Adeno-Associated Virus–Mediated Delivery of a Mutant Endostatin in Combination with Carboplatin Treatment Inhibits Orthotopic Growth of Ovarian Cancer and Improves Long-term Survival

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Abstract
A human ovarian cancer cell line, which migrates to mouse ovaries and establishes peritoneal carcinomatosis, was used to evaluate the cooperative effect of an antiangiogenic gene therapy combined with chemotherapy. The ovarian carcinoma cell line MA148 was genetically modified by “Sleeping Beauty” transposon-mediated delivery of DsRed2 fluorescent protein. Stable, high-level expression of DsRed protein enabled in vivo imaging of peritoneal dissemination of ovarian cancer. Both external and internal imaging, along with histopathology, showed migration of i.p. injected human ovarian cancer cell line to mouse ovaries. Using this model, we evaluated the effect of adeno-associated virus (AAV)–mediated expression of a mutant endostatin either alone or in combination with carboplatin treatment. A single i.m. injection of recombinant AAV (rAAV)–mutant human endostatin with P125A substitution (P125A-endostatin) showed sustained expression of mutant endostatin. Antiangiogenic gene therapy inhibited orthotopic growth of ovarian cancer and resulted in 33% long-term tumor-free survival. A single cycle of carboplatin treatment combined with mutant endostatin gene therapy resulted in 60% of the animals remaining tumor free for >200 days, which was significantly better than rAAV-LacZ and/or carboplatin. Combination treatment delayed tumor appearance in 40% of the animals, wherein the residual tumors were smaller in size with limited or no peritoneal metastasis. These studies suggest that AAV-mediated gene therapy of P125A-endostatin in combination with carboplatin is a useful method to inhibit peritoneal dissemination of ovarian carcinoma. (Cancer Res 2006; 66(8): 4319-28)

Introduction
Ovarian cancer is associated with the highest rate of mortality among the gynecologic malignancies (1) with a 5-year survival of ~50% (2). Most of the ovarian cancers arise from the surface epithelium, and a smaller fraction originates from the granulosa cells and, even more rarely, from the stroma or germ cells (3). Ovarian cancer growth is essentially confined to the ovary at stage I (International Federation of Gynecology and Obstetrics). During progression, tumor cells are shed into the peritoneum, which results in the seeding of the peritoneal wall, omentum, diaphragm, and other peritoneal sites along with the development of malignant ascites (4). Standard care for ovarian cancer is surgical debulking and adjuvant chemotherapy using a combination of a platinum compound and Taxol. Recurrent disease is often treated with additional drug regimens, including gemcitabine and cyclophosphamide (5). Ovarian cancer growth and peritoneal dissemination are angiogenesis dependent (6, 7). Development of malignant ascites is a main cause of morbidity in ovarian cancer (8). Vascular endothelial growth factor (VEGF) plays a critical role in tumor angiogenesis and induces hyperpermeability of the peritoneal vasculature. Increased vascular leak in combination with blockade of lymphatic drainage leads to the formation of malignant ascites in ovarian cancer (9–12). Therefore, inhibition of angiogenesis and neutralization of VEGF can be used to complement existing methods of treatment (13). Recently, we have shown that a mutant endostatin, containing a single amino acid substitution at position 125, resulted in a better binding to the endothelial surface and increased antiangiogenic activity compared with the native protein (14, 15). Adeno-associated virus (AAV)–mediated delivery of the mutant endostatin inhibited s.c. growth of ovarian cancer cells in athymic nude mice (16). Because the peritoneal microenvironment is different from the s.c. site and can significantly influence therapeutic response, we investigated the effect of gene therapy with mutant human endostatin with P125A substitution (P125A-endostatin) in an orthotopic model. To continuously monitor i.p. growth, an ovarian cancer cell line, MA148, was stably transduced to express DsRed protein by using the nonviral “Sleeping Beauty” transposon system. Transplantation of MA148 expressing DsRed protein enabled in vivo imaging of tumor growth and metastasis. Unlike many other human ovarian cancer cell lines, i.p. injection of MA148 cells resulted in colonization of mouse ovaries and peritoneal metastasis. OVCA-3, OVCAR-5, and SKOV-3 cell lines generally produce large quantities of ascites, whereas the MA148 cells generated smaller amounts of ascites. Imaging methods allowed accurate determination of tumor burden and the effect of antiangiogenic gene therapy. A significant fraction of animals treated with a single dose of recombinant AAV (rAAV)-P125A-endostatin remained tumor free for >200 days. Then, we investigated whether chemotherapy can further potentiate the effect of antiangiogenic gene therapy. A single cycle of carboplatin and rAAV-P125A-endostatin treatment resulted in higher fraction of long-term survival and marked inhibition of peritoneal carcinomatosis. These studies show that antiangiogenic gene therapy can be combined effectively with chemotherapy to inhibit orthotopic growth of ovarian cancer.

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Materials and Methods

Cell culture. MA148 cells were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Cellgro, Mediatech, Washington, DC) and 1% streptomycin and penicillin (Cellgro, Mediatech). Adherent human embryonic kidney (Ad HEK293) and human umbilical vascular endothelial cells (HUVEC) were grown as described previously (16). Preparation and titration of rAAV-P125A-endostatin and rAAV-LacZ have been described previously (16).

Transfection of MA148 cells. MA148 cells were transfected with Sleeping Beauty transposon expressing DsRed2 using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate liposomal transfection reagent (Boehringer Mannheim, Indianapolis, IN). The plasmid used for stable transfection was a cis construct containing both Sleeping Beauty transposase (version 10) under cytomegalovirus (CMV) promoter expression control and DsRed2 expressed from a chicken β-globin and CMV composite promoter (CAGGS; refs. 17–19). These cells were sorted using fluorescence-activated cell sorter (FACS Diva, Becton Dickinson, San Jose, CA) and limiting dilution assays. Positive colonies were selected under the Nikon (Melville, NY) Eclipse TE200 inverted fluorescence microscope (×400) and expanded in culture.

Animal studies. Exponentially growing human ovarian epithelial carcinoma cell line transfected with DsRed (MA148-DsRed) were harvested and resuspended at 4 × 10^6/mL in serum-free RPMI 1640. Suspension (500 μL) was then injected i.p. into 6- to 8-week-old female athymic nude mice (n = 15; three independent experiments with five mice per group; National Cancer Institute, Bethesda, MD). Two days later, mice were injected i.m. with either 1 × 10^9 viral particles rAAV-LacZ (100 μL) or 1 × 10^9 viral particles rAAV-P125A-endostatin (100 μL). In experiments involving combination therapy, six groups of animals were used. Each group had five animals. The first group of animals was left untreated. The second group of animals was treated with carboplatin at a dosage of 32.5 mg/kg s.c. every 3rd day (10 total doses). The first dose of the drug was given on the 7th day after the implantation of the tumor cells. The third and fourth groups of animals were treated with rAAV-LacZ (1 × 10^9 viral particles) on day 2. The fifth and sixth groups of animals were treated with rAAV-P125A-endostatin (1 × 10^9 viral particles) on day 2. In addition to the virus injection, the fourth and sixth groups of animals were also treated with carboplatin at the same dose and schedule as described previously.

Imaging of the mice with ovarian cancer. The in vivo imaging of the mice was done under Leica (Bannockburn, IL) dissection microscope under the green filter (excitation, 546/10 nm; dichroic, 565 LP; emission, 590 LP), and the digital images were acquired by a Nikon camera. Representative whole-body images of mice taken at different time points after tumor cell injection. A, day 2; B, day 16; C, day 35. Ovaries, along with uterine horns, were surgically removed. D, day 2; E, day 16; F, day 35. G, whole-body image of a mouse taken on day 42. Metastasis of GIT (H), ovaries (I), and tumor cells recovered from the ascites (J). Yellow arrows, normal ovarian tissue; blue arrows, tumor lesion.
antibody-FITC conjugate (Sigma Chemical, St. Louis, MO). Statistical significance was calculated by the Student’s t test. Tissue samples (tumor, liver, diaphragm, ovary, and kidney) were fixed in 10% formalin and processed for H&E staining.

Quantitative real-time PCR. Total RNA was extracted from frozen tumor tissues, and reverse transcription was done as described previously (15). Real-time PCR was carried out by using PCR Master Mix (LightCycler FastStart DNA Master SYBR Green I, Roche Diagnostics GmbH, Mannheim, Germany) in a LightCycler. Previously validated primer sequences (21) specific for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CD31 were used for real time-PCR (RT-PCR). The conditions used for PCR are denaturation at 95°C for 10 minutes, annealing at 60°C for 10 seconds, and chain extension at 72°C for 10 seconds for 40 cycles. Relative mRNA equivalent for each sample was calculated after normalizing the values to mouse GAPDH level. Two independent experiments were carried out, and the statistical significance was calculated by Student’s t test.

Cytotoxicity assay. Effect of carboplatin and P125A-endostatin either alone or in combination on endothelial cell proliferation was determined by isobolographic analysis. Endothelial cells (HUVEC, 5 × 10² per well) were seeded into gelatin-coated 96-well plates. Recombinant P125A-endostatin expressed in Ad HEK293 cells was purified from the culture supernatants by heparin-Sepharose affinity chromatography. P125A-endostatin (3, 30, and 300 ng/mL) and carboplatin (2, 10, and 20 μg/mL) were added in an array to quadruplicate sets of cultures. Cultures were first treated with carboplatin for 1 hour, and P125A-endostatin was added. Cell proliferation was determined by bromodeoxyuridine incorporation (Roche Diagnostics, Mannheim, Germany) in a LightCycler. Previously validated primer sequences (21) for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CD31 were used for real time-PCR (RT-PCR). The conditions used for PCR are denaturation at 95°C for 10 minutes, annealing at 60°C for 10 seconds, and chain extension at 72°C for 10 seconds for 40 cycles. Relative mRNA equivalent for each sample was calculated after normalizing the values to mouse GAPDH level. Two independent experiments were carried out, and the statistical significance was calculated by Student’s t test.

Results

MA148 is an epithelial ovarian carcinoma cell line established from a stage III serous adenocarcinoma patient. MA148 cells expressing DsRed protein was established by the Sleeping Beauty transposon-mediated gene transfer system. DsRed transduction did not affect the growth rate of this cell line in vitro (data not shown). Lp. injected MA148 cells consistently migrated to mouse ovaries and established large tumor masses in addition to the peritoneal metastasis. Both control and DsRed-transduced cells seeded the mouse ovaries with similar rate of growth and tumor burden (data not included). In addition to seeding the ovaries, MA148 cells also disseminated into the peritoneal cavity resembling the clinical presentation of the disease. Therefore, we used the MA148 cells to study the orthotopic growth and effect of antiangiogenic gene therapy in this tumor model.

Noninvasive imaging of ovarian tumor growth and metastases. To monitor the extent of tumor growth and metastases, three mice were imaged at weekly intervals. Animals were first imaged under anesthesia and then sacrificed to explore the peritoneum in greater detail. Ovaries, along with the uterine horns, were removed and imaged separately to determine colonization of the ovaries. There was no peritoneal seeding at the early time points (day 2), which was confirmed by whole-mouse imaging as well as by imaging the ovaries separately (Fig. 1A and D). Over time, the ovarian seeding of the cells was readily observed. As shown in Fig. 1B and E, by day 16, mouse imaging exhibited seeding of MA148-DsRed cells to the ovary and tumor dissemination into the peritoneum. By day 35, ovarian tumors were 10 times larger in size compared with the normal ovaries with extensive peritoneal tumor implants (Fig. 1C and F). In addition to ovarian colonization, MA148-DsRed cells also metastasized to diaphragm, gastrointestinal tract (GIT; Fig. 1H), peritoneal wall, omentum, spleen, and liver. In many animals, tumor growth was seen in both ovaries (Fig. 1I). The MA148-DsRed cells recovered from the ascites fluid showed typical spheroids (Fig. 1J). The VEGF level in ascites showed an increase from 156.3 ng/mL at the 2nd week to 4,500 ng/mL at the 5th week.

Histopathology of the ovaries. To determine whether the MA148-DsRed cells actually invaded the ovaries and grew inside,
tissue samples obtained at different time points were histologically analyzed. Sections of the ovaries on days 2 and 7 showed histologically intact ovarian tissue with secondary follicles and corpora lutea. On day 16, there was a minute ovarian subcapsular nodule composed of pleomorphic epithelial cells with enlarged, hyperchromatic, and atypical nuclei and a high nuclear-to-cytoplasmic ratio, consistent with carcinoma. On day 23, there were larger ovarian tumors composed of similar cells, clearly representing poorly differentiated carcinoma. On day 30, the scant residual nonneoplastic ovarian tissue could be identified only with difficulty. Instead, the ovaries were, for the most part, replaced by carcinoma, showing solid and trabecular architecture and areas of perivascular pseudorosette formation (Fig. 2A). The tumor cell nuclei had prominent nucleoli, often multiple, and there were frequent mitotic figures. Sections of the diaphragm showed nodules of carcinoma attached to the peritoneal surface and focially invading the diaphragm and infiltrating among the myofibers. Sections of the pancreas and spleen showed a minute subcapsular focus of metastatic carcinoma in a peripancreatic lymph node (Fig. 2B). In all cases, the tumor was a poorly differentiated carcinoma that histologically did not resemble closely to a human serous or endometrioid carcinoma, although it was perhaps morphologically slightly closer to a poorly differentiated serous carcinoma.

**Inhibition of orthotopic ovarian cancer growth in mice.**

Three independent experiments were carried out to investigate the effect of rAAV-P125A-endostatin-mediated gene therapy. In each experiment, groups of five mice were used. In one of the experiments, mice were observed for tumor development up to 300 days. We have earlier established that the mutant endostatin expression following a single i.m. injection of the virus was delayed by 3 weeks (data not shown). Based on these studies, gene therapy was initiated on day 2. Under these conditions, tumors should have been well established at the ovaries by the time the P125A-endostatin begins to be secreted from the transduced myocytes.

Control animals were treated with rAAV-LacZ virus at a similar dose. Figure 3A shows survival curve of mice treated with antiangiogenic gene therapy. rAAV-LacZ-injected mice showed significant tumor burden with a median survival (50%) of 56 days, and they all died by day 84. A single i.m. injection of rAAV-P125A-endostatin significantly delayed the tumor growth. Median survival of the treated group was on day 70 ($P < 0.02$). One third (5 of 15) of the treated mice showed long-term tumor-free survival. In one of the experiments, mice were followed for >300 days, and at that point, mice were sacrificed to image the i.p. sites for micrometastasis. Long-term survivors did not show any tumor nodules either in the ovaries or in any of the peritoneal sites. Tumors were resected from the remaining animals at the time of death to accurately determine tumor burden. Wet weight of the tumor tissue was compared between control and rAAV-P125A-endostatin-treated groups to determine the effect of gene therapy on total tumor burden (Fig. 3B). Mean tumor burden in the rAAV-LacZ-treated control group was 6.85 g (mean of 15 animals). The average tumor burden from the 10 mice that developed tumors in the rAAV-P125A-endostatin-injected mice was 3.4 g ($P < 0.02$). There was a 50.3% reduction in the tumor burden in mice injected with rAAV-P125A-endostatin compared with mice injected with rAAV-LacZ. Moreover, 33% of the treated mice did not show any tumors at the time of termination of the experiment. Residual tumors obtained from the mice were processed for microvessel density using anti-CD31-PE conjugate. Morphometric analyses of CD31 staining showed significant inhibition in tumor vasculature in
Increased tumor-free survival by antiangiogenic gene therapy in combination with chemotherapy. After evaluating the efficacy of rAAV-P125A-endostatin gene therapy as a single agent, we then investigated whether the inclusion of a chemotherapeutic drug can further improve the effect of antiangiogenic gene therapy. The treatment schedule was followed as described in Materials and Methods. Mice were observed for tumor growth and survival. Figure 4A shows the survival curve of untreated controls and treated mice. The control untreated group of animals started to die on day 42. By day 73, all the animals in this group died due to tumor burden. Similarly, 100% of the rAAV-LacZ-treated animals died by day 70 ($P = 0.193$). The animals treated with rAAV-P125A-endostatin alone showed 20% long-term tumor-free survival ($P < 0.02$) in this experiment. Carboplatin treatment either alone ($P \leq 0.05$) or along with AAV-LacZ ($P \leq 0.05$) showed 20% (1 of 5) long-term survival. However, when the chemotherapy was given along with rAAV-P125A-endostatin treatment, 60% (3 of 5) of the animals remained tumor free for >200 days ($P < 0.02$). Images of the animals taken at the time of necropsy are shown in Fig. 4B. One of the animals with a smaller tumor and a tumor-free animal from the combination treatment arm are shown in (Fig. 4B). To further validate the efficacy of combined treatment on ovarian cancer growth, ovarian tissues were examined histologically. Figure 5A shows histopathology of ovaries obtained at the time of necropsy. Ovaries from the control untreated group showed extensive tumor burden. Carboplatin-treated group also showed ovarian tumors interspersed with normal ovarian stroma and follicles. rAAV-P125A-endostatin-treated animals have localized ovarian tumors. However, ovaries from the animals treated with rAAV-P125-endostatin and carboplatin appeared histologically normal, containing several of developing follicles. There were no detectable tumor lesions. At the end of the experiment, mice were terminally bled, and serum samples were collected. The mean endostatin levels in the serum of group of mice injected with rAAV-P125A-endostatin and rAAV-P125A-endostatin combined with carboplatin was 12.8 ($P < 0.02$) and 7.5 ($P < 0.02$) ng/mL, respectively. The other groups showed no significant levels of endostatin.

Inhibition of tumor neovascularization. We wanted to further investigate whether the decrease in tumor growth is associated with a reduction in the tumor angiogenesis. Residual tumor specimens obtained at the time of necropsy were snap frozen and processed for immunofluorescence studies to localize blood
vessels. Representative images of CD31-positive endothelial cells from the frozen sections are shown in Fig. 5B. Figure 5B (top) shows CD31-positive endothelial cell–lined blood vessels in tumor sections counterstained with 4',6-diamidino-2-phenylindole (DAPI). These images were used for the morphometric analysis to accurately determine vessel density. Figure 5B (middle) shows CD31-positive images, which were then binarized to characterize vessel architecture (Fig. 5B, bottom). There was a significant reduction in the tumor blood vessels in mice treated with rAAV-P125A-endostatin alone (Fig. 5B, 7–9) or in combination with carboplatin (Fig. 5B, 16–18). The effect of antiangiogenesis was enhanced in the group of mice treated with rAAV-P125A-endostatin in combination with carboplatin. To further confirm the reduction in microvasculature due to antiangiogenic gene therapy, we determined the transcript levels for CD31 in tumor samples obtained from the control and treated animals using RT-PCR. As shown in Fig. 5C, there was a 65% \( (P = 0.02) \) reduction in the amount of CD31 mRNA level in the antiangiogenic gene therapy combined with carboplatin treatment group compared with the control untreated group. rAAV-P125A-endostatin-treated mice also showed ~21% inhibition in the transcript levels for CD31 compared with rAAV-LacZ-injected mice. These results suggest that the combination treatment is more effective in reducing the number of endothelial cells infiltrating the tumors.

**Characterization of blood vessels.** The characteristics of blood vessels from the residual tumors were analyzed by dual immunofluorescence staining for PECAM1 (endothelial cells) and \( \alpha \)-smooth muscle actin (pericytes). Figure 5D shows representative images from control and treated animals. About 8 to 10 nonoverlapping areas were imaged under green and red filter sets to determine vascular density and pericyte coating. Total blood vessels and pericyte-coated vessels were differentially quantified by morphometric analysis (20). These results are summarized in Fig. 5E. Control animals treated with rAAV-LacZ showed that majority of the blood vessels were devoid of pericytes (65% of the total vasculature), indicating that the vast majority of the vessels were immature. Treated animals showed lesser degree of angiogenesis in the residual tumors. Interestingly, P125A-endostatin treatment reduced the relative amount of immature vessels to ~45% of the total vasculature. The tumors from the mice that were treated with carboplatin had equal amounts of pericyte-positive and pericyte-negative vessels (50%). However, a combination of carboplatin and P125A-endostatin treatment completely eliminated all the immature blood vessels in the tumor. These results imply that the combination of rAAV-P125A-endostatin with carboplatin not only reduces the vessel density but also differentially eliminates the immature vasculature in the tumors.

**Inhibition of ascites.** The ascites levels were estimated at the end of the experiment. As shown in Fig. 6A, the ascites levels were significantly reduced in the mice injected with rAAV-P125A-endostatin compared with mice in the control untreated group \( (P = 0.02) \). The average volume of ascites in mice injected with rAAV-P125A-endostatin was 0.17 mL compared with 0.32 mL in the mice injected with rAAV-LacZ. As shown in Fig. 6A, the ascites levels were less in the mice injected with carboplatin either alone (0.19 mL) or in combination with rAAV-LacZ (0.14 mL; \( P \leq 0.02 \)) or rAAV-P125-endostatin (0.16 mL; \( P \leq 0.05 \)) compared with the control untreated animals with 0.41 mL ascites. Combination treatment with rAAV-P125A-endostatin and carboplatin did not decrease the ascites volume any further (0.16 mL ascites volume). These results suggest that either the chemotherapy or the antiangiogenic gene therapy is sufficient to reduce the ascites volume.

To determine whether the combination treatment affects proangiogenic microenvironment, VEGF secreted by the tumor cells was measured in the serum and tumor extracts. Control group of animals showed a mean serum level of 658.2 ng/mL human
VEGF (Fig. 6B). rAAV-P125A-endostatin-treated animals had 13.3-fold less VEGF in the serum compared with the control group ($P = 0.05$). Carboplatin treatment reduced the serum levels of VEGF by 7.3-fold (89.55 ng/mL). About 23-fold reduction in serum VEGF was seen in the combination treatment group (28.3 ng/mL mean serum level; $P = 0.03$) compared with the serum levels of VEGF in the control group (rAAV-LacZ). In addition, VEGF content in the residual tumor tissues was also determined. Similar to the serum profile, VEGF amount was affected at the tissue level as well (data not shown).

Reduced VEGF levels in the tumor tissue can "normalize" interstitial pressure and thereby facilitate efficient delivery of
chemotherapeutic drugs. Direct measurement of platinum levels following antiangiogenic gene therapy will provide a definitive answer to this question. Whereas improved delivery can potentiate antitumor effects, chemotherapeutic agents can modulate sensitivity of endothelial cells to antiangiogenic molecules. Indeed, carboplatin sensitized the endothelial cells to endostatin treatment. Isobolographic analysis (data not shown) of in vitro cytotoxicity data suggests synergy between carboplatin and P125A-endostatin. For example, 3.5 μg/mL carboplatin and 110 ng/mL P125A-endostatin were expected to induce 30% inhibition of HUVEC proliferation (additive inhibition). However, 30% inhibition was observed at a lesser concentration of P125A-endostatin (30 ng/mL) in the presence of carboplatin, indicating synergistic inhibition.

Discussion

Ovarian cancer growth and peritoneal dissemination are angiogenesis dependent. Angiogenic phenotypes, such as increased secretion of VEGF and tumor-associated microvessels, are prognostic indicators of ovarian cancer outcome (23). Inhibition of angiogenesis is therefore a viable complimentary approach to improve conventional chemotherapy-based treatment of ovarian cancer. Earlier studies have shown that adenoviral vectors encoding antiangiogenic proteins, such as soluble FLT-1, can inhibit ascites formation and tumor growth and prolong the survival in treated animals (24). Adenovirus-mediated gene expression is however transient, and its use in long-term therapy is limited by immune response. AAV-based gene therapy approach can overcome some of these limitations. Successful antiangiogenic therapy requires sustained secretion of the protein for longer periods of time. For example, better tumor growth inhibition was observed when endostatin was microencapsulated into alginate beads than by bolus injection of similar doses (25). Endostatin, a proteolytic fragment of collagen type XVIII, and angiotatin, a fragment of plasminogen, are effective antiangiogenic molecules (26, 27). Several studies have shown efficacy of endostatin against ovarian cancer growth. Most of these studies used s.c. models to assess the effect of angiogenesis inhibitors. A mutant endostatin (P125A) was characterized earlier, which showed significantly improved antiangiogenic activities (15). AAV-mediated delivery of P125A-endostatin construct inhibited s.c. growth of ovarian cancer (16). These studies are currently extended to an i.p. model to investigate the therapeutic efficacy in a biologically relevant tumor microenvironment. Several human ovarian cancer cell lines (e.g., OVCAR-3, OVCAR-5, SKOV-3, and HEY) have been used in xenograft models using athymic mice. Lai et al. (28) established platinum-resistant ovarian cancer cell lines, which are again widely used in tumor models to establish metastatic growth in the peritoneum. None of these cell lines, however, seeds the mouse ovaries after i.p. injection. MA148 cell line, on the other hand, showed consistent homing to the mouse ovaries after i.p. injection. To visualize the tumor cell seeding and tumor burden, we established clonal lines of MA148 cells stably expressing DsRed protein. This allowed accurate determination of tumor burden as well as live imaging using noninvasive methods. Tumor cells were found in the ovaries as early as 2 weeks after i.p. injection. At the same time, peritoneal seeding along with metastasis of GIT and diaphragm also occurred. Histopathology showed tumor nodules attached to the surface of liver. Either single or both ovaries were seeded by MA148 cells. DsRed construct used to label the MA148 cells had no signal sequence, but it is possible to establish secretory constructs that will allow monitoring serum levels of the protein as a surrogate tumor marker. Indeed, a similar strategy has been used to establish ovarian cancer cell lines secreting heat-stable alkaline phosphatase, which was used to evaluate tumor burden and response to chemotherapy (29, 30). Epithelial ovarian cancers do not develop spontaneously in mice, although tissue-specific expression of viral T antigens in transgenic mice have resulted in spontaneous tumorigenesis, some of which showing epithelial origin of tumors (31). These models have not yet been used to determine the efficacy of antiangiogenic therapies in preventing the development of ovarian cancer.

Several studies have shown that AAV-mediated delivery of endostatin is effective in various tumor models (32–34). Earlier, we showed that AAV-mediated delivery of a mutated endostatin could inhibit s.c. tumor growth in a mouse xenograft model. A single i.m. injection of rAAV-P125A-endostatin construct showed sustained secretion of P125A-endostatin for up to 9 weeks (16). AAV can be used to express endostatin i.p. (data not shown) or i.m. with equal efficacy. Present studies show that a single i.m. injection was able to inhibit i.p. growth of ovarian cancer. AAV construct was injected 2 days following the tumor cell transplantation. Because
AAV-mediated gene delivery is associated with delayed expression of transgene as late as 3 weeks, tumor should have been well established in this model system. rAAV-P125A-endostatin treatment showed ~33% long-term tumor-free survival. Comparable level of long-term survival was observed in a recently published study, wherein AAV was used to express both angiostatin and endostatin (35). In these studies, virus was injected 3 weeks before tumor cell transplantation; therefore, this model can be considered as prevention rather than intervention model. Similarly, AAV can be used to express soluble FMS-like tyrosine kinase-1 receptor to inhibit the growth of SKOV-3.ip1 xenograft in vivo in a prevention model (36). A combination of angiostatin and endostatin can synergistically inhibit tumor angiogenesis (22, 32). Current studies show that AAV-mediated P125A-endostatin expression can be effective as an interventional therapy as well. Antiangiogenic gene therapy either alone or in combination with chemotherapy not only reduced the vascular density in the residual tumor tissue but also differentially affected the immature blood vessels. Combination treatment almost completely eliminated immature vessels (pericyte-negative blood vessels). These results agree with earlier studies by Jain (37) and Winkler et al. (38), such that antiangiogenic agents reduce vessel density by reducing vascular permeability, destroying “less mature” vessels, and increasing the recruitment of pericytes to stabilize other vessels in tumors. This apparent stabilization has been termed the normalization window, defined as a period where tumor blood flow and oxygenation transiently increase, thus providing an opportunity to better deliver chemotherapeutic agents and radiation therapy. Antiangiogenic agents can synergize with cytotoxic drugs and radiation to improve therapeutic efficacy (39). Such strategies can also be used to minimize toxicity because it can reduce the doses of chemotherapy needed. Ovarian cancer patients are generally treated with a combination of platinum and Taxol in adjuvant settings (40, 41). Although we used carboplatin alone as a single chemotherapy regimen, a combination with Taxol is likely to render similar or better therapeutic benefit. Carboplatin is used as a single agent to treat ovarian cancer in some European centers. When rAAV-P125A-endostatin therapy was combined with carboplatin, there was a significant improvement in tumor-free survival. Treated animals (60%) remained tumor free. Interestingly, the remaining animals showed tumors restricted to the ovaries with minimal or no peritoneal spread of the cancer. Whereas a single cycle of carboplatin is able to improve antiangiogenic gene therapy, it may be possible to increase the response rate by multiple cycles of carboplatin either alone or in combination with Taxol.

At least four different mechanisms may contribute to the synergistic inhibition of i.p. ovarian cancer growth by rAAV-P125A-endostatin and carboplatin. First of all, the combination treatment targets two distinct population of cells (i.e., tumor and endothelial cells). Independent targeting can amplify the total effect of either of the treatment alone. Secondly, in vitro cytotoxicity assays using isobolographic analysis suggest that carboplatin sensitizes endothelial cells to P125A-endostatin. Thirdly, the differential elimination of immature vasculature by the combination treatment can normalize interstitial fluid pressure so that chemotherapeutic drugs could now reach the target cells more efficiently. Finally, endostatin binds to ovarian cancer cells directly via α5/β3 integrin and thereby prevents peritoneal seeding and co-option of microvasculature. Our studies suggest that antiangiogenic gene therapy and chemotherapy can synergistically inhibit i.p. ovarian cancer growth.

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