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INTRODUCTION

One of the major implications of prostate cancer progression is bone metastasis. Primary therapies for neoplastic prostate disease have been prostectomy followed by chemotherapy and radiation therapy. Although these forms of palliative therapies have been successful in early detected prostate cancers, a problem in majority of the treated cases is the growth of radiation/chemotherapy resistant tumor cells, which become refractory to treatment and exhibit an aggressive growth and metastatic profile. Thus, novel therapies that will control the process of recurrence and metastasis will have a profound clinical implication in the management of prostate cancer patients who undergo primary therapies.

An interesting new target for prostate cancer therapy is tumor angiogenesis, which is vital for tumor growth and metastasis. Since anti-angiogenic therapy targets normal endothelial cells that form neovasculature, long term sustained presence of anti-angiogenic factors is critical for therapeutic significance. Although few drugs and purified proteins have shown preclinical efficacy of this form of therapy, a long-term application of these therapies have been associated with systemic toxicity, limited half life and increasing cost. Thus, stable long-term therapies without these effects would be highly beneficial. Gene therapy approach using recombinant adeno-associated virus vectors (rAAV) encoding anti-angiogenic factors is a very promising form of therapy for prostate cancer recurrence and metastasis. Major advantage of rAAV vectors are 1) long-term transgene expression 2) stable integration, 3) low-immunogenicity or toxicity and 4) non-pathogenicity.

Our recent preclinical evaluation using rAAV encoding angiostatin, endostatin and soluble vascular endothelial growth factor receptor (sFlt-1) indicated long-term protection of mice against the growth of a human angiogenesis-dependent ovarian cancer cells as xenograft. Sustained expression of the anti-angiogenic factors was detected over four months without any systemic toxicity. Based on these data, we proposed in our funded application to evaluate the potential of rAAV-mediated anti-angiogenic gene therapy in a transgenic adenocarcinoma mouse prostate (TRAMP) model, which exhibits most of the pathological features seen in human prostate cancer including a progressive angiogenic phenotype with advancing stages of the disease, bone metastasis and refractiveness of androgen depletion over time. New experiments will include the analysis of bone metastasis of prostate cancer cells following rAAV-mediated anti-angiogenic gene therapy. Further, we will also determine the effects of long-term expression of murine osteoprotegrin as primary and an adjuvant to anti-angiogenic gene therapy for the inhibition of bone metastasis of malignant prostate disease in the TRAMP model.

The proposed specific aims of the project are:

1. To determine long-term therapeutic potential of rAAV-mediated anti-angiogenic gene therapy in bone metastasis of neoplastic prostate disease in the transgenic adenocarcinoma mouse prostate (TRAMP) model *in vivo*.
2. To determine the adjuvant effects of long-term anti-angiogenic gene therapy and osteoprotegrin therapy for androgen-independent recurrence of prostate cancer in the TRAMP model.

BODY

Construction of recombinant adeno-associated virus vectors:

Our recent studies demonstrated the efficacy or rAAV in controlling the growth of human epithelial ovarian cancer as a xenograft (1). These studies formed the basis of the current proposal Construction of recombinant adeno-associated virus vectors encoding osteoprotegrin, murine angiostatin, endostatin, endostatin + angiostatin, kringle 1-5.

Since the present work will evaluate the effects of rAAV vectors in a transgenic adenocarcinoma mouse prostate model (TRAMP), which is an immunocompetent model, it was essential for us to generate rAAV encoding mouse versions of the therapeutic transgenes. In the last 10 months, we have finished the cloning of the following vectors:

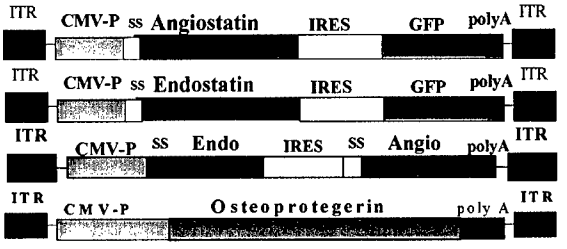

Figure 1. Schematic representation of rAAV encoding endostatin and angiostatin. rAAV containing mouse angiostatin and endostatin genes in different vectors or as bicistronically expressed proteins, and that encoding murine osteoprotegerin were subcloned under the control of the CMV promoter. Secretory signal sequences of either IL-2 or human plasminogen gene was included upstream of the angiostatin and endostatin genes for systemic secretion. ITRs represent the inverted terminal repeat sequences of AAV.

In addition to the angiostatin and endostatin genes, we also obtained a cDNA encoding mouse kringle 1-5 region of plasminogen, known to have increased anti-angiogenic effect (2). The recombinant plasmids were packaged into mature virions in 293 cells in a helper virus-free packaging system that is routinely used in our laboratory.

Optimization of conditions for the bone-specific delivery of mouse osteoprotegerin gene using rAAV:

We have planned to determine the effects of rAAV-mediated gene therapy for prostate cancer bone metastasis through stable expression of osteoprotegerin, which is known to inhibit ostaoclastogenesis and eventually the osteolytic bone degradation. Towards this pursuit, we plan to test the effects of both intramuscular administration of the rAAV vector for stable systemic levels of osteoprotegrerin and bone-specific delivery of the vector by using cellular mesenchymal stem cell vehicle.

Transient ectopic expression of α4β1 integrin enhances bone-specific homing of culture-expanded MSC. In the last several months, we conducted experiments to determine if transient ectopic expression of α4β1 integrin on MSC prior to transplantation would enrich bone-specific homing. A plasmid vector encoding murine α4β1was obtained from Dr. David Rowe (University of Connecticut Health Science Center, CT) and subcloned in the eukaryotic expression vector pCI (Promega, WI) to yield pCMV-α4β1. The expression and ectopic expression of α4β1 on mouse mesenchymal stem cells surface was confirmed by RT-PCR analysis and flow cytometry, respectively (Fig. 2).

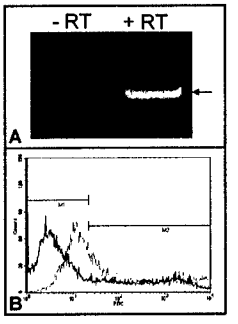

Figure 2. RT-PCR and flow cytometry analysis of mMSC transfected with a plasmid expressing α4β1 integrin. Total RNA isolated from mMSC transfected with the plasmid pCMV-α4β1 was reverse-transcribed using oligo-dT primer. After second strand synthesis, PCR amplification of α4β1 DNA region was performed (A). Ectopic expression of α4β1 integrin was confirmed by flow cytometry using an antibody specific for mouse α4β1 integrin. A shift in the signal towards right side is indicative of α4β1 expression (B).

To determine if transient ectopic expression α4β1 integrin will increase the bone homing of the mesenchymal stem cells, the cells were obtained from male C57BL/6J mice were cultured in the presence of FGF2. Twenty four-hours prior to transplantation, the cells were either mock-transfected or transfected with the plasmid pCMV-α4β1. The cells were injected into female C57Bl/6J mice by tail vein. Each recipient mouse received $1x10^6$ MSC. The mice were sacrificed 3 weeks after transplantation and *in situ* hybridization was performed in bone sections to enumerate the homing of transplanted MSC. Results of these studies indicated a significant increase in the number of donor cell homing to bone following transient expression of α4β1 integrin (Figure 3).

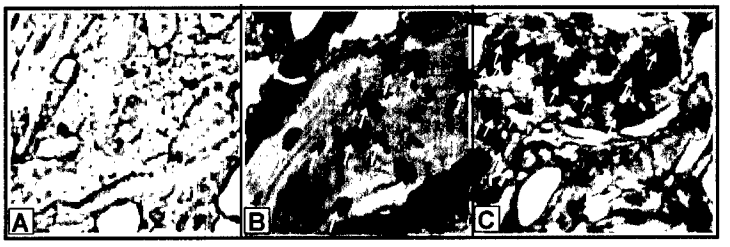

Figure 3. Increased homing efficiency of murine MSC following ectopic expression of α4β1 integrin. One million culture-expanded mMSC from male C57Bl/6 mice, that were untransfected (B) or transfected with a plasmid encoding α4β1 integrin (C) were transplanted to female recipients. Three weeks after, the mice were sacrificed and bone sections were hybridized *in situ* with Y-chromosome-specific probe. Bone section from a female mice, which did not receive donor cells was included as a negative control (A). Magnificationx40.

KEY RESEARCH ACCOMPLISHMENTS

Constructed four different rAAV vectors encoding mouse osteoprotegerin, endostatin, angiostatin and endostatin + angiostatin.

Generated high-titer recombinant AAV vectors encoding the indicated transgenes in a helpervirus-free packaging system.

Established conditions to achieve bone enriched homing of mesenchymal stem cell vehicles.

Established that long-term expression of angiostatin and endostatin significantly inhibits the growth of a human epithelial ovarian cancer cell line in combination with chemotherapy to increase survival rate, indicating the benefit of a combination therapy.

REPORTABLE OUTCOMES

(Papers published or communicated)

Ponnazhagan, S. Mahendra, G., Kumar, S., Shaw, D., Meleth, S., Stockardt, R., and Grizzle, W.E. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. *Cancer Res.* 2004, 64: 1781-1787.

Ponnazhagan, S. Parvovirus vectors for cancer gene therapy. *Expert. Opin. Biol. Ther.*, 2004, 4: 53-64.

Ponnazhagan, S., and Hoover, F. Delivery of DNA to tumor cells in vivo using adeno-associated virus. *Meth. Mol. Biol.* 2004, 246:237-243.

Mahendra, G., Mahasreshti, P., Curiel, D.T., Stockardt, R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., and Ponnazhagan, S. Anti-angiogenic gene therapy through adeno-associated virus 2-mediated stable expression of soluble Flt-1 receptor *Cancer Gene Ther.* 2004 (communicated).

(Results presented in conferences)

Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D., Stockard, C.E., and Grizzle, W.E. Adeno-associated virus 2-mediated gene therapy: long-term efficacy of a combination vector over individual therapy. 94th Annual Meeting of the American Society for Cancer Research, Washington D.C., 2003.

Zinn, K.R, Chaudhuri, T.R., Stargel, A., Kumar, S., and Ponnazhagan, S. Gamma camera imaging of Tc-99m-labeled AAV vector followed by bioluminescence imaging of luciferase expression. 6th Annual Meeting of the American Society for Gene Therapy, Washington D.C., June 2003.

Ponnazhagan, S., Mahendra, G., Lima, J., Aldrich, W., Jenkins, C., Ren, C., Kallman, L., Strong, T., Shaw, D., and Triozzi, P. Augmentation of anti-tumor activity of a recombinant adeno-associated virus carcinoembryonic antigen vaccine with plasmid adjuvants 95th Annual Meeting of the American Society for Cancer Research, Orlando, FL, April 2004.

Chaudhuri, T.R., Cao, Z., Ponnazhagan, S., Stargel, A., Simhadri, P.L., Zhou, T., LoBuglio, A.F., Buchsbaum, D.J., and Zinn, K. Detection of disseminated breast cancer growth and treatment response using non-invasive bioluminescence imaging (BI). 40th Annual meeting of the American Society of Clinical Oncology, New Orleans, LA, June 2004.

CONCLUSIONS

In the first year of the project, we finished construction and production of recombinant AAV that will be used in the in vivo studies. Further we optimized conditions for bone-specific delivery of rAAV encoding anti-metastatic proteins through mesenchymal stem cell vehicle. Further, work done during this period of funding with rAAV containing anti-angiogenic genes in a xenograft model indicated both the efficacy of this therapy and the increased survival when combined with chemotherapy.

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2. Cao, A., Wu, H-L., Veitonmaki, N, Linden, P., Farnebo, J., Shi, G-Y., and Cao, Y. Suppression of angiogenesis and tmor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. Proc. Natl. Acad. Sci. USA. 1999, 96: 5728-5733.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.
Diptiman Chanda, Ph.D.
Gene Siegal, MD., Ph.D.

APPENDICES

Copies of the following manuscripts enclosed:

Ponnazhagan, S. Mahendra, G., Kumar, S., Shaw, D., Meleth, S., Stockardt, R., and Grizzle, W.E. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. *Cancer Res.* 2004, 64: 1781-1787.

Ponnazhagan, S., and Hoover, F. Delivery of DNA to tumor cells in vivo using adeno-associated virus. *Meth. Mol. Biol.* 2004, 246:237-243.

Mahendra, G., Mahasreshti, P., Curiel, D.T., Stockardt, R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., and Ponnazhagan, S. Anti-angiogenic gene therapy through adeno-associated virus 2-mediated stable expression of soluble Flt-1 receptor *Cancer Gene Ther.* 2004 (communicated).

[CANCER RESEARCH 64, 1781–1787, March 1, 2004]

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.

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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 endonucelases 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

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′-TCGAGATGGAACATAAGGAAGTG-GTTCTTCTACTTCTTTTATTTCTGAAATCAGGTCAAG-3 and 5′-GATCCTTGACCTGATTTCAGAAATAAAAGAAGTAGAAGAACCA-CTTCCTTATGTTCCATC-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 μl 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 5×10^3 cells/well and grown in EGM-2 medium containing hydrocortisone, human fibroblast growth factor β, VEGF, ascorbic acid, heparin, human epidermal growth factor, and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). Twenty-four h later, 100 μl of fresh medium containing 1, 10, or 25 μl 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 (3×10^{11}) of rAAV encoding GFP, endostatin, angiostatin, or endostatin plus angiostatin, in normal saline, were injected in a volume of 100 μl 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 $mm^3 = (L \times W^2)/2$. When tumor growth exceeded 1800 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 μg/ml in borate saline buffer (BS; pH 8.6). Next day, the antibody was discarded and wells blocked with 150 μl 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 μg/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^{Plus} (Roche Molecular Diagnostics, Indianapolis, IN) following manufacturer's protocol. Formalin-fixed tissues were sectioned at 5-μm 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 (Biomeda, 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-μm 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 H_2O_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 μg/ml) or angiostatin (clone 79735, 5 μg/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 χ^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. 1*A*). 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. 1*A*), 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 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.

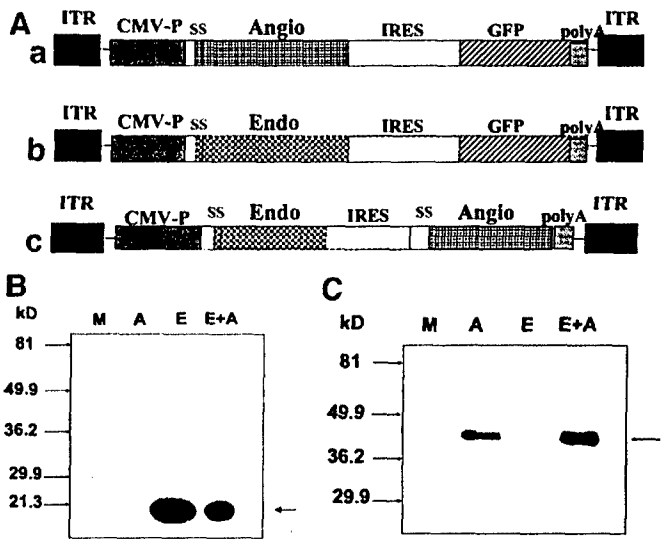

Fig. 1. Recombinant adeno-associated virus 2 (rAAV) encoding endostatin and/or angiostatin and Western blot analysis for transgene expression. *A*, rAAV containing human angiostatin (*Angio*) or endostatin (*Endo*) gene either in different vectors (*a* & *b*), or as bicistronically expressed proteins 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.

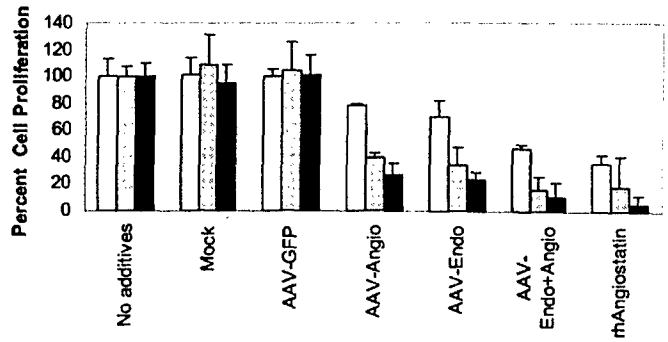

Fig. 2. Endothelial cell proliferation assay to determine the biological activity of endostatin (Endo) and angiostatin (Angio) produced after recombinant adeno-associated virus 2 (rAAV) transduction. rAAV encoding green fluorescent protein (GFP), Endo, Angio, or Endo + Angio was transduced into 293 cells at a multiplicity of 100. Forty-eight h later, 1 (□), 10 (□), or 25 (■) μl 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.

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 μl 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-

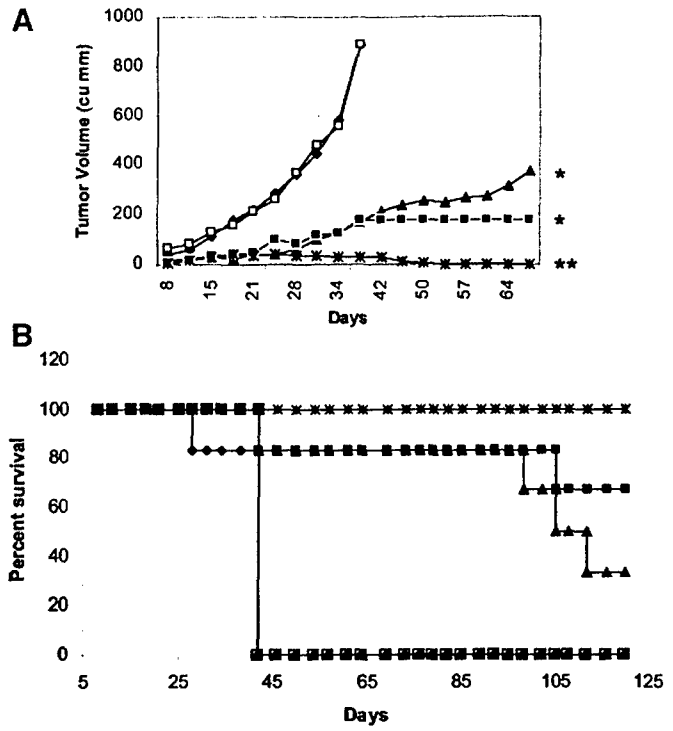

Fig. 3. Growth of SKOV3.ip1 tumor xenografts in athymic nude mice after injection of recombinant adeno-associated virus 2 (rAAV) encoding endostatin and angiostatin. Mice received i.m. injection with saline (♦), or with 3×10^{11} particles of AAV-GFP (□), AAV-endostatin (▲), AAV-angiostatin (■) or AAV-endostatin + 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 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. 3*A*, and tumor-free survival of mice is presented in Fig. 3*B*. 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. 3*B*). 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 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. 4*A*). Serum endostatin levels were determined using a commercial kit and demonstrated a pattern of endostatin expression that was similar to that for angiostatin (Fig. 4*B*). 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,

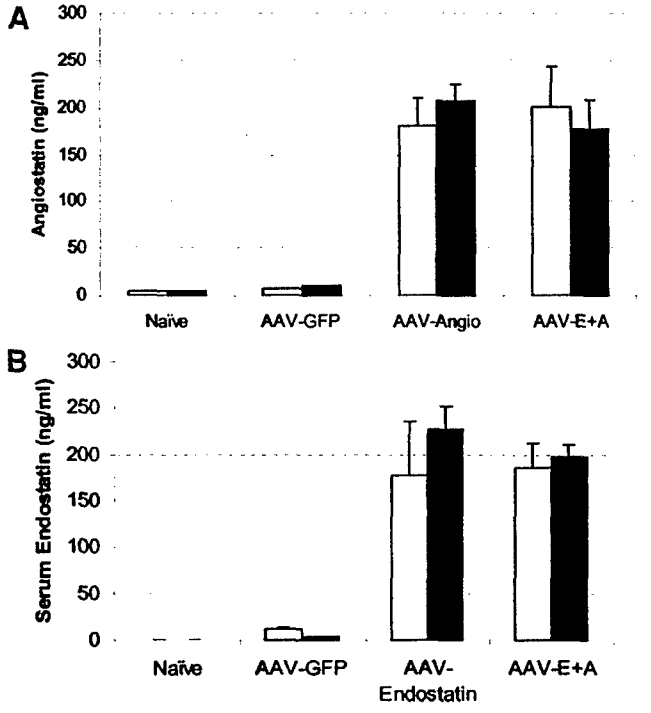

Fig. 4. Levels of human endostatin and angiostatin in mouse serum after injection of recombinant adeno-associated virus 2 (rAAV) encoding green fluorescent protein (GFP), endostatin (E), or angiostatin (Angio or A). Serum samples were obtained before tumor challenge (□) and at the time of sacrifice of 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 ± SD of calculated concentrations of each factor (mean of triplicates).

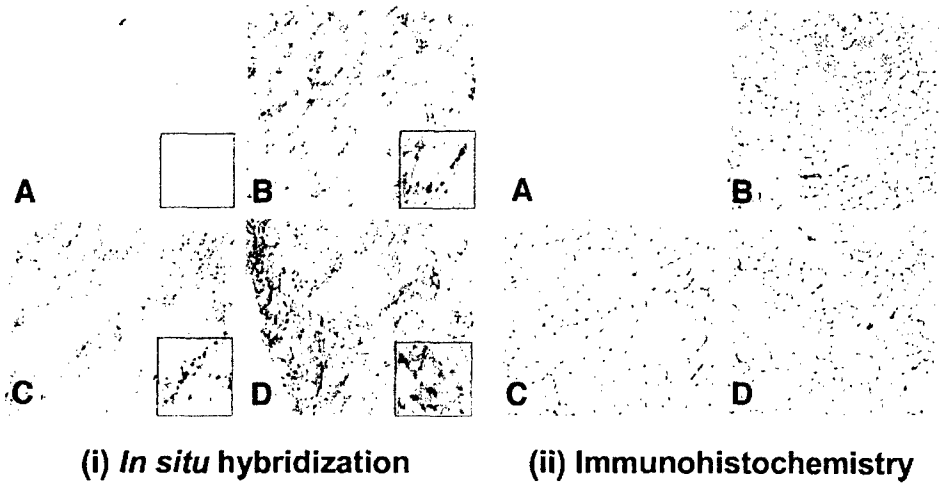

Fig. 5. *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Five-μm sections of injected muscles were used for *in situ* hybridization (*left panels*) and immunohistochemistry (*right panels*). *A*, uninjected. *B*, AAV-endostatin. *C*, AAV-angiostatin. *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 $\alpha v\beta 3$ 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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Delivery of DNA to Tumor Cells In Vivo Using Adeno-Associated Virus

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1. Introduction

The number of published studies on transduction of tumor cells in vivo using recombinant adeno-associated virus (rAAV) vectors is very limited compared with those that have been published on targeting normal cells. A major reason for this can be attributed to the biology of the vector itself. AAV, being a nonpathogenic vector capable of providing transgene integration and long-term expression, is ideally suited for the correction of metabolic defects either to replace a defective protein/enzyme or to elevate their otherwise suboptimal levels in the system. However, increased understanding of both the biology of tumor progression and potential utility of AAV-based vectors suggests that this vector can also be wisely used for cancer gene therapy.

1.1. Possible Approaches for Cancer Gene Therapy Using AAV Vectors

The anti-oncogenic properties of wild-type AAV (wtAAV) have been observed long before the realization of the potential of this vector for gene therapy *(1)*. Until recently, this property was attributed to the nonstructural protein of AAV, which is also toxic to cells *(2)*. However, in a recent report, Raj et al. described selective killing of tumor cells that contain mutant p53 through initiation of apoptotic signals following the introduction of single-stranded AAV genome. This suggested a new approach to gene therapy against tumors that are positive for mutant p53 expression *(3)*. Although stable integration and long-

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term expression capabilities of AAV vectors may not be a desirable/beneficial characteristic for therapy targeting the tumor cells directly, these features can be advantageous for targeting normal cells of the body that can exert influence on the control of tumor growth. Activation of the host immune system against tumor growth or arresting the growth of tumor neovasculature through anti-angiogenesis by targeting endothelial cells of tumor origin are some examples of targeting normal cells for which AAV can be used *(4)*. These strategies can be employed by transducing the vector either directly at the site of tumor or transducing normal tissue such as muscle and systemically expressing the therapeutic protein in secreted form. The main advantage of vector administration to tumor cells is the vicinity of target cells, which not only includes tumor cells themselves but also other target cells such as tumor endothelium in anti-angiogenic therapy or immunomodulation of the host system by cytokine gene transfer or chemoattracting antigen-presenting cells (APCs) to the site of tumor for the uptake, processing, and presentation of tumor antigens for tumor-specific T-cell response.

Prior to the application of intratumoral AAV administration in human patients, it is important to investigate the efficacy, delivery, and therapeutic impact in preclinical animal models. Thus, procedural details given in this chapter may benefit those in this field of study. A vast majority of preclinical evaluation of gene-therapy vectors by intratumoral administration have been done in subcutaneously grown syngeneic or xenograft cell lines. Hence, the present protocol will describe a method of intratumoral rAAV administration in tumors developed as xenografts in rodents. This protocol can, however, be modified to target tumors that are grown in other organs. All animal protocols must be approved by Institutional Animal Care and Use Facility or other appropriate local, state, and federal regulations.

2. Materials

1. rAAV containing a gene of interest, which can be a reporter gene such as β-galactosidase (β-gal), green fluorescent protein (GFP), or luciferase, or a therapeutic gene such as an enzyme, growth factor, or cytokine. Several methods are currently being used to prepare rAAV. The general principle of rAAV preparation involves rescue and packaging of mature virions from a plasmid vector containing the gene of interest cloned within the inverted terminal repeats (ITRs) of AAV. We currently use a discontinuous density-gradient centrifugation followed by heparin-agarose affinity column purification *(5)*. Quantitation of the vector can be done either by slot-blot analysis, by infectious center assay *(5)* or by real-time polymerase chain reaction (PCR) *(6)*. The vector is resuspended in PBS for intratumoral administra-

tion. The vector can be stored at 4°C up to 4 wk. For longer storage, we recommend storage at −80°C.
2. Tumor cell lines (Example: U-87MG, ATCC, Manassas, VA).
3. Microinjection pump Model UMP2-1 (World Precision Instruments Inc., Sarasota, FL).
4. Matrigel (Collaborative Research, Bedford, MA).
5. Nude rats (Rowett, Aberdeen, Scotland).
6. Stereotactic animal frame (Model 900, David Kopf Instruments, Tujunga, CA).
7. Bulldog clamps (Algaier Instruments GmbH, Tuttlingen, Germany).

3. Methods

3.1. Development of Subcutaneous Tumors

Human tumor cell lines can be implanted in 4–6-wk-old nude mice (athymic nude or SCID mice). Depending on the growth characteristics of a particular cell line, the number of cells to be implanted can vary. For example, when we administer between 0.5–1 × 10^7 SKOV3.ip1 cells (a human ovarian cancer cell line), palpable tumors appear in approx 10 d. If the growth rate of a tumor cell line is not known, we recommend conducting a pilot study with different amounts of cells before performing an experiment with the vector.

3.1.1. Preparation of Tumor Cells

1. Prepare a fresh culture of the cell line to be implanted using appropriate media and other additives such as serum, antibiotics, cytokines, and so on.
2. Expand cultures by splitting the cells before they reach confluence because overcrowding may retard subsequent growth characteristics.
3. On the day of tumor cell implantation, harvest the cells by using trypsin-ethylenediaminetetraacetic acid (EDTA) to detach the cells; use one-tenth the volume of trypsin-EDTA compared to the total volume of medium used to maintain the culture.
4. Incubate the cells at 37°C until they begin to round up (once again, the time of trypsin treatment varies between cell lines). Tap the vial to detach the cells. Do not incubate the cells in trypsin for an extended period of time because it will decrease the viability.
5. Stop trypsin activity by adding 10 volumes of medium containing 10% serum.
6. Collect the cells in sterile centrifuge tubes and pellet at 500*g* for 5 min.
7. Remove the supernatant and resuspend the pellet in 10–20 mL of PBS.
8. Pellet the cells as before.

9. Resuspend the cells in cold PBS or 50% Matrigel in PBS at a concentration of 1–2 × 10^8 cells/mL. Addition of matrigel allows the implanted cells to remain clustered at the site of injection and minimizes dispersion.

3.1.2. Implantation of Tumor Cells

1. Anesthetize the mice by subcutaneous administration of ketamine (100 mg/kg body weight) and xylazine (15 mg/kg body weight), abdominally.
2. Using a sterile alcohol swab, clean the area of the flank region.
3. Using a 1 cc syringe with a 25 G needle, deliver the required number of cells/tumor nodule subcutaneously (sc) in a volume of 50–100 µL: insert the needle carefully approx 1 cm under the skin between the skin and muscle, then inject the cells (*see* **Note 1**).
4. Observe the animal for proper recovery following the injection.
5. Once the tumor starts growing to a palpable size, make measurements at least three times every week. Measure the tumor volume with digital calipers for two-dimensional longest axis (L in mm) and shortest axis (W in mm) and calculate the tumor size using the formula:

$$\text{Volume in mm}^3 = (L \times W^2)/2$$

3.2. Intratumoral Injection of rAAV

The volume of virus to be injected depends on the size of tumor. More care is needed when the vector is delivered to a tumor of less than 100 mm^3. Hence, we recommend that the volume of virus suspension be kept as small as possible, ideally around 25 µL for sc developed tumors.

1. Prepare the virus in PBS to be injected in a 0.5-cc insulin syringe with a 26 G. Use a separate syringe for injection into each tumor nodule.
2. Anesthetize the mice with ketamine and xylazine
3. Clean the area of tumor with a sterile alcohol swab.
4. Gently insert the needle into the tumor nodule so that the tip of the needle stays approximately at the center of the tumor. Use the caliper reading from each tumor to empirically determine the required distance to insert the needle.
5. Slowly inject the virus (up to 25 µL) with constant pressure (*see* **Note 2**).
6. Monitor the animals for recovery after the procedure and observe them, daily for any complications. If they appear ill or fail to recover properly, they should be humanely euthanized.

3.3. Development of Intracranial Tumors

3.3.1. Implantation of Tumor Cells

A larger animal such as a rat will be easy to handle if the xenografts are developed in the brain. Xenografts into young male and female nude rats can be generated according to a protocol modified from Engebraaten et al. *(7)*. This procedure can be adapted for other cell lines to be grown as xenografts in rodent brain by varying the number of cells injected. Rats weighing 150–200 g should be kept on an ad libitum standard pellet diet with unlimited access to water and caged at constant temperature (21°C) and humidity in rooms with a 12 h light/12 h dark cycle.

1. Anesthetize rats by subcutaneous injection of Midazolam 0.2 g/100 g, Fentanyl Citrate 0.0126 g/100 g, and Fluanizone 0.4 g/100 g into the intraperitoneal cavity and mounted in a stereotactic frame. An additional injection of local anesthesia (1 mL of xylocaine [10 mg/mL] can be delivered sc to the incision area).
2. Make a 2.5-cm mid-sagittal incision on the skull spread the surrounding tissue, held open with bulldog clamps.
3. Position a surgical drill 1 mm posterior to the bregma suture and 3 mm to the right of the midline suture and carefully drill a small burrhole approx 2 mm in diameter. Take care to avoid rupturing the underlying blood vessels.
4. Implant 10–20 tumor spheroids using a 25-µL Hamilton syringe with a 22 G needle 2.5 mm deep into in the cerebral cortex (measured from the dura) using slow, steady hand pressure.
5. Close the wound with thread suture (Ethylon, polyamide 6).
6. Place the animals in a 35°C incubator until they regain consciousness and then return them to their cage. Observe daily.
7. To verify the presence of tumor, 2–4 wk after implantation, perform magnetic resonance imaging (MRI) using Siemens Magnetom Vision Plus 1.5T scanner and a small loop finger coil. Establish the location of the tumor for injection (*see* **Note 3**).

3.3.2. Intratumoral Injection of rAAV

We describe below intratumoral injection of rAAV in brain of nude or athymic rats, which can be modified according to the animal size.

1. Typically, animals should be anesthesized and secured in the stereotaxic frame and viral vector injected into the same coordinates after re-opening the original incision under anesthesia.
2. Inject the virus using a micro-pump connected to a 10-µL Hamilton syringe with a 26 G needle. Inject 10^8–10^9 viral particles over a period of 1 h in a

maximum volume of 8 µL *(8)*. Infusion rates can vary between 33 nL/min and 133 nL/min. It should be emphasized that intra-cranial injections by slow-push hand pressure using a Hamilton syringe give variable and inconsistent results in our experience.

3. As a positive control for infectious virus, inject small amounts of vector (1–2 µL) into the tibialis anterior muscle, in the hind leg of a different animal, through the skin using slow push hand pressure and a 10-µL Hamilton syringe. To aid in identification of the area, measurements can be made from below the knee to the approximate site of injection. Skeletal muscle is chosen because it is easily accessible and is permissive for most AAV serotypes examined (Hoover, unpublished results).
4. Following wound closure, place the animal in a 35°C incubator until it regains consciousness, then return it to its original cage.
5. If animals with tumor implants develop neurological symptoms resulting from tumor burden and tumor phenotype, they should be sacrificed immediately when symptoms such as weight loss, disorientation, lack of movement, or loss of appetite are observed.
6. Following intratumoral delivery of the vector by the aforementioned procedures, the animals can be used for monitoring the effects of gene transfer based on transgene expression analysis if a reporter gene is used, or other end-point measurements such as reduction in tumor size or delayed growth kinetics based on therapeutic efficacy of the transgene used.

3.4. Conclusion

The procedures described can be modified based on the application and growth characteristics of different tumor types and location. Because AAV-based vectors infect a variety of cells, it is possible to get "leakage" of the intratumorally administered vector to other organs as well as through systemic circulation from the tumor site. Thus, depending on the type of study, rAAV may be constructed with tumor-specific expression if transgene expression is deleterious in other organs. An additional concern is the variable infectivity of rAAV among different tumor cells. Thus, prior to conducting intratumoral studies with AAV vectors, it is important to determine the ability of AAV to infect the tumor of interest in vitro.

4. Notes

1. Care should be taken to avoid intra-dermal invasion of the needle. Slow release of the cells minimizes dispersion from the site of injection. Also, the use of matrigel helps in the retention of tumor cells in one region. To determine if implanted cells form tumors, MRI can be performed as described in **Subheading 3.3.1** and **Note 3**.

2. Hold the tumor in one hand so that the needle does not slip and the virus is injected into the tumor. Inject the virus slowly, avoiding any jerking motion.
3. For MRI, anesthetize the rat and place it in a polystyrene immobilizing tube. Obtain coronal T1 (TR 400 ms, TE 14 ms, slice thickness 2 mm, slice center distance 2 mm, totaling 13 coronal slices covering the forebrain) and coronal T2 (TR 4000 ms, TE 96 ms, slice thickness 2 mm, slice center distance 2 mm, totaling 19 coronal slices covering the forebrain) images prior to and 10 min following sc injection of contrast agent (1 mL of 0.5 m*M* Gadolinium).

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Anti-angiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble FMS-like tyrosine kinase-1 receptor

(*Short title*: AAV2-mediated sFlt-1 cancer gene therapy)

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Abstract

Anti-angiogenic gene transfer has the potential to be more efficacious than protein-based therapies or pharmacotherapies for the control of solid tumor growth, invasion and metastasis. For a sustained anti-angiogenic effect, a vector capable of long-term expression without vector-associated immunity or toxicity is advantageous. The present study evaluated the potential of a recombinant adeno-associated virus-2 (rAAV) encoding the human soluble FMS-like tyrosine kinase receptor 1 (sFlt-1), which functions by both sequestering vascular endothelial growth factor (VEGF) and forming inactive heterodimers with other membrane-spanning VEGF receptors, *in vitro* and *in vivo*. Results indicated significant growth inhibitory activity of the transgenic factor in a human umbilical vein endothelial cell proliferation assay *in vitro* and protection against the growth of an angiogenesis-dependent human ovarian cancer cell line, SKOV3.ip1, xenograft *in vivo* with increased disease-free survival. Stable expression of the secretory factor and transgene persistence were confirmed by immunohistochemistry and *in situ* hybridization analyses respectively. Increased therapeutic effects on both the growth index of the implanted tumor cells and tumor-free survival also correlated with an increasing dose of the vector used. These studies indicate that rAAV-mediated sFlt-1 gene therapy may be a feasible approach for inhibiting tumor angiogenesis, particularly as an adjuvant/preventive therapy.

Introduction

A better understanding of the interaction of normal host and tumor cells has provided clues towards developing novel therapies for cancer. Cancer gene therapy targeting non-neoplastic cells has recently shown greater potential against tumor growth and metastasis in preclinical models. One of the promising areas of cancer therapy is targeting the growth of tumor-associated endothelium, which provides anchorage and nourishment for the growth of solid tumors through the process of angiogenesis (1). It has been clearly established that in the absence of angiogenesis, tumors fail to grow beyond 2-3 mm^3 in size and remain dormant (1,2). Thus, by providing anti-angiogenic signals through sustained systemic expression of the inhibitory factors at therapeutic levels, it may be possible to control the growth of solid tumors. Further, anti-angiogenic gene therapy can be combined with conventional therapies such as radiation or chemotherapy or newer therapies including immunotherapy for synergistic effects (3).

For sustained expression of anti-angiogenic factors at a therapeutic level by gene transfer, an important requirement is the use of vectors capable of stable expression without the possibility of elimination of transgene-positive cells by T cell-mediated cytolysis. Adeno-associated virus (AAV)-based vectors have shown the potential for long-term expression of therapeutic genes without such vector-associated immunity or toxicity (4-7). Since anti-angiogenic gene therapy mandates a sustained expression of the anti-angiogenic factors at a therapeutic level, rAAV is an ideal vector to accomplish this.

Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) is a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt-1, lacking the transmembrane and cytoplasmic domains (8). VEGF is a potent angiogenic factor, and overexpression of VEGF has been reported to be associated with poor prognosis in many human cancers (9-13). The VEGF antagonistic activity of sFlt-1 is effected both by forming inactive heterodimers with membrane-spanning VEGF receptor and by sequestering VEGF in a dominant negative manner thereby inhibiting the downstream VEGF signaling cascade following receptor-mediated internalization (8). Thus, use of sFlt-1 for downregulating VEGF signaling at two different steps would maximize the process of inhibiting tumor neovascularization and associated tumor growth. The present study demonstrates that stable expression of sFlt-1 following rAAV-mediated gene transfer provides significant protection against the growth of an angiogenesis-dependent human ovarian cancer cell line in a mouse xenograft model suggesting its potential application in anti-angiogenic cancer gene therapy in humans.

Materials and Methods

Cells and reagents. The human embryonic kidney cell line, 293, was purchased from the ATCC and maintained in Iscove's Modified Essential Medium supplemented with 10% fetal bovine serum. Human ovarian cancer cell line SKOV3.ip1 was a kind gift of Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX) and was maintained as before (14). Primary human umbilical cord vein endothelial cells (HUVEC) were obtained from Dr. Francoise Booyse (The University of Alabama at Birmingham, AL) and maintained as before (15). Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corporation (Madison, WI). A mouse monoclonal antibody, which recognizes the human sFlt-1 was purchased from Sigma Chemicals (St. Louis, MO). Secondary antibodies and color reagents were purchased from Amersham, Piscataway, NJ. Purified recombinant human sFlt-1 was purchased from R&D Systems Inc., Minneapolis, MN.

Construction of recombinant plasmids, production and purification of rAAV. All rAAV plasmids were constructed using pSub201 as the backbone (16). cDNA encoding human sFlt-1 was cloned from a human placental cDNA library as described (17). The coding sequences were initially subcloned in a mammalian expression vector pCI (Promega, Madison, WI) under the control of the CMV promoter and a cassette comprising the CMV promoter, sFlt-1 gene and polyadenylation signal sequence was isolated and subcloned in pSub201,

replacing the AAV-2 genes. Packaging of rAAV-sFlt-1 was accomplished in an adenovirus-free system as described (18). Purification of the virions was carried out in a discontinuous iodixanol gradient centrifugation followed by affinity purification in heparin affinity columns. The particle titer of the purified virions was determined by quantitative slot blot analysis as described (19,20).

Western blot analysis. Western blot analysis was performed using conditioned media obtained from rAAV-transduced 293 cells. Briefly, conditioned media collected following mock-transduction or rAAV-sFlt1 transduction was concentrated 5-fold and 20 µl from each aliquot was electrophoretically separated in a 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). The gel was transferred to a PVDF membrane and immunodetection of the proteins was performed using a monoclonal antibody directed against the extracellular region of the human sFlt-1. A goat anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP), was used as a secondary antibody. Detection of the bands was performed using an enhanced chemiluminescent (ECL) system as described (21).

Endothelial cell proliferation assay. Early passage HUVEC were seeded in gelatin-coated 96-well tissue culture plates at a density of $5x10^3$ cells per well and grown in EGM-2 medium containing hydrocortisone, human FGF-β, VEGF, ascorbic acid, heparin, human EGF and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). Twenty-four hr later, the medium was changed and 100

µl of fresh medium containing the same additive plus 20 µl of conditioned medium obtained from 293 cells that were either mock-transduced or transduced with 100 MOI of rAAV-sFlt-1. As a positive control, purified recombinant human angiostatin (R&D Systems, Minneapolis, MN) was added in a separate well at a concentration of 20 µg/ml. Seventy-two hours later, the cells were fixed in 10% buffered-formalin and stained with 1% crystal violet in 70% ethanol as described (22) to analyze the rate of proliferation.

***In vivo* studies.** Six-week old female athymic nude mice were purchased from the Fredericks Cancer Institute, NCI, and housed in the animal facility of the University of Alabama at Birmingham (UAB). Maintenance of the animals was performed following the guidelines of the Institutional Animal Care and Use Committee (IACUC) after all experimental procedures were approved by both the UAB-IACUC and the Occupational Health and Safety Department. The animals were divided into six groups consisting of six mice per group. $3x10^{11}$ particles of rAAV encoding either GFP, or sFlt-1, suspended in normal saline, were injected in a volume of 50-100 µl in the quadriceps muscle of the hind limb. Naïve animals did not receive any vector. Three weeks after vector administration, each mouse was implanted with 10^7 SKOV3.ip1 cells, subcutaneously. A total of two injections were delivered per mouse, one in each flank. Tumor size was measured twice a 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 formula: volume in $mm^3 = (L \times W^2)/2$. If the tumor growth exceeded 1800 mm^3,

the animals were euthanized. The surviving mice were sacrificed by the end of 130 days after tumor cell implantation, and the experiment terminated. At the time of sacrifice either due to tumor burden or termination of the experiment, both the liver and regions of the quadriceps muscle at the site of sham or vector injection were isolated and processed for total DNA isolation, histology, immunohistochemistry and *in situ* hybridization.

Histological analyses of liver tissue. Liver tissues were harvested from naïve or rAAV-sFlt1 treated mice. The tissues were immediately fixed in 10% buffered-formalin (pH 7.0) and embedded in paraffin following standard processing methods. Sections of 5 μm thickness were cut, deparaffinized in xylene and dehydrated in alcohol. The slides were H&E stained and mounted with cover slips. Analysis of the stained sections was carried out by a senior histopathologist by standard bright-field microscopy (23).

PCR analysis for vector genome. Total DNA was isolated from naïve or rAAV-sFlt1 administered mouse muscle using Trizol reagent (GIBCO-BRL). PCR amplification was carried out in a 30 cycle reaction using a primer-pair, specific for the amplification of the vector genome. The forward primer consisted sequence of CMV promoter and the reverse primer that of the human sFlt-1. The primer sequences were: forward primer 5'-TAAGCAGAGCTCGTTTAGTGAACCGT-3' and reverse primer, 5'-TACTCACCATTTCAGGCAAAGACCAT-3'. The amplified products (548 bp)

were electrophoretically separated on 1% agarose gels and the bands visualized by ethidium bromide staining.

Immunohistochemistry. The quadriceps muscle of mice were harvested under anesthesia and fixed immediately in alcoholic-formalin (PenFix; Richard-Allan, Kalamazoo, MI) for 18 hrs at room temperature. The tissues were dehydrated in graded alcohol and embedded in paraffin. Five micrometer sections, prepared on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling the sections 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 H_2O_2 for 5 min to quench endogenous peroxidase. Sections were then treated with 3% goat serum for 1 hr at room temperature to reduce non-specific staining followed by 1 hr incubation with a human monoclonal anti-VEGF receptor-1 antibody, which recognizes the extracellular domain. The antibody was used at a concentration of 10 µg/ml. The remainder of staining procedure was performed using a Universal Mouse Kit (Biogenex, Sam Ramon, CA), which contained biotinylated goat linking antibody to mouse immunoglobulins and a HRP-streptavidin complex. Diaminobenzidine tetrahydrochloride (DAB) was used as a substrate for the visualization of antigen-antibody complex. Slides were minimally counterstained with hematoxylin.

***In situ* hybridization.** A digoxigenin (DIG)-labeled DNA probe containing the CMV promoter sequence was generated by PCR using the PCR-DIG labeling

mix^{Plus} (Roche Molecular Diagnostics) following the manufacturer's protocol. Formalin-fixed tissues were sectioned to five micron thickness, deparaffinized in xylene and rehydrated through a series of graded-ethanol and PBS. Slides were then treated with 0.01M citrate buffer, pH 6.0 at 42°C for 3 hrs. Prehybridization was performed at 65°C for 2 hrs in hybridization solution (ULTRAhyb^{TM}, Ambion, TX). The hybridization reaction was carried out with approximately 400 ng/ml of the 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, Indianapolis, IN). Counterstaining of the slides was carried out with a diluted eosin solution for 1-2 minutes and then mounted with Crystal/Mount (Biomeda, Forest City, CA).

Statistical analyses. The association of the presence or absence of tumor with differing treatment conditions was tested for statistical significance by using the Chi-square test. The distribution of the tumor volume was measured against a normal (Poisson's distribution) following log transformation. In a general linear mixed model of the data, the dependent variable was the logged tumor volume and the predictors were treatment, day after treatment and "an interaction event" between treatment and day 0. All three were subsequently shown to be statistically significant predictors. *P* values <0.05 were considered to indicate significant difference between data sets.

Results

Generation of high-titer rAAV encoding human sFlt-1 for skeletal muscle injection. Previous studies have established sustained expression of rAAV transgenes in skeletal muscle without diminution of expression or deterioration of transgene-expressing cells by cytolytic T cells. Hence, in the present study, we have chosen skeletal muscle as a platform for rAAV administration and production of sFlt-1 as a secretory protein. The human sFlt-1 cDNA was isolated from a HUVEC cDNA library (17) and subcloned in an rAAV vector under the control of the human cytomegalovirus immediate early promoter (CMV) (Figure 1). The rAAV was packaged in a helper virus-free system and purified using discontinuous iodixanol gradient centrifugation and affinity chromatography. The titer of the vectors ranged between 1-5x10^{12} particles/ml.

Determination of sFlt-1 expression as a secretory protein. The rAAV-sFlt-1 was tested in 293 cells for the extracellular secretion of the factor. Since the cDNA of cloned sFlt-1 contained the native VEGF receptor secretory signal, no additional modifications were performed to achieve extracellular transport of sFlt-1 from the transduced cells. The cells were either mock-transduced or transduced with 100 multiplicity of infection (MOI) of the rAAV encoding sFlt-1. The conditioned medium, obtained 48 hrs after transduction, was concentrated five-fold and separated on SDS-PAGE and Western blot analysis performed using a monoclonal antibody, which recognizes human sFlt-1. Results, shown in Figure 2, clearly indicated a high-level expression of human sFlt-1 as a secretory

protein following rAAV transduction in 293 cells. The antibody, recognizing sFlt-1 in supernatants obtained only from the rAAV-transduced 293 cells but not from the mock-transduced cells also indicated the specificity of detection.

Determination of biological activity of sFlt-1 following rAAV transduction. In the next set of experiments, we determined the biological activity of rAAV-expressed sFlt-1. We based our therapeutic approach on the well established property of rAAV transgene expression as a secretory protein in skeletal muscle. Thus, the *in vitro* evaluation of the biological activity was performed mimicking the *in vivo* strategy. The rAAV containing sFlt-1 gene was transduced into 293 cells and the transgene product was obtained as a secreted protein in the supernatant. The supernatant was added to early passage HUVEC, grown in the presence of 10 ng/ml VEGF. Seventy two hours later, the cells were fixed and stained with crystal violet. The proliferation index was determined by microphotography. Results, given in Figure 3, indicated a significant inhibition of HUVEC proliferation following the addition of supernatant from rAAV-sFlt1 transduced cells but not from the mock-transduced cells. As a positive control, purified recombinant human angiostatin was used at a concentration of 20 μg/ml, which also showed a significant inhibition of HUVEC proliferation.

Development of a xenograft tumor model and vector treatment. As a model system to evaluate the *in vivo* anti-angiogenic effects of rAAV-mediated long-term expression of sFlt-1, we developed subcutaneous tumors with an

angiogenesis-dependent human ovarian cancer cell line SKOV3.ip1. Our pilot studies indicated that palpable tumors begin to appear 8-10 days following subcutaneous implantation of 10^7 SKOV3.ip1 cells in nude mice (data not shown). Since we based the present studies to evaluate the potential of AAV-mediated sFlt-1 gene therapy as a possible preventive therapy against the growth and recurrence of solid tumors, the animals were first injected with rAAV encoding sFlt-1. Also because optimal expression of rAAV transgenes is not achieved until at least two weeks after vector administration, tumor challenge was performed 3-weeks after vector administration. Each animal received an injection in both flanks. All the naïve animals and rAAV-GFP administered animals developed palpable tumors by 8 days after injection. The animals that developed tumors were monitored until the tumor volume reached 1800 mm^3 and euthanized according to the IACUC approved guidelines. Tumor-free animals were monitored up to 130 days before terminating the experiment.

Evidence of transgene persistence and expression following rAAV-sFlt1 therapy *in vivo.* A major advantage of using rAAV in muscle-based gene therapy is the stable retention of transgene(s) as either integrated or episomal copies for long-term expression. Since anti-angiogenic gene therapy is directed towards inhibiting proliferation of tumor neovasculature and not tumor cells directly, an important requirement for anti-angiogenic therapy is stable expression of these factors at therapeutic levels. Studies have reported that administration of rAAV in muscle not only results in long-term expression of the

transgene but also that vector administered in this way does not elicit any host immune response against the transgenic protein, which would otherwise eliminate transgene-expressing cells (4,24). Thus, in the present study, we sought to achieve this result by using skeletal muscle as a target tissue for stable integration of rAAV and systemic secretion of sFlt-1. To demonstrate the long-term persistence of the AAV transgene, we performed genomic PCR and *in situ* hybridization, and to determine the systemic secretion of sFlt-1 from transduced muscle, we performed immunohistochemistry.

PCR analysis of vector genome was performed using genomic DNA isolated from naïve or rAAV-sFlt-1 treated mouse muscle. The forward and reverse primers consisted sequences of CMV promoter and human sFlt-1 coding sequences respectively. An amplification product of 548 base-pairs, only in rAAV-sFlt-1 treated mice [Figure 4(i)] confirmed the persistence of vector genome. For *in situ* hybridization analysis, we used a digoxigenin (DIG)-labeled CMV promoter sequence as the probe. Results, shown in Figure 4(iii), also demonstrate the persistence of the AAV transgene in the skeletal muscle of only the vector-treated mice after 130 days. The use of CMV-promoter sequence as a probe in the *in situ* hybridization experiment also eliminated the possibility of hybridization signal from transgenic mRNA transcripts as well as cellular Flt-1 genomic DNA. Although the results of PCR or *in* situ hybridization do not confirm the integration of the rAAV genome into the host, increasing evidence indicate that only a small amount of rAAV transgenes integrate *in vivo* (25). Nonetheless,

in skeletal muscle, concatemerization of the rAAV genome allows long-term retention of transgenes as circular episomes (6,7).

Immunohistochemistry was performed in paraffin sections of the transduced muscle to determine the expression of human sFlt-1. Data, shown in Figure 4B, indicates systemic secretion of human sFlt-1 from the quadriceps muscle, only from rAAV-sFlt1-treated mice.

Stable expression of sFlt-1 inhibits tumor growth *in vivo.* The results of the *in vivo* studies on growth kinetics of SKOV3.ip1 cells and tumor-free survival with a vector dose of $3x10^{11}$ particles are given in Figures 5 and 6 respectively. However, when a vector dose of $3x10^{10}$ particles was used, although there was a modest increase in tumor-free survival and a lesser mean tumor volume was noted, these effects were not statistically significant (data not shown). Palpable tumors started to develop 8 days after tumor challenge in naïve animals. When a dose of $3x10^{10}$ particles of rAAV-sFlt1 was given, tumor-free survival was seen in >33% of the animals ($p>0.05$) whereas a dose of $3x10^{11}$ particles protected 83% of the mice ($p<0.005$). At a higher vector dose, there was also a significant difference in the mean tumor volume between different treatments (naïve or GFP versus sFlt-1, $p<0.0001$). There was a slight delay in the appearance of tumors in the rAAV-GFP treated group compared to naive animals, which did not receive any vector.

rAAV-sFlt-1 therapy does not cause liver damage. Previous studies have indicated that higher levels of sFlt1 in mouse liver cause significant hepatotoxicity associated with hemorrhage of blood vessels in the liver (14). VEGF has been reported to regulate the proliferation and survival of the sinusoidal endothelial cells acting through the VEGF receptor Flt-1 and Flk-1 (26,27). Blockade of the receptors, resulting from higher concentrations of sFlt-1 leads to such damage. Thus overexpression of sFlt1 from systemically delivered adenovirus vector, which endogenously targets liver, has been reported to result in similar damage (14). To determine if stable systemic expression of sFlt-1 by rAAV leads to liver toxicity, liver tissues were macroscopically and microscopically examined following rAAV-sFlt-1 treatment. Hematoxylin and eosin (H&E) stained sections from naïve and AAV-sFlt-1 treated mice showed no cytomorphological signs of hepatotoxicity strongly suggesting that systemic expression of sFlt-1 from skeletal muscle does not result in liver toxicity (Figure 7). Further, immunohistochemical staining for human sFlt-1 in the liver sections did not reveal accumulation of systemically expressed sFlt-1 (data not shown) indicating that delivery of rAAV-sFlt-1 in skeletal muscle and systemic secretion of the factor at stable levels would be advantageous. Also, several studies using rAAV encoding a variety of therapeutic proteins have shown stable systemic expression of transgenic factors following intramuscular vector administration.

Discussion

Recent studies have indicated the potential of sFlt-1 gene therapy in murine models of human diseases using viral vectors (15,28-32). Whereas studies using adenoviral and retroviral vectors have reported the efficacy of sFlt-1 gene therapy in inhibiting the growth of human tumors, that using AAV reported the effects in retinal diseases and *in vivo* growth of a stably transduced cell line (31,32). The present study demonstrates that stable expression of sFlt-1 as a secretory protein from skeletal muscle by rAAV provides significant protection and long-term survival of mice against the growth of a human tumor xenograft suggesting its potential in cancer gene therapy.

The potential use of rAAV-sFlt1 as a vector for anti-angiogenic therapy of solid tumors is multifold. Since tumor angiogenesis is mediated by a cascade of signals provided by cells of both tumor and endothelial origin, which ultimately results in the growth and differentiation of endothelial cells forming the tumor neovasculature, therapy targeting such an event should be sustained to effect a maximal tumoristatic response. Stable expression of transgenic factors as secretory proteins by administering rAAV in skeletal muscle has been well established in preclinical models (6,7,33-37) and formed the basis for a human clinical trial (38). Thus, the proven efficiency of muscle-based administration of rAAV-sFlt-1 should provide sustained anti-angiogenic effects. Since sFlt-1 is a native protein, there is no expected host immune response against the transgenic factor when used for therapy in humans. Further, the cytolytic T-cell response against rAAV-transduced muscle is known to be minimal due to both

low-immunogenicity against the vector and poor transduction of rAAV to dendritic cells (24). Thus, the potential of muscle-based rAAV-sFlt-1 therapy should be advantageous. Although several drugs have shown promise in controlling tumor neovasculature, a major problem in pharmacotherapy is the profound side effects of constant administration due to their limited half-life (39). Gene therapy, on the other hand, offers advantages of maximizing cost effectiveness while maintaining sustained levels of anti-angiogenic factors.

Although many factors are known to play important roles in new blood vessel formation, a key molecule promoting the growth of tumor neovasculature is VEGF, which has been considered a predictive marker in many human cancers (9-13). Further, overexpression of VEGF mRNA and elevated serum VEGF levels have been correlated with decreased survival in many neoplastic conditions including ovarian cancer (40). Despite higher levels of plasma VEGF levels observed in many human cancers, there was no concomitant increase in sFlt-1 levels in cancer patients indicating an imbalance in the native anti-angiogenic signal pathway (41). In patients who showed earlier relapse of breast cancer, tumor VEGF levels were higher than in patients with a longer disease-free survival and the rate of response to chemotherapy decreased with higher VEGF levels (42). Hence, a stable gene therapy approach targeting VEGF as an adjuvant therapy offers the promise of increased survival in patients.

Inhibition of endogenous VEGF levels by the administration of VEGF antibody alone or in combination of topoisomerase inhibitors has resulted in effective immunotherapy and reduction of Wilms' tumor respectively in murine

models (43,44). Unlike other biologically-driven anti-angiogenic factors such as angiostatin and endostatin whose mechanism of action are not fully elucidated despite their anti-angiogenic effects, the biological properties of sFlt-1 are well known. The sFlt-1 acts by both sequestering VEGF and blocking VEGF receptors from binding to VEGF (8). Thus, therapy targeting VEGF will have specific effects on tumor growth inhibition and metastasis.

We have recently reported that intravenous administration of adenovirus encoding sFlt-1 results in a high level of systemic toxicity due to vector accumulation and maximal transgene expression in the liver (14). Using skeletal muscle as a platform to achieve systemically therapeutic levels of sFl-1, rAAV can overcome such a deleterious effect in addition to offering the advantage of long-term expression. Further, we also histopathologically evaluated the liver of mice, treated with sFLt-1 and observed no cytomorphic damage.

Results of our studies additionally indicated that significant therapeutic effect was seen only with a vector dose of $3x10^{11}$ genomic particles in mice. Thus, a clinical translation of these findings would require careful determination of optimal vector dose. Although we did not quantitatively determine the systemic levels of sFlt-1 following therapy, at the therapeutically efficacious dose, immunohistochemical analysis of rAAV-sFlt-1 injected muscle and adjacent blood vessels clearly indicated abundant expression and secretion of sFlt-1 suggesting that delivery of the sFlt-1 transgene in muscle is a safer method of overcoming any potential toxicity due to systemic delivery of a recombinant adenovirus encoding sFlt-1.

A recent study on the potential use of rAAV encoding sFlt-1 in ovarian cancer reported that transduction of the human ovarian cancer cell line, RMG-1, with AAV-sFlt1 *in vitro* followed by intraperitoneal administration in nude mice resulted in a decrease in proliferative and metastatic indices, further suggesting the feasibility of localized AAV-sFlt-1 anti-angiogenic gene therapy (32). However, a major limitation of intratumoral delivery is the limited transduction efficiency and dispersion of the vector within the tumor cells. Also, unlike certain genetic metabolic diseases, which require only partial amounts of the deficient protein/enzyme for phenotypic correction of the disease, tumor therapy requires inhibition of the tumor growth *in toto*. Anti-angiogenic therapy, in particular, requires a constant level of the inhibitory factor(s) for a sustained therapeutic effect. Thus, the strategy that we adapted in the present study will likely have greater potential and translational utility for human cancers, in particular, as an adjuvant therapy against tumor recurrence. Although several preclinical studies have shown that stable levels of systemically secreted proteins using rAAV resulted in the phenotypic correction of inherited metabolic defects (6,7), accumulation of anti-angiogenic factors in other organs due to unregulated expression may lead to ischemic conditions or impair wound healing. Thus, future studies are warranted to test the efficacy of regulated expression of these factors by using inducible promoters, for a safe muscle-based rAAV anti-angiogenic gene therapy.

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Figure Legends

Figure 1. Recombinant AAV encoding human sFlt-1. rAAV containing human sFlt1 was subcloned under the control of the CMV promoter. Poly A represents the SV40 late polyadenylation signal sequence and ITR represents the inverted terminal repeat sequence of AAV.

Figure 2. Western blot analysis of rAAV-sFlt-1 expression. 293 cells were either mock-transduced (2) or transduced with rAAV encoding sFlt-1 (3). Forty-eight hrs after transduction, the supernatant was analyzed by Western blotting using a monoclonal antibody against human sFlt-1. A recombinant purified sFlt-1 protein, fused to the Fc-portion of immunoglobulin was used as a positive control (lane 1).

Figure 3. Endothelial cell proliferation assay to determine the biological activity of rAAV-sFlt-1. rAAV encoding sFlt-1 was transduced into 293 cells at an MOI of 100. Forty-eight hours later, 25 µl of supernatant from mock-transduced (A) or rAAV-sFlt-1transduced (B) cells were tested on HUVEC. Cell proliferation was determined by fixing and staining the HUVEC with crystal violet. Purified recombinant human angiostatin was used at a concentration of 20 µg/ml (C) as a positive control. Magnification x20.

Figure 4. PCR, *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Top Pane: PCR was performed

using genomic DNA isolated from naïve or rAAV-sFlt-1 injected muscle using a primer pair specific for the amplification of the transgene. Whereas no amplification product was seen in DNA from naïve mice, (lanes 1 & 2), a 548 bp fragment, specific for the transgene, was amplified from rAAV-sFlt-1 treated mice [lanes 3 & 4, (i)]. Sham- or rAAV-transduced muscle tissues were harvested and immediately fixed in buffered-formalin for 24 hr and then embedded in paraffin. *In situ* hybridization was performed using a DIG-labeled DNA probe on sections obtained from naïve (ii), or rAAV-sFlt1 (iii) administered muscle tissues. A two-fold magnification of a specific area is indicated in the box, showing positive signal only in the vector-transduced group. **Bottom Panel:** Immunohistochemistry was performed using a human VEGF receptor-1 antibody, which recognizes the extracellular domain of VEGF receptor. Extracellular secretion of the transgenic factor from the vector-transduced muscle tissue is evident in rAAV-sFlt1 treated animal (B) compared to naïve mouse (A) .

Figure 5. Growth characteristics of subcutaneously implanted SKOV3.ip1 cells in athymic nude mice following therapy with rAAV-sFlt-1. Three weeks after sham (♦), $3x10^{11}$ AAV-GFP (□), or rAAV-sFlt1 (▲) injection, 10^7 SKOV3.ip1 cells were subcutaneously implanted. Tumor size was measured using a digital caliper. The average tumor volume in each group is given in mm^3. * indicates *P* value <0.0001, compared to naïve and AAV-GFP groups.

Figure 6. Tumor-free survival of mice following rAAV-sFlt1 therapy. $3x10^{11}$ particles of rAAV encoding GFP, or sFlt1 were injected into the quadriceps muscle in the hind limb of athymic nude mice. Three weeks after the vector injection, the animals were challenged with 10^7 SKOV3.ip1 cells in each flank subcutaneously and tumor-free survival recorded. The mean tumor-free survival of naïve (♦), rAAV-GFP (□), or rAAV-sFlt1 (▲) is given above.

Figure 7. Absence of hepatotoxicity following rAAV-sFlt1 therapy. Livers from naïve or rAAV-sFlt-1 treated mice were formalin-fixed, sectioned to 5 μm thickness and stained with H&E. The stained slides were analyzed by light microscopy for hepatotoxicity including hemorrhage and congestion, focal necrosis with minimal inflammation, hepatocyte dropout, or increased apoptosis. There was no difference that existed in the histopathology of liver sections between naïve mice (A) and rAAV-sFlt1-treated mice (B).

Figure 1

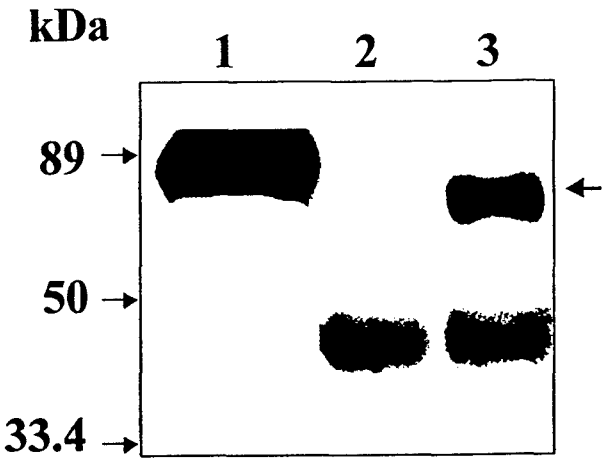

Figure 2

Figure 3

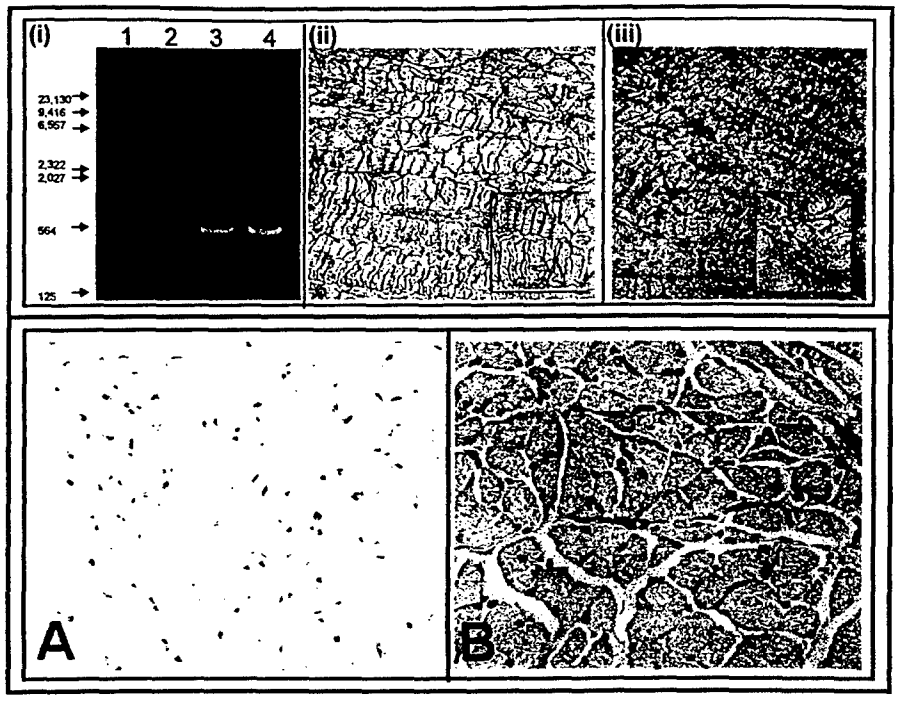

Figure 4

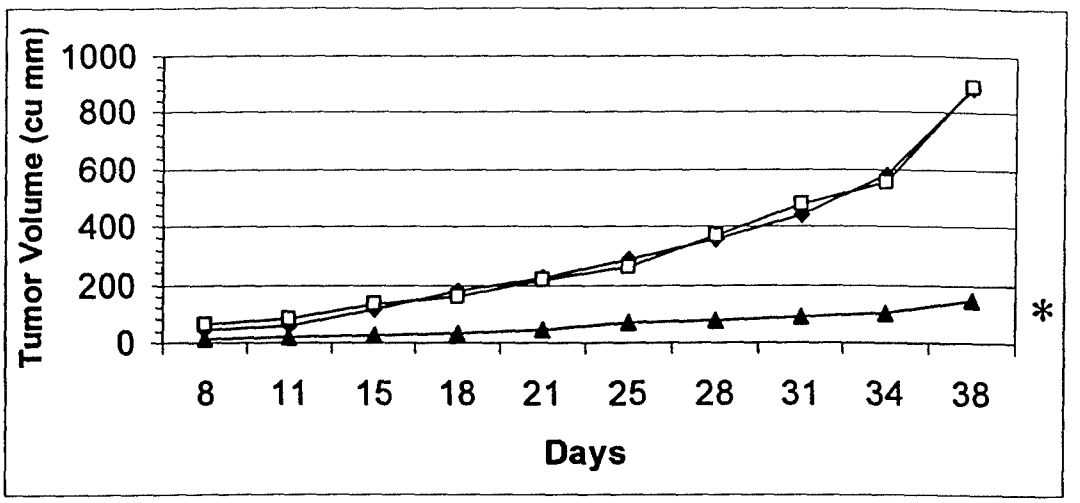
Tumor Volume (cu mm)
1000
800
600
400
200
0
8
11
15
18
21
25
28
31
34
38
Days
*

Figure 5

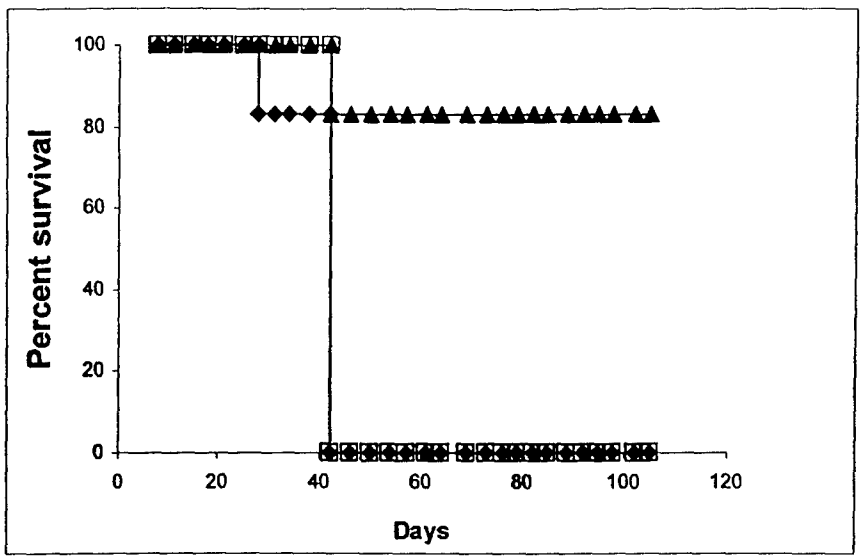

Figure 6

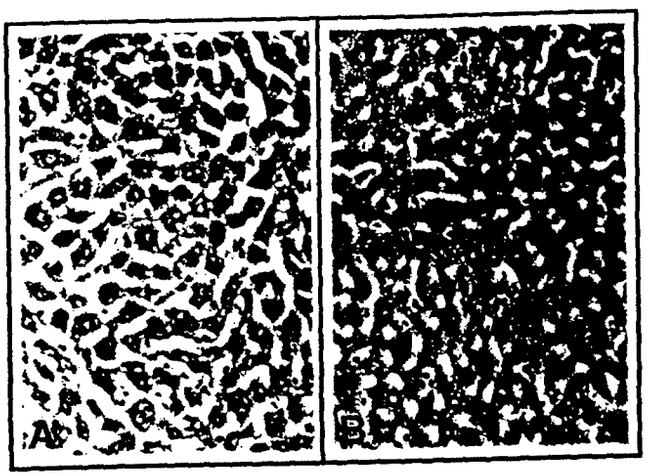

Figure 7