

Recombinant Adeno-Associated Virus 2-Mediated Antiangiogenic Prevention in a Mouse Model of Intraperitoneal Ovarian Cancer

Tatyana Isayeva, Changchun Ren, and Selvarangan Ponnazhagan

Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama

ABSTRACT

Purpose: In the present study, we sought to determine the potential of sustained transgene expression by a single i.m. administration of recombinant adeno-associated virus 2 (rAAV) encoding angiostatin and endostatin in inhibiting i.p. ovarian cancer growth and dissemination in a preclinical mouse model.

Experimental Design: Cohorts of female athymic nude mice received either no virus or 1.2×10^{11} particles of rAAV encoding green fluorescence protein or endostatin plus angiostatin, i.m. Three weeks later, the mice were i.p. injected with 10^6 human epithelial ovarian cancer cell line SKOV3.ip1. As a measure of effectiveness of the therapy, tumor weight, abdominal distension, ascites volume and vascular endothelial growth factor level, and tumor weight were determined. Immunohistochemistry was done to determine tumor cell apoptosis and endothelial cell proliferation following the therapy. Tumor-free survival was recorded as the end point.

Results: Results indicated a significant tumor-free survival ($P < 0.003$) following therapy with rAAV encoding endostatin and angiostatin compared with untreated or rAAV-green fluorescence protein-treated mice. Ascites volume in rAAV endostatin and angiostatin-treated mice was significantly lower than naive mice and contained less hemorrhage and tumor conglomerates. The level of vascular endothelial growth factor in the ascites of antiangiogenic vector treated mice was also significantly less compared with the untreated mice. Immunohistochemical analyses indicated increased tumor cell apoptosis and decreased blood vasculature following rAAV endostatin and angiostatin treatment.

Conclusion: The results indicate that antiangiogenic genetic prevention from stable systemic levels of angiostatin and endostatin by i.m. administration of rAAV can be used

for the treatment of i.p. ovarian cancer growth and dissemination.

INTRODUCTION

Ovarian cancer is the second most common gynecologic malignancies in women (1). Because ovarian carcinoma frequently remains clinically silent, a majority of patients with the disease have advanced i.p. dissemination during diagnosis. The mean survival rate for disseminated ovarian cancer is <5 years (2, 3). Despite a better understanding of the disease pathology, surgery, and chemotherapy remain the major therapeutic interventions for ovarian cancer. Like most of the solid tumors, ovarian cancer growth and metastasis is dependent on new blood vessel formation by the process of angiogenesis (4, 5). Thus, therapies targeting angiogenesis are promising for the control of tumor growth in patients with ovarian cancer. Because the tumorstatic antiangiogenic therapy targets endothelial cells, effects of this therapy should be sustained without toxicity. Gene therapy approaches seem promising for this purpose.

We recently showed using a recombinant adeno-associated virus (rAAV) that stable systemic expression of antiangiogenic factors following i.m. vector administration results in significant inhibition of a human epithelial ovarian cancer cell line, SKOV3.ip1, grown as a s.c. xenograft in nude mice (6). Although this study showed the effects of rAAV antiangiogenic gene therapy, unlike s.c. tumors, the growth of epithelial ovarian cancer is highly disseminative, accompanied by excessive i.p. ascites and exfoliation of tumor cells in the peritoneal cavity, which limits the efficacy of drugs and other therapeutic molecules from reaching tumor cells. The presence of excess ascites at the time of laparotomy for ovarian cancer patients has also been associated with poor prognosis (7) and the amount of vascular endothelial growth factor (VEGF) in ascites correlates to the disease pathology (8, 9).

Thus, in the present study, we sought to determine if rAAV-mediated expression of angiostatin and endostatin as secretory factors following i.m. administration of the vector will reduce VEGF levels, ascites burden, and prolong survival in a preclinical mouse model of i.p. ovarian cancer. The results indicate the effectiveness of this molecular therapy.

MATERIALS AND METHODS

Cells and Reagents. Human embryonic kidney cell line 293 was purchased from American Type Culture Collection (Manassas, VA) and maintained in Iscove's modified essential medium supplemented with 10% newborn calf serum. The human epithelial ovarian cancer cell line SKOV3.ip1 was a gift from Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX) and was maintained in Eagle's MEM containing nonessential amino acids and 1 mmol/L sodium pyruvate supplemented with 10% fetal bovine serum. The cells were cultured in 37°C with 5% CO₂. The SKOV3.ip1 cells were harvested from

Received 7/12/04; revised 11/2/04; accepted 11/11/04.

Grant support: NIH grants R01CA90850 and R01CA98817 and U.S. Army Department of Defense grants BC010494 and PC020372.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Selvarangan Ponnazhagan, Department of Pathology, LHRB 513 701, 19th Street South, University of Alabama at Birmingham, Birmingham, AL 35294-0007. Phone: 205-934-6731; Fax: 205-975-9927; E-mail: sponnazh@path.uab.edu.

©2005 American Association for Cancer Research.

subconfluent culture by brief exposure to 0.25% trypsin, stopped with medium, containing 10% serum, were washed twice, and resuspended in PBS. Only single-cell suspension with >95% viability was used for *in vivo* injection.

Restriction endonucleases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Co. (Madison, WI). A mouse monoclonal antibody for Ki67 (clone SP6) and a rabbit polyclonal poly(ADP-ribose) polymerase (PARP) p85 fragment were obtained from Research Diagnostics, Inc. (Flanders, NJ) and Promega, respectively. Secondary antibodies and color reagents were purchased from Amersham (Piscataway, NJ). The mouse VEGF ELISA kit was purchased from R&D System, Inc. (Minneapolis, MN).

Recombinant Plasmids, Production, and Purification of rAAV. Construction of recombinant plasmids containing secretable form of human angiostatin and endostatin as bicistronically expressed proteins and that encoding green fluorescence protein (GFP) was recently published (6). Production and purification of rAAV was done by transient transfection in 293 cells followed by iodixanol gradient centrifugation and heparin affinity column chromatography (10). The particle titer of purified virions was determined by quantitative slot blot analysis (11).

***In vivo* Studies.** Six-week-old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD). All the animal studies were done in accordance with 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. Approximately 10^{11} genomic particles of rAAV encoding GFP or endostatin and angiostatin, in normal saline, were injected in a volume of 100 μ L in the quadriceps muscle in the hind limbs. Naive animals did not receive any vector. Each group consisted of 10 mice. Three weeks after vector administration, each mouse received 10^6 SKOV3.ip1 cells by i.p. injection. The onset of i.p. tumors was determined based on significantly increased abdominal circumference due to ascites. Comparisons were made between animals in the control (mice without AAV or tumor challenge), naive (mice with SKOV3.ip1 cells but no rAAV), and rAAV (rAAV GFP/endostatin + angiostatin + SKOV3.ip1 challenge) groups for abdominal volume. The abdominal area was carefully checked to detect palpable tumors in these groups. The animals were monitored twice every week for body weight and tumor formation in the peritoneal cavity and were euthanized when they become moribund, the day of euthanasia considered as the limit of survival. Ascites fluid and peritoneal tissues were harvested for further analyses. The weight of solid peritoneal tumors and ascites volume were recorded. Blood samples were collected from all animals before vector administration, before tumor cell implantation, and at sacrifice.

Immunohistochemistry. Immunohistochemical studies were done in 5- μ m sections of paraffin-embedded tumor tissues using antibodies for Ki67 and anti-PARP p85 for the determination of proliferation and apoptosis indices respectively. Antigen retrieval was achieved by incubating the slides

in 0.05% trypsin for 20 minutes at 37°C and endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 10 minutes in room temperature. The anti-Ki67 monoclonal antibody was used in a working dilution of 1:50, and the anti-PARP p85 polyclonal antibody in a dilution of 1:50. Furthermore, the slides were stained with a donkey anti-rabbit horseradish peroxidase-linked secondary antibody (1:500 dilution). To determine the proliferation and apoptotic indices, stained slides were examined under high power ($\times 40$). A minimum of 10 randomly chosen fields were counted to determine the total number of cells and that stained positive in each field. The percentage proliferation and apoptosis was calculated using the formula: (number of positively stained cells/total number of cells in a field) \times 100. The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride, and tissues were counterstained minimally with hematoxylin.

ELISA. Ascites fluid was harvested on the day of sacrifice of the animals due to tumor burden and the volume measured. The ascites fluid was briefly centrifuged to remove loose cells and the supernatant frozen at -80°C until analysis. The VEGF levels in ascites were determined using a commercial ELISA kit (R&D System), which recognized the 164 and 120 amino acid residues of mouse VEGF.

Toxicity Analysis. Hepatic toxicity was assessed by quantitative measurement of serum alanine aminotransferase using a commercial kit (TECO Diagnostics, Anaheim, CA), and histopathology of serial liver sections by H&E staining. The effects of rAAV treatment in other major peritoneal organs, including kidney, ovary, and spleen were also determined histopathologically by H&E staining of tissues obtained from control and rAAV-treated mice.

Statistical Analysis. Data were compiled as mean \pm SE in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student's *t* test was done. *P*s < 0.05 were considered to indicate significant difference between data sets.

RESULTS

Development of Mouse Model of i.p. Ovarian Cancer.

An experimental model of ovarian cancer was developed with 6-week-old female athymic nude mice. To establish i.p. tumor, each mouse was injected with 10^6 SKOV3 i.p.1 cells i.p. Mice that developed ascites or lost 10% of body weight were euthanized. With this amount of tumor cells, mice developed palpable tumor between days 15 and 20. Upon sacrifice, in the peritoneum, visible tumors were found under the diaphragm, intestine, and in the peritoneal cavity. The mean survival of untreated mice was found to be 45 days after tumor cell implantation.

Treatment with rAAV Endostatin and Angiostatin Significantly Decreases Ascites Volume and VEGF Levels.

Because SKOV3.ip1 cells form tumors around 2 weeks following i.p. administration and that optimal expression of rAAV transgenes does not occur until 2 to 3 weeks after vector injection, the present study was designed to pretreat the mice with rAAV before tumor challenge. Based on the kinetics of rAAV transgene expression, we evaluated the therapeutic

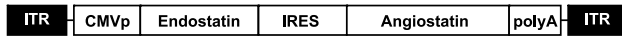


Fig. 1 Recombinant AAV encoding endostatin and angiostatin as bicistronically expressed proteins. rAAV containing human endostatin and angiostatin as a bicistronic cassette was subcloned under the control of the CMV promoter. *PolyA*, SV40 late polyadenylation signal sequence; *ITR*, inverted terminal repeat sequence of AAV.

efficacy of a one-time i.m. injection of rAAV endostatin and angiostatin (Fig. 1) before tumor cell implantation. Cohorts of mice were given 10^{11} genomic particles of rAAV encoding endostatin and angiostatin as bicistronically expressed proteins from a single vector or rAAV encoding GFP to determine vector-related nonspecific effects.

By serum ELISA, we determined that this dose of vector produced a mean 196 ng/mL endostatin and 227 ng/mL angiostatin systemically beginning 3 weeks. The peak level expression achieved at 3 weeks after vector injection was found to be stable for over 4 months without any diminution or toxicity. The SKOV3.ip1 cells were implanted i.p. at a dose of 10^6 cells per mouse. Approximately 2 weeks following tumor cell injection, abdominal distension was noted in the group of mice, which received no treatment. However, in the group of mice treated with rAAV endostatin and angiostatin, there was a significant decrease in the ascites volume ($P < 0.05$) as shown in Fig. 2. In addition, the ascites from naive mice was more hemorrhagic, whereas that from rAAV endostatin and angiostatin treated group was clear. The VEGF levels in ascites fluid also showed a significant decrease in the group of mice treated with rAAV endostatin and angiostatin ($P < 0.05$) indicating the specificity of the antiangiogenic effect (Fig. 3).

Recombinant AAV Endostatin Plus Angiostatin Therapy Decreases Tumor Cell Proliferation and Increases Apoptosis. The mean tumor weight in rAAV endostatin and

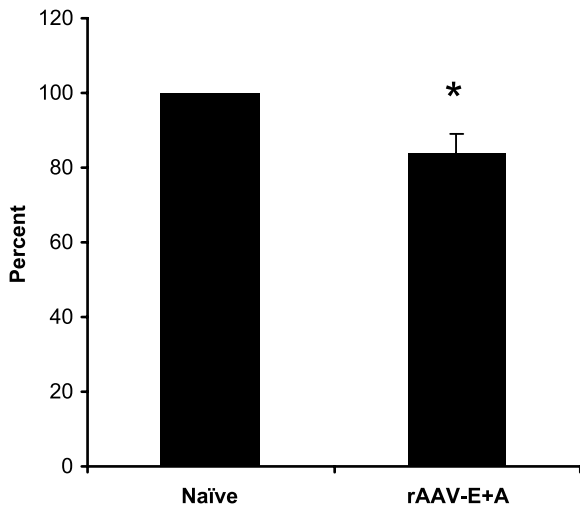


Fig. 2 Effects of rAAV endostatin + angiostatin therapy in ascites volume. The mean ascites volume was determined in naive and rAAV endostatin + angiostatin-treated mice following peritoneal lavage and expressed as percentage relative to levels obtained in naive mice. *, $P < 0.05$ compared with naive animals.

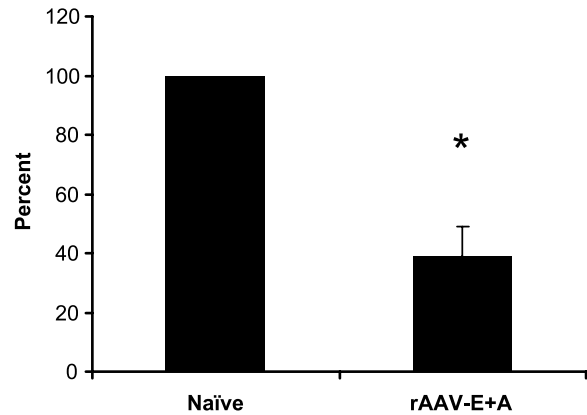


Fig. 3 Ascites VEGF levels following rAAV endostatin + angiostatin therapy in ascites volume. VEGF level in the ascites was determined by ELISA. Columns, mean compared with that obtained in naive mice as 100%; bar, \pm SE. *, $P < 0.0001$ compared with naive mice.

angiostatin-treated group was significantly less ($P < 0.0002$) compared with naive mice as shown in Fig. 4. To determine if stable systemic levels of endostatin and angiostatin from rAAV transgene effects on the proliferation and apoptosis of i.p. ovarian cancer cells, immunohistochemistry was done with Ki67 and anti-PARP antibodies respectively. The *in situ* effects of angiostatin and endostatin gene transfer are illustrated in immunohistochemical analysis of tumor cell proliferation and apoptosis compared with naive animals (Fig. 5). Whereas $>30\%$ of implanted tumor cells in the rAAV-treated group were apoptotic compared with animals in the naive group, no significant differences was found between the naive and rAAV-GFP-treated animals ($P > 0.05$, data not shown) indicating the specificity of the transgenic factors. The median proliferation index of tumor cells in rAAV endostatin

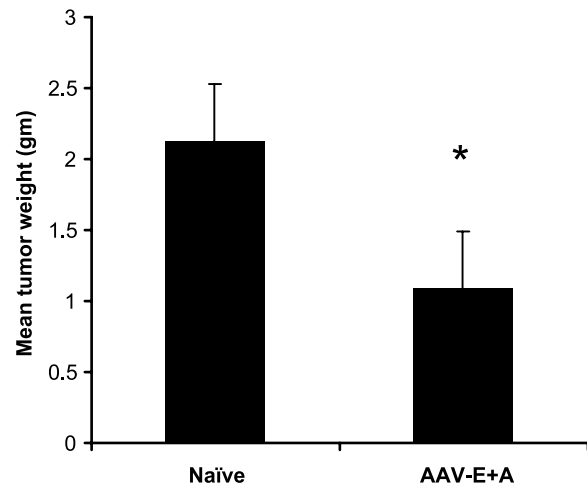


Fig. 4 Effects of rAAV endostatin + angiostatin gene therapy in tumor burden. Tumor nodules from the peritoneal cavity of naive and rAAV endostatin + angiostatin-treated mice were collected and weighed. Columns, mean weight of tumors. *, $P < 0.0002$ compared with naive animals.

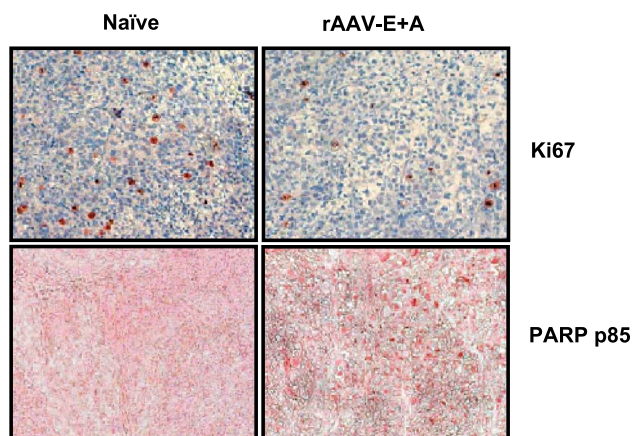


Fig. 5 Immunohistochemical analysis of tumor tissues for tumor cell proliferation and apoptosis. I.p. tumor nodules were isolated during sacrifice and fixed in buffered formaldehyde. Immunohistochemical staining to determine tumor proliferation and apoptosis was done in 5- μ m sections using the Ki67 and anti-PARP antibody, respectively, and visualized under a light microscope. Magnification $\times 20$.

and angiostatin treated mice was 22%, significantly less than in control group, which showed a proliferation index of 61%.

Stable Antiangiogenic Gene Transfer Increases Tumor-free Survival of Mice. Following tumor cell implantation, mice were monitored at least twice every week as described in Materials and Methods for tumor growth up to 150 days when the experiments were terminated. The result of tumor-free survival is shown in Fig. 6. There was a significant protective effect of AAV-mediated antiangiogenic gene expression with both endostatin and angiostatin compared with control or rAAV-GFP-treated animals ($P < 0.003$). There were no apparent effects such as loss of body weight, general mobility, or food uptake in the long-term surviving rAAV- endostatin and angiostatin-treated mice compared with untreated mice that were not tumor challenged. Serum alanine aminotransferase

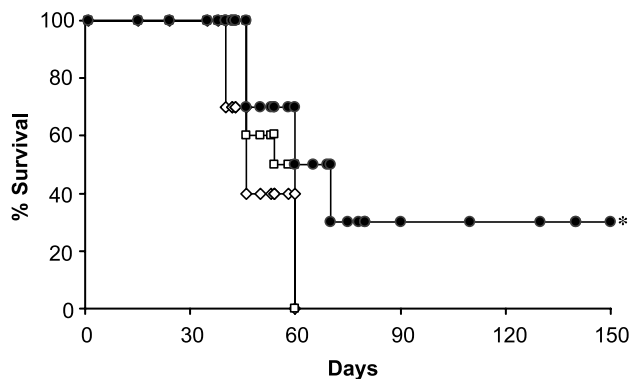


Fig. 6 Tumor-free survival following rAAV therapy. Following no treatment (\diamond) or i.m. injection of 10^{11} particle of rAAV encoding GFP (\square) or endostatin + angiostatin (\bullet), 10^6 SKOV3.ip1 cells were i.p. injected. The animals were monitored for body weight, abdominal distension, and peritoneal tumor growth. When the animals became moribund or the tumor size/abdominal volume reached Institutional Animal Care and Use Committee stipulated limits, they were euthanized. Tumor-free animals were maintained for 150 days before terminating the experiment. *, $P < 0.003$ compared with naive and rAAV-GFP-treated animals.

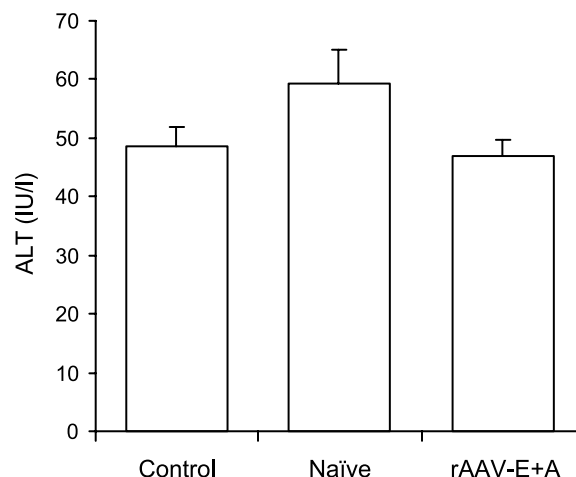


Fig. 7 Serum alanine aminotransferase (ALT) measurement following rAAV therapy. Serum samples from control (untreated and unchallenged), naive (no treatment but challenged with SKOV3.ip1 cells), and rAAV (endostatin + angiostatin and challenged with SKOV3.ip1) were used to determine the ALT levels by a colorimetric method. Points, mean \pm SD.

ase levels, as a measure of hepatotoxicity, suggested no difference between control and rAAV treatment groups (Fig. 7). The absence of microscopic abnormalities in liver, ovary, spleen, and kidney in rAAV-treated group was also confirmed by histopathology (data not shown).

DISCUSSION

Although the growth of ovarian cancer depends on neovasculature, unlike organ-localized solid tumors, the growth of i.p. ovarian cancer is highly disseminative. The end-stage disease is characterized by the accumulation of i.p. ascites fluid, decreasing intracellular levels of given drugs, posing a great challenge. We recently showed that production of systemically stable levels of angiostatin and endostatin by rAAV gene transfer abrogated the growth of s.c. implanted human ovarian cancer cell line SKOV3.ip1 (6). As a logical extension of these studies, we determined the effects of stable systemic expression of angiostatin and endostatin following a single i.m. administration of the vector on i.p. growth and dissemination of SKOV3.ip1 cells.

Results indicated a significant protective effect of rAAV-mediated expression of endostatin and angiostatin against i.p. growth of ovarian cancer in nude mice. Peritoneal dissemination of ovarian cancer originates by their release into ascites, which initiates the process of metastasis. Many reports have suggested that the major angiogenic factor which plays an important role in the vascularization of neoplastic tissue and ascites formation is VEGF, also known as vascular permeability factor. The VEGF induces ascites accumulation by increasing the permeability of diaphragmatic and tumor-associated vasculature (12, 13).

Previous studies have reported that administration of monoclonal antibody to human VEGF can prevent ascites formation in a mouse model of i.p. ovarian carcinoma with SKOV3 cells (14). Compared with protein or pharmacotherapies, gene transfer approach provides greater benefit of stable

systemic levels of the antiangiogenic factors. The advantages of using rAAV over other vectors are nonpathogenicity, long-term transgene expression, and absence of vector-related cellular immune response (15, 16). Our studies established that a single i.m. administration of rAAV encoding angiostatin and endostatin results in systemic levels of these factors between 177 to 277 and 176 to 206 ng/mL, respectively, in serum after 3 weeks and remained stable for over 4 months without any apparent toxicity.

The antiangiogenic mechanism of endostatin and angiostatin are beginning to be discovered. Although angiostatin seems to exert antiangiogenic effect by primarily inhibiting the proliferation and invasion of endothelial cells (17, 18) and inducing endothelial cell apoptosis (19), endostatin reduces endothelial cell proliferation (20) and migration (21) and significantly reduces the invasion of endothelial as well as tumor cells into the reconstituted basement membrane (22). Thus, a combination of angiostatin and endostatin is likely to increase the inhibitory effect on ascites formation as observed in the present studies.

The results of these studies indicate that antiangiogenic gene therapy by stable systemic levels of angiostatin and endostatin following i.m. administration of rAAV can be used for the treatment of i.p. ovarian cancer growth and dissemination. This strategy may be combined with other therapies, including chemotherapy to increase the survival of ovarian cancer patients. A recent study involving i.m. administration of rAAV encoding angiostatin in human glioma xenografts (23) and i.m. administration of rAAV endostatin in a colorectal cancer model have also shown therapeutic efficacy (24). However, the growth characteristics of i.p. ovarian cancer limit intratumoral administration due to dissemination of the tumor cells and poor transduction efficiency of the primary tumors. Thus, administration of the vector in skeletal muscle may result in a therapeutically stable systemic level of the factors. Due to the lack of an effective screening method, insidious onset and nonspecific symptoms, a majority of women present with late-stage disease. In this situation, cytoreductive surgery and chemotherapy form the major therapeutic interventions. Although these therapies have increased the initial clinical response rates, recurrent disease remains a formidable challenge. As in the primary disease, the growth of recurring ovarian cancer is highly dependent on angiogenesis. Hence, antiangiogenic prevention of recurring cancer may prove beneficial in extending survival rates. Furthermore, ~10% to 15% of the patients develop the disease due to genetic predisposition. To such "at-risk" population, this approach could possibly help to delay or prevent the onset of the primary cancer.

Although in our studies we did not observe any toxicity following rAAV therapy, if constant systemic levels of the antiangiogenic factors prove toxic to patients, the approach presented in this study may be improved to achieve localized production of the factors within the tumor. Furthermore, although several preclinical studies have shown that stable levels of systemically secreted proteins using rAAV resulted in the phenotypic correction of inherited metabolic defects (25), accumulation of antiangiogenic 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 antiangiogenic gene therapy.

REFERENCES

- Shih IeM, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *Am J Pathol* 2004;164:1511–8.
- Ozols RF. Optimum chemotherapy for ovarian cancer. *Int J Gynecol Cancer* 2000;10:33–7.
- Thulesius HO, Lindgren AC, Olsson HL, Hakansson A. Diagnosis and prognosis of breast and ovarian cancer: a population-based study of 234 women. *Acta Oncol* 2004;43:175–81.
- Kikkawa F, Arii Y, Kawai M, Mizutani S. Randomized trial of cisplatin, vinblastine and bleomycin in ovarian cancer. *Gynecol Oncol Investig* 2000;50:269–74.
- Salvesen HB, Gulluoglu MG, Stefansson I, Akslen LA. Significance of CD 105 expression for tumour angiogenesis and prognosis in endometrial carcinomas. *APMIS* 2003;111:1011–8.
- Ponnazhagan S, Mahendra G, Kumar S, et al. Adeno-associated virus 2-mediated antiangiogenic cancer gene therapy long-term efficacy of a vector encoding angiostatin and endostatin over vector encoding a single factor. *Cancer Research* 2004;64:1781–7.
- Puls LE, Duniho T, Hunter JE, Kryscio R, Blackhurst D, Gallion H. The prognostic implication of ascites in advanced-stage ovarian cancer. *Gynecol Oncol* 1996;61:109–12.
- Paley PJ, Staskus KA, Gebhard K, et al. Vascular endothelial growth factor expression in early stage ovarian carcinoma. *Cancer* 1997;80:98–106.
- Shen GH, Ghazizadeh M, Kawanami O, et al. Prognostic significance of vascular endothelial growth factor expression in human ovarian carcinoma. *Br J Cancer* 2000;83:196–203.
- Zolotukhin S, Byrne B, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 1999;6:973–85.
- Ponnazhagan S, Erikson D, Kearns WG, et al. Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum Gene Ther* 1997;8:275–84.
- Shibuya M, Luo JC, Toyoda M, Yamaguchi S. Involvement of VEGF and its receptors in ascites tumor formation. *Cancer Chemother Pharmacol* 1999;43:S72–7.
- Doldi N, Bassan M, Gulisano M, Broccoli V, Boncinelli E, Ferrari A. Vascular endothelial growth factor messenger ribonucleic acid expression in human ovarian and endometrial cancer. *Gynecol Endocrinol* 1996;10:375–82.
- Hasumi Y, Mizukami H, Urabe M, et al. Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. *Cancer Res* 2002;62:2019–23.
- Davidoff AM, Nathwani AC, Spurbeck WW, Ng CY, Zhou J, Vanin EF. rAAV-mediated long-term liver-generated expression of an angiogenesis inhibitor can restrict renal tumor growth in mice. *Cancer Res* 2002;62:3077–83.
- Sarukhan A, Camugli S, Gjata B, von Boehmer H, Danos O, Jooss K. Successful interference with cellular immune responses to immunogenic proteins encoded by recombinant viral vectors. *J Virol* 2001;75:269–77.
- Cao Y, Ji RW, Davidson D, et al. Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells. *J Biol Chem* 1996;271:29461–7.
- Redlitz A, Daum G, Sage EH. Angiostatin diminishes activation of the mitogen-activated protein kinases ERK-1 and ERK-2 in human dermal microvascular endothelial cells. *J Vasc Res* 1999;36:28–34.
- Claesson-Welsh L, Welsh M, Ito N, et al. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc Natl Acad Sci U S A* 1998;95:5579–83.

20. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277–85.
21. Yamaguchi N, Anand-Apte B, Lee M, et al. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. *EMBO J* 1999;18:4414–23.
22. Kim YM, Jang JW, Lee OH, et al. Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase. *Cancer Res* 2000;60:5410–3.
23. Ma HI, Guo P, Li J, et al. Suppression of intracranial human glioma growth after intramuscular administration of an adeno-associated viral vector expressing angiostatin. *Cancer Res* 2002;62:756–63.
24. Shi W, Teschendorf C, Muzyczka N, Siemann DW. Adeno-associated virus-mediated gene transfer of endostatin inhibits angiogenesis and tumor growth *in vivo*. *Cancer Gene Ther* 2002;9:513–21.
25. Ho TT, Maguire AM, Aguirre GD, Surace EM, Anand V, et al. Phenotypic rescue after adeno-associated virus-mediated delivery of 4-sulfatase to the retinal pigment epithelium of feline mucopolysaccharidosis VI. *J Gene Med* 2002;4:613–321.