few human clinical studies, a majority of these applications have been in the context of genetic metabolic defects to provide long-term expression of defective enzymes/factors (33, 44). However, a few studies with antiangiogenic factors have provided promising preclinical data, indicating the potential of long-term gene therapy targeting the inhibition of tumor neovasculature (45, 46). Our

data provide evidence that synergy between angiostatin and endostatin delivered by AAV-dependent gene transfer will be clinically relevant to control recurrence and metastasis of primary cancers.

Almost all early preclinical studies of antiangiogenic tumor gene therapy have been performed in immunodeficient mice and, hence, cannot predict the role of host immunity on long-term transgene expression. A potential advantage of rAAV is the proven long-term *in vivo* expression of AAV-encoded transgenes administered in skeletal muscle of immunocompetent individuals. Because rAAV does not encode any viral proteins, host immune response against the vector is minimal (31, 32). In addition, potent antigen presenting cells, especially dendritic cells, are not transduced efficiently by rAAV. Undiminished expression of AAV-transgene as a secreted protein in muscle has been recorded for over 4 years in immunocompetent animals (47).

Persistence and stable expression of AAV-encoded antiangiogenic factors is evident from our results of ELISA for serum levels and *in situ* DNA hybridization studies of injected muscle tissue. Recent studies using intratumoral administration of rAAV encoding endostatin in a mouse glioma model and i.m. administration of rAAV-endostatin in a colorectal cancer model have also shown therapeutic efficacy (45, 46). A limitation in the intratumoral administration of rAAV is the poor transduction efficiency in primary tumors as well as the lack of accessible tumor sites for treatment in many patients. Thus, administration of the vector in skeletal muscle may represent a preferred approach, especially for treatments in the setting of minimal residual disease.

On the other hand, if constant systemic levels of antiangiogenic factors become toxic to the experimental animals or patients, the approach presented in this study may not prove superior over localized production of the factors within tumors. In such situations, development of targeted AAV with increased infectivity to tumor cells may be highly beneficial. Accumulation of antiangiogenic factors in other organs because of unregulated expression may also lead to ischemic conditions or impair wound healing. Thus, future studies will be necessary to test the safety of long-term expression of angiostatin and endostatin, and the development of vectors allowing regulated expression of transgenes by inducible promoters, for example, may be warranted for full development of this genetic therapy (48, 49).

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## REFERENCES

- Sharma, R. A., Harris, A. L., Dalglish, A. G., Steward, W. P., and O'Byrne, K. J. Angiogenesis as a biomarker and target in cancer chemoprevention. *Lancet Oncol.*, 2: 726–732, 2001.
- Teo, N. B., Shoker, B. S., Martin, L., Sloane, J. P., and Holcombe, C. Angiogenesis in pre-invasive cancers. *Anticancer Res.*, 22: 2061–2072, 2002.
- Kieran, M. W., and Billett, A. Antiangiogenesis therapy. Current and future agents. *Hematol. Oncol. Clin. N. Am.*, 15: 835–851, 2001.
- Carmeliet, P., and Jain, R. K. Angiogenesis in cancer and other diseases. *Nature (Lond.)*, 407: 249–257, 2000.
- Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst. (Bethesda)*, 82: 4–6, 1990.
- Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.*, 29: 15–18, 2002.
- O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by Lewis lung carcinoma. *Cell*, 79: 315–328, 1994.
- O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, 88: 277–285, 1997.
- Moses, M. A., and Langer, R. A. J. A metalloproteinase inhibitor as an inhibitor of neovascularization. *J. Cell. Biochem.*, 47: 230–235, 1991.
- Rastinejad, F., Polverini, P. J., and Bouck, N. P. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell*, 56: 345–355, 1989.
- Boehm, T., Folkman, J., Browder, T., and Reilly, M. O. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature (Lond.)*, 390: 404–407, 1997.
- Sim, B. K.-L., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lapcevic, R., and Nancy, C. A. A recombinant humanangiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res.*, 57: 1329–1334, 1997.
- Moulton, K. S., Heller, E., Konerding, M. A., Flynn, E., Palinski, W., and Folkman, J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation*, 99: 17261–17232, 1999.
- Yokoyama, Y., Dhanabal, M., Griffioen, A. W., Sukhatme, V. P., and Ramakrishnan, S. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. *Cancer Res.*, 60: 2190–2196, 2000.
- Perletti, G., Concar, P., Giardini, R., Marras, E., Piccinini, F., Folkman, J., and Chen, L. Antitumor activity of endostatin against carcinogen-induced rat primary mammary tumors. *Cancer Res.*, 60: 1793–1796, 2000.
- Kisker, O., Becker, C. M., Prox, D., Fannon, M., D'Amato, R., Flynn, E., Fogler, W. E., Sim, B. K., Allred, E. N., Pirie-Shepherd, S. R., and Folkman, J. Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. *Cancer Res.*, 61: 7669–7674, 2001.
- Marshall, E. Cancer therapy. Setbacks for endostatin. *Science (Wash. DC)*, 295: 2198–2199, 2002.
- Twombly, R. Clinical trials of endostatin yield lukewarm results. *J. Natl. Cancer Inst. (Bethesda)*, 94: 1520–1521, 2002.
- Thomas, J. P., Arzoumanian, R. Z., Alberti, D., Marnocha, R., Lee, F., Friedl, A., Tutsch, K., Dresen, A., Geiger, P., Pluda, J., Fogler, W., Schiller, J. H., and Wilding, G. Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors. *J. Clin. Oncol.*, 21: 223–231, 2003.
- Herbst, R. S., Hess, K. R., Tran, H. T., Tseng, J. E., Mullani, N. A., Charnsangavej, C., Madden, T., Davis, D. W., McConkey, D. J., O'Reilly, M. S., Ellis, L. M., Pluda, J., Hong, W. K., and Abbruzzese, J. L. Phase I study of recombinant human endostatin in patients with advanced solid tumors. *J. Clin. Oncol.*, 20: 3792–3803, 2002.
- Bleizinger, P., Wang, J., Gondo, M., Quezada, A., Mehrens, D., French, M., Singhal, A., Sullivan, S., Rolland, A., Ralston, R., and Min, W. Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene. *Nat. Biotechnol.*, 17: 343–348, 1999.
- Kuo, C. J., Farnebo, F., Yu, E. Y., Christofferson, R., Swearingen, R. A., Carter, R., von Recum, H. A., Yuan, J., Kamihara, J., Flynn, E., D'Amato, R., Folkman, J., and Mulligan, R. C. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc. Natl. Acad. Sci. USA*, 98: 4605–4610, 2001.
- Matsuda, K. M., Madoiwa, S., Hasumi, Y., Kanazawa, T., Saga, Y., Kume, A., Mano, H., Ozawa, K., and Matsuda, M. A novel strategy for the tumor angiogenesis-targeted gene therapy: generation of angiostatin from endogenous plasminogen by protease gene transfer. *Cancer Gene Ther.*, 7: 589–596, 2000.
- Dmitriev, I., Kashentseva, E., Rogers, B. E., Krasnykh, V., and Curiel, D. T. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. *J. Virol.*, 74: 6875–6884, 2000.
- Samulski, R. J., Chang, L.-S., and Shenk, T. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised *in vitro* and its use to study viral replication. *J. Virol.*, 61: 3096–3101, 1987.
- Zolotukhin, S., Byrne, B., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R., and Muzyczka, N. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.*, 6: 973–985, 1999.
- Ponnazhagan, S., Erikson, D., Kearns, W. G., Zhou, S. Z., Nahreini, P., Wang, X. S., and Srivastava, A. Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum. Gene Ther.*, 8: 275–284, 1997.
- Ponnazhagan, S., Mukherjee, P., Yoder, M. C., Wang, M. C., Zhou, S. Z., Kaplan, J., Wardsworth, S., and Srivastava, A. Adeno-associated virus 2-mediated gene transfer *in vivo*: organ-tropism and expression of transduced sequences in mice. *Gene*, 190: 203–210, 1997.
- Ponnazhagan, S., Mukherjee, P., Wang, X. S., Kurpad, C., Qing, K., Kube, D., Mah, C., Yoder, M. C., Srouf, E. F., and Srivastava, A. Adeno-associated virus 2-mediated transduction of primary human bone marrow derived CD34+ hematopoietic progenitor cells: donor variation and correlation of expression with cellular differentiation. *J. Virol.*, 71: 8262–8267, 1997.
- Ponnazhagan, S., Mahendra, G., Kumar, S., Thompson, J. A., and Castillas, M., Jr. Conjugate-based targeting of recombinant adeno-associated virus type 2 vectors by using avidin-linked ligands. *J. Virol.*, 76: 12900–12907, 2002.
- Jooss, K., Yang, Y., Fisher, K. J., and Wilson, J. M. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J. Virol.*, 5: 4212–4223, 1998.
- Fisher, K. J., Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E., and Wilson, J. M. Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.*, 3: 306–312, 1997.
- Ponnazhagan, S., Curiel, D. T., Shaw, D. R., Alvarez, R. D., and Siegal, G. P. Adeno-associated virus for cancer gene therapy. *Cancer Res.*, 61: 6313–6321, 2001.
- Folkman, J. Anti-angiogenic gene therapy. *Proc. Natl. Acad. Sci. USA*, 95: 9064–9066, 1998.
- Indraccolo, S., Gola, E., Rosato, A., Minuzzo, S., Habeler, W., Tisato, V., Roni, V., Esposito, G., Morini, M., Albini, A., Noonam, D. M., Ferrantini, M., Amadori, A.,

- and Chieco-Bianchi, L. Differential effects of angiostatin, endostatin and interferon- $\alpha(1)$  gene transfer on *in vivo* growth of human breast cancer cells. *Gene Ther.*, *9*: 867–878, 2002.
36. Jin, X., Bookstein, R., Wills, K., Avanzini, J., Tsai, V., LaFace, D., Terracina, G., Shi, B., and Nielsen, L. L. Evaluation of endostatin antiangiogenesis gene therapy *in vitro* and *in vivo*. *Cancer Gene Ther.*, *8*: 982–989, 2001.
  37. Tarui, T., Majumdar, M., Miles, L. A., Ruf, W., and Takada, Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. *J. Biol. Chem.*, *277*: 33564–33570, 2002.
  38. Tarui, T., Miles, L. A., and Takada, Y. Specific interaction of angiostatin with integrin  $\alpha(v)\beta(3)$  in endothelial cells. *J. Biol. Chem.*, *276*: 39562–39568, 2001.
  39. Dhanabal, M., Ramachandran, R., Waterman, M. J., Lu, H., Knebelmann, B., Segal, M., and Sukhatme, V. P. Endostatin induces endothelial cell apoptosis. *J. Biol. Chem.*, *274*: 11721–11716, 1999.
  40. Hanai, J., Dhanabal, M., Karumanchi, S. A., Albanese, C., Waterman, M., Chan, B., Ramachandran, R., Pestell, R., and Sukhatme, V. P. Endostatin causes G<sub>1</sub> arrest of endothelial cells through inhibition of cyclin D1. *J. Biol. Chem.*, *277*: 16464–16469, 2002.
  41. Lee, S. J., Jang, J. W., Kim, Y. M., Lee, H. I., Jeon, J. Y., Kwon, Y. G., and Lee, S. T. Endostatin binds to the catalytic domain of matrix metalloproteinase-2. *FEBS Lett.*, *519*: 147–152, 2002.
  42. Chen, C. T., Lin, J., Li, Q., Phipps, S. S., Jakubczak, J. L., Stewart, D. A., Skripchanko, Y., Forry-Schaudies, S., Wood, J., Schnell, C., and Hallenbeck, P. L. Antiangiogenic gene therapy for cancer via systemic administration of adenoviral vectors expressing secreted endostatin. *Hum. Gene Ther.*, *11*: 1983–1996, 2000.
  43. Eisterer, W., Jiang, X., Bachelot, T., Pawliuk, R., Abramovich, C., Leboulch, P., Hogge, D., and Eaves, C. Unfulfilled promise of endostatin in a gene therapy-xenotransplant model of human acute lymphocytic leukemia. *Mol. Ther.*, *5*: 352–359, 2002.
  44. Snyder, R. O. Adeno-associated virus-mediated gene delivery. *J. Gene Med.*, *1*: 166–175, 1999.
  45. Ma, H. I., Lin, S. Z., Chiang, Y. H., Li, J., Chen, S. L., Tsao, Y. P., and Xiao, X. Intratumoral gene therapy of malignant brain tumor in a rat model with angiostatin delivered by adeno-associated viral (AAV) vector. *Gene Ther.*, *9*: 2–11, 2002.
  46. Shi, W., Teschendorf, C., Muzyczka, N., and Sieman, D. W. Adeno-associated virus-mediated gene transfer of endostatin inhibits angiogenesis and tumor growth *in vivo*. *Cancer Gene Ther.*, *9*: 513–521, 2002.
  47. High, K. Gene-based approaches to the treatment of hemophilia. *Ann. N. Y. Acad. Sci.*, *953*: 64–74, 2001.
  48. Ye, X., Rivera, V. M., Zoltick, P., Cerasoli, F., Jr., Schnell, M. A., Gao, G., Hughes, J. V., Gilman, M., and Wilson, J. M. Regulated delivery of therapeutic proteins after *in vivo* somatic cell gene transfer. *Science (Wash. DC)*, *283*: 88–91, 1999.
  49. Rivera, V. M., Ye, X., Courage, N. L., Sachar, J., Cerasoli, F., Jr., Wilson, J. M., and Gilman, M. Long-term regulated expression of growth hormone in mice after intramuscular gene transfer. *Proc. Natl. Acad. Sci. USA*, *15*: 8657–8662, 1999.