Endostatin Binding to Ovarian Cancer Cells Inhibits Peritoneal Attachment and Dissemination

Yumi Yokoyama, Gerald Sedgewick, and S. Ramakrishnan

Abstract

Ovarian cancer cells use integrins to attach to the peritoneal wall. Integrin $\alpha_h\beta_1$ is also the target for the angiogenesis inhibitor, endostatin. Therefore, the ability of endostatin to competitively inhibit tumor cell seeding of the peritoneum was investigated. An imaging method was developed to determine early phases of peritoneal dissemination of ovarian cancer cells. Using this method, endostatin was found to bind ovarian cancer cells through integrin $\alpha_h\beta_1$ and inhibit vessel cooption efficiently. Although both angiotatin and endostatin are potent inhibitors of tumor angiogenesis, peritoneal attachment and vessel cooption was blocked only by the endostatin. Knocking down the expression of integrins $\alpha_5$ and $\beta_1$ in ovarian cancer cells interfered with endostatin-mediated inhibition of peritoneal seeding. Furthermore, adenovirus-mediated in situ expression of endostatin either inside the peritoneum or by the ovarian tumor cells inhibited peritoneal seeding and dissemination in vivo. Endostatin treatment also prevented primary ovarian cancer cells from attaching to mouse peritoneal wall. These studies show a paraendothelial mechanism by which endostatin can inhibit peritoneal dissemination of ovarian cancer cells and raises the possibility of intraperitoneal expression of endostatin to reduce recurrence. [Cancer Res 2007;67(22):10813–22]

Introduction

Ovarian cancer is estimated to occur in 22,430 women in the year 2007 and remains to be the leading cause of death among gynecologic cancers (1). The majority of ovarian tumors arise from the single epithelial layer covering the ovaries, which are contiguous with the mesothelium (2). During progression, tumor cells breach the ovarian capsule and reach the peritoneal cavity. The free tumor cells then seed the peritoneal wall by integrin-mediated attachment to extracellular matrix (ECM; ref. 3) and disseminate throughout the peritoneal cavity, posing a major challenge in treating ovarian cancer patients. Therefore, preventing peritoneal dissemination can improve the survival of ovarian cancer patients. In many tumor models, it has been observed that tumor cells that adhere closer to blood vessels (vessel cooption) have a survival advantage, and are thus selected for further growth (4). Vessel cooption is followed by angiogenic switch and neoangiogenesis, leading to invasive cancers (5).

Endostatin is a COOH-terminal fragment of collagen XVIII and is a potent angiogenesis inhibitor (6). Several putative receptors for endostatin have been identified (7–11). Integrin $\alpha_h\beta_1$ is the major target for endostatin-mediated inhibition of endothelial cell proliferation and migration (9). Because ovarian cancer cells also interact with ECM via the integrin $\alpha_h\beta_1$, we hypothesized that endostatin can competitively inhibit $\alpha_h\beta_1$-mediated attachment of tumor cells to the peritoneum. In a recent study, we described that endostatin can directly bind to ovarian cancer cells through integrin $\alpha_h\beta_1$, and that down-regulation of integrin $\alpha_h\beta_1$ by small interfering RNA (siRNA) significantly reduced binding of endostatin to ovarian cancer cells (12). Furthermore, ovarian cancer cell attachment to fibronectin-coated plates was inhibited by preincubation with endostatin. Based on these results, we investigated whether binding of endostatin to ovarian cancer cells could inhibit their attachment to peritoneal wall in vivo. For this purpose, an imaging method was developed to quantify peritoneal attachment and vessel cooption of ovarian cancer cells in athymic mice. Endostatin treatment of ovarian cancer cells and i.p. expression of endostatin prevented ovarian cancer cell attachment and peritoneal seeding.

Materials and Methods

**Cells.** Six ovarian cancer cell lines (OVCAR5, A2780, OVCAR3, SKOV3, OC495, and MA148) are used in this study. Human umbilical vein endothelial cells (HUVEC) and MA148, a human epithelial ovarian carcinoma cell line, have been previously described (13). A2780 was provided by Dr. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). OVCAR3 and SKOV3 were obtained from American Type Culture Collection. OC495 was provided by Dr. Martinez-Maza (University of California, Los Angeles, Los Angeles, CA). Human melanoma cell line (WM35) was provided by Dr. Iida (University of Minnesota, Minneapolis, MN). Ovarian cancer cells and melanoma cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS; Invitrogen). HUVEC was maintained in endothelial growth medium (Lonza). Primary ovarian cancer cells were obtained from the ascites of three patients (MA160, MA161, and MA162) diagnosed with stage III ovarian cystadenocarcinoma. Tumor cells were purified from the ascites (14) and provided by Dr. A. Skubitz (University of Minnesota).

**Antibodies.** Monoclonal antibodies to integrin $\alpha_h\beta_3$ (LM609), $\alpha_h\beta_1$ (JBS5), and $\alpha_h\beta_5$ (P1F6) were obtained from Chemicon. Monoclonal antibody to integrin $\alpha_h\beta_1$ (PG9) was obtained from Biomeda. Rabbit anti-integrin $\alpha_h$ polyclonal antibody (Chemicon) and monoclonal antibody to $\beta$-actin (Sigma) were used for Western blot. Control mouse IgG and secondary antibodies (rabbit anti-mouse IgG-FITC, rabbit anti-mouse IgG-peroxidase, and goat anti-rabbit IgG-peroxidase) were obtained from Sigma.

**Mice.** Female, athymic nu/nu mice (ages 4–6 weeks) were obtained from the National Cancer Institute and housed in microisolators until the age of 8 weeks before using them in experiments. Animal use was in compliance with University of Minnesota guidelines and protocols approved by the Institutional Animal Care and Use Committee.

**Flow cytometric analysis.** Expression levels of integrins on cells were analyzed by flow cytometry as described before (12).
Cell attachment assay. Details of cell attachment assay have been previously described (15). One nanomole of endostatin per well was used to coat 96-well plates and then blocked with 2% bovine serum albumin in PBS for 2 h at 37°C. Cells were labeled with 5 μM/L 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA; Molecular Probes), and added to the wells at a density of 40,000 per well. After 1 h incubation at 37°C, the plates were washed twice with HBSS and read in a fluorescence plate reader (CytoFluor II; PerSeptive Biosystems; excitation 485 nm, emission 530 nm).

Preparation of green fluorescent protein–expressing cell lines. Green fluorescent protein (GFP) expression vector pEGFP was purchased from BD Biosciences Clontech. For long-term experiments, OVCAR5 cells were stably transfected with pEGFP using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. GFP-positive cells were selected by a cell sorter (FACSDiva, BD Bioscience).

Purification of recombinant proteins. Endostatin and angiostatin were purified from yeast as previously described (16–18).

Attachment of ovarian cancer cells to mouse peritoneal wall. Cells (3 × 10⁵ per mouse) were prelabeled with 5(6)-CFDA. GFP-expressing cells were used in long-term studies. Labeled cells were incubated with human endostatin (200 μg/3 × 10⁵ cells) or HBSS (control) at 37°C for 1 h, and then the cells were injected into athymic nude mice i.p. Five mice were used for each group per experiment. From day 2 through day 7, PBS or endostatin (20 mg/kg/d) was injected daily s.c. At different time points (5 h, 1 day, 2 days, or 1 week for OVCAR5; 2 days for OVCAR3 and WM35), RITC-dextran was injected i.v. Peritoneal membranes were surgically removed and observed under a fluorescence dissection microscope (Leica) at ×100 magnification. Tumor cell attachment to diaphragm was also determined.

Quantification of ovarian cancer dissemination on peritoneal walls. Images (10–20 fields per mouse) of the fluorescence-labeled cells attached to the peritoneal membranes were digitally recorded. Random fields covering the entire area were chosen instead of hotspots. Images were analyzed using Adobe Photoshop (Adobe Systems, Inc.), Image Processing Toolkit (RGi, Inc.), and Metamorph image analysis software (Universal Imaging Corp.). Color images were converted to grayscale mode (Adobe command, image: mode: grayscale). Images were quantified by the Metamorph program using a protocol in which all individual objects are counted and then summed.

Effect of endostatin on SKOV3 transfected with siRNA to integrins α5 and β1. siRNAs specific for integrins, and control scrambled siRNA with and without fluorescence tag were purchased from Santa Cruz Biotechnology. FITC–conjugated control siRNA was used to evaluate the transfection efficiency (12). Cells were seeded into 100-mm dishes (6 × 10⁵ per dish) in DMEM medium with 10% FBS 24 h before transfection. siRNAs were added at a concentration of 40 μM/l well with X-tremeGENE siRNA transfection reagent (8 μl/mL; Roche). Four days after transfection, cells were harvested by 2 mM/L EDTA in PBS and used for in vivo assay. Expression levels of integrin α5 siRNA treatment were determined by flow cytometry (12) and Western blot. Cells were lysed with lysis buffer [50 mM/L Tris-HCl (pH 7.6), 1% BRJ35, 0.14 mol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L MnCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, one tablet per 50 ml of protease inhibitor cocktail without EDTA (Roche)], and 20 μg of cell lysate were used for Western blot. For in vivo studies, siRNA-transfected cells were prelabeled with 5(6)-CFDA and injected into athymic mice i.p.

Adenoviral construct of endostatin inhibition of ovarian cancer cell attachment in vivo. Human endostatin cDNA was subcloned into an adenoviral shuttle vector (Stratagene) containing the cytomegalovirus promoter; Igs–chain leader sequence for protein secretion was cloned into the vector (19). Adenoviral vector encoding LacZ gene was used as a control. Expression of endostatin was measured using an enzyme-linked immunosorbent assay (Cytomine). Endostatin expressed in HEK293 conditioned medium was purified by heparin ceramic column (17). Purified protein was analyzed by SDS-PAGE (15%) and Western blot using a polyclonal antibody generated against human endostatin (18). Endostatin expressed by AdEndo-infected cells was measured by ELISA (Cytomine). OVCAR5 cells were first infected with adenovirus in vitro at 200 multiplicity of infection (MOI). Three days after infection, 3 × 10⁶ cells were labeled with CFDA and injected into five mice per group i.p. Mice were sacrificed after 3 days and the peritoneal dissemination was examined. In the next series of experiments, mice were i.p. injected with AdEndo virus at a dose of 1.5 × 10⁸ plaque-forming units per mouse. Three days after virus infection, CFDA-labeled OVCAR5 cells were injected i.p. (n = 5). AdLacZ was used as a control. Two days after the cell injection, the peritoneal attachment of tumor cells was determined.

Ex vivo attachment of primary ovarian cancer cells to mouse peritoneal wall. CFDA-labeled primary ovarian cancer cells were used in ex vivo cell attachment assays (12). Image acquisition methods and processing have been described previously.

Results

Integrin-mediated attachment to the peritoneal wall is important for the dissemination of ovarian cancer. Earlier, we have shown that human endostatin can bind to ovarian cancer cells and inhibit cell attachment to fibronectin-coated plates in vitro. To further understand which one of the integrin complex is involved in this process, a panel of ovarian cancer cell lines was compared for integrin expression and endostatin binding.

Correlation between integrin α5β1 expression and endostatin binding to ovarian cancer cells. Six different human ovarian cancer cell lines (OVCAR5, OVCAR3, SKOV3, A2780, MA148, and OC495), primary endothelial cells (HUVEC), and a melanoma cell line (WM35) were examined for their expression levels of integrins α5β1, α1β3, α1β5, and α5β1 using flow cytometry. All the eight cell types were investigated for their ability to bind endostatin in a cell attachment assay. These results are shown in Fig. 1A to D. Only integrin α5β1 expression levels positively correlated with cell attachment to endostatin-coated wells (Fig. 1A; correlation efficiency 0.795). WM35 melanoma cell line is used as a negative control and does not express any detectable levels of integrin α5β1. Integrins α1β3, α1β5, and α5β1 expression levels did not correlate with attachment to endostatin-coated wells (Fig. 1B–D, respectively). These results suggest that endostatin binding to ovarian cancer cells is directly related to the levels of integrin α5β1 expression.

A method to quantify peritoneal adhesion of ovarian cancer cells. An imaging method was developed to determine time-dependent changes in the number of ovarian cancer cells bound to the peritoneal wall (Fig. 2). Figure 2A shows a representative image of tumor cells attached near peritoneal blood vessels. Fluorescence images were captured under green and red filters. The split image of green fluorescence (labeled tumor cells) was converted to gray scale (Fig. 2B). Then, a “high-pass” filter (Fig. 2C) and “threshold” levels were selected to eliminate background noise. The high-pass filter accentuates intensity changes in an image by modifying pixel value to exaggerate intensity differences from its neighbors. As a final step, images were inverted to facilitate counting the number of cells by using Metamorph software (Fig. 2D). The same high-pass and threshold levels were used for processing all images to minimize intra-assay variations.

Endostatin treatment inhibits peritoneal seeding and vessel cooption of ovarian cancer cells in vivo. The following experiments were carried out to determine whether endostatin could inhibit ovarian cancer cell attachment and peritoneal dissemination in vivo. Labeled cells were preincubated with endostatin for 30 min, and then i.p. injected into female athymic mice. Mice were also treated s.c. with 20 mg/kg of endostatin daily. This dose had been previously found to have therapeutic effect in...
inhibiting ovarian cancer growth. Immediately after i.p. injection, OVCAR5 cells were evenly distributed as single cells on the peritoneal surface (Fig. 3A, column 1). By day 1, the randomly distributed tumor cells were found to be clustered into discrete nodules localized closely to the vascular bed. By day 2, coopted tumor cell clusters grew further in size whereas the number of randomly attached single cells decreased in the peritoneum. Tumor cell nodules grew bigger and denser by 1 week after i.p. injection in the control group of mice.

OVCAR5 cells pretreated with endostatin showed a significant difference in peritoneal seeding (Fig. 3A, column 2). In contrast to the control group, OVCAR5 cells treated with endostatin failed to adhere to the peritoneal wall even at the earliest time point checked (4 h). Twenty-four hours later, a few areas of single cells were found on the peritoneal surface. At 1 week time point, there was no distinct tumor nodules in the endostatin-treated group, indicating that endostatin can interfere with ovarian cancer cell seeding of the peritoneum in vivo. To determine whether peritoneal attachment and vessel cooption of ovarian cancer cells was a general effect mediated by all antiangiogenic molecules, we investigated the effect of angiostatin in this model system.

Although endostatin is derived from collagen XVIII, one of the ECM components of the vessel wall, angiostatin is produced by the proteolytic cleavage of plasminogen. The mechanism of action of endostatin and angiostatin on endothelial cells is much different from each other. Angiostatin binds to ATP synthase localized onto the endothelial cell surface (20). Previously, we showed that ovarian cancer cells do not attach to angiostatin-coated wells (12). Unlike endostatin treatment, angiostatin did not inhibit peritoneal attachment of OVCAR5 cells (Fig. 3A, column 3). On day 1, a higher number of tumor cells were found near peritoneal vasculature in the angiostatin-treated group. Interestingly, continued treatment with angiostatin for 1 week showed significant reduction in the number of established tumor nodules around the peritoneal vasculature, suggesting that angiostatin inhibits later stages of angiogenesis distinct from the vessel cooption phase. Peritoneal seeding was quantified and summarized in Fig. 3B. Peak levels of tumor cell attachment were seen on day 1. The transient reduction seen on day 2 suggests selection pressure leading to the elimination of tumor cells, which failed to coopt peritoneal vasculature. The transient reduction was followed by a progressive increase in the number and the size of tumor nodules in the control group, whereas endostatin and angiostatin-treated groups showed progressively decreased number of tumor nodules.

Because ovarian cancer cells are also known to metastasize to diaphragm, we determined the effect of endostatin treatment on ovarian cancer cell seeding of diaphragm as well. Furthermore, to examine whether endostatin-mediated inhibition of vessel cooption and peritoneal dissemination is dependent on integrin α5β1 expression and applicable to other ovarian cancer cells, two other cell lines (OVCAR3 and SKOV3) were tested in this model. OVCAR3 cells express similar levels of integrin α5β1 when compared with OVCAR5, whereas SKOV3 cells showed higher integrin levels. As a negative control, WM35 melanoma cells that have almost no detectable expression of integrin α5β1 was used (12). Endostatin treatment inhibited OVCAR3 cells attaching to the peritoneum and diaphragm significantly. Attachment of WM35 to both peritoneum

Figure 1. Correlation between integrin expression and ovarian cancer cell binding to endostatin. Integrin expression was determined by flow cytometry. A, α5β1. B, αvβ3. C, αvβ5. D, α4β1. Cells were prelabeled with CFDA and used in attachment assays. Points, mean of three independent assays. HUVEC (○); ovarian cancer cell lines MA148 (○). OVCAR5 (○), OVCAR3 (△), SKOV3 (●), and OC495 (▲); A2780 (▼); and melanoma cell line, WM35 (△).
and diaphragm was ~4-fold less than OVCAR3 cells. Endostatin and angiostatin treatment did not inhibit WM35 melanoma cell attachment to the peritoneum. In fact, angiostatin-treated cells showed a marginal increase in the number of cells attached to both tissues, which was, however, not statistically significant (Supplementary Data 1). These studies show that α5β1 is important for peritoneal seeding and that endostatin can inhibit tumor cell attachment in vivo.

Effect of integrins α5 and β1 knockdown in SKOV3 cells. As a further confirmation that endostatin inhibits ovarian cancer cell seeding of the peritoneum through α5β1 integrin, siRNA-mediated knockdown experiments were carried out. A third ovarian cancer cell line, SKOV3, expresses higher levels of integrin α5β1 when compared with OVCAR5 and OVCAR3. siRNA specific to integrins α5 and β1 were used to knock down expression of integrin α5β1 in SKOV3. Reduction in the integrin expression following siRNA treatment was confirmed by Western blotting (Fig. 4A). Both integrin α5β1 and α3 levels were significantly reduced after siRNA treatment. Scrambled siRNA-treated cells were used as a control. Control and siRNA-treated cells were then labeled and used in peritoneal attachment studies. Scrambled siRNA-treated cells showed an average of 450 SKOV3 cells attached to the peritoneal wall. Knocking down of integrins α5 and β1 by siRNA decreased the peritoneal attachment of SKOV3 by 50% (Fig. 4B and C). These results indicate that integrin α5β1 is important for the peritoneal attachment of SKOV3 cells. Endostatin treatment inhibited the peritoneal attachment of SKOV3 cells transfected with scrambled siRNA by 75% (Fig. 4B and C). The residual attachment of ovarian cancer cells treated with siRNA is likely due to the incomplete inhibition of α5β1 and involvement of other integrins, such as integrin α3β1. The SKOV3 transfected with siRNA to integrins α5 and β1 was not further inhibited by endostatin treatment (Fig. 4B and C). These studies confirm that ovarian cancer cells attach to the peritoneal wall mainly through integrin α5β1, and that endostatin can inhibit ovarian cancer cells that are positive for integrin α5β1 expression.

In situ expression of endostatin by adenovirus inhibits vessel cooption and dissemination of ovarian cancer cells. As a further evidence to support that endostatin expression in the tumor microenvironment could affect cancer cell seeding of the peritoneum, we first engineered an adenovirus construct (AdEndo) to express endostatin transiently in ovarian cancer cells. Endostatin cDNA was cloned into an adenovirus vector and evaluated for mammalian cell expression using HEK293 and OVCAR5 cells. AdEndo-infected HEK293 cells secreted 344 ng/mL of endostatin into the culture medium. Subsequently, OVCAR5 cells were infected with AdEndo virus at a MOI of 200. OVCAR5 cells secreted 90.0 ng of endostatin/10^6 cells 3 days after AdEndo infection. Data in Fig. 5A show purification and characterization of adenovirus-mediated expression of endostatin in OVCAR5 cells. Western blot showed a distinct band similar to the endostatin purified from yeast. The purified endostatin secreted by OVCAR5 cells was found to be biologically active in inhibiting endothelial cell proliferation in vitro (data not shown). As a control, human endostatin expressed in yeast was used.

Two series of experiments were carried out to determine the effect of in situ expression of endostatin on peritoneal seeding. First, we determined the effect of endostatin secreted by the ovarian cancer cells on peritoneal attachment of tumor cells. OVCAR5 cells were infected with AdEndo virus, and 3 days after infection adenovirus-infected cells were injected i.p. An adenovirus construct containing LacZ (AdLacZ) was used as a negative control in parallel experiments. Tumor cells that had attached to the peritoneal wall along the vascular bed were determined by the imaging method described before. OVCAR5 cells infected with AdLacZ showed 135 ± 86 cells per field on day 3.
Endostatin-expressing OVCAR5 cells showed a significant reduction in the number of cells attached (42 ± 39 per field) to the peritoneal wall on day 3 (Fig. 5B). These studies show that tumor cell–derived endostatin is capable of inhibiting cell-matrix interaction and thereby reducing the number of tumor cell seeding of the peritoneal wall.

Next, we tested whether the peritoneal tissue of the host can be transduced with endostatin-secreting adenoviral construct and evaluated the effect of i.p. secretion of endostatin on ovarian cancer cell seeding. AdLacZ-injected mice showed 193 ± 40 cells attached to the peritoneal wall (Fig. 5C and D). In comparison, the AdEndo-injected mice showed significantly reduced peritoneal attachment of OVCAR5 cells. Mice expressing endostatin inside the peritoneum showed a mean number of 50 ± 15 cells per field on day 2 (Fig. 5C and D). Serum samples obtained at the time of sacrifice showed that mice injected with AdEndo virus had 117.9 ± 41.7 ng/mL of endostatin in circulation. Control mice injected with AdLacZ virus at a comparable dose showed no detectable level of endostatin in the serum. These data clearly show that the in situ secretion of endostatin either at the target host tissues or by the tumor cells can inhibit peritoneal seeding and dissemination.

**Figure 3.** Endostatin treatment inhibits peritoneal attachment of OVCAR5 cells in vivo. A, CFDA-labeled (4 h, 1 d, and 2 d) or GFP-transduced OVCAR5 cells (1 wk) were pretreated with recombinant endostatin or angiostatin and then injected into the peritoneum of female, athymic mice. Mice were subsequently treated with daily injections of endostatin or angiostatin (s.c.), RITC-dextran (red) was injected i.v. before sacrifice for visualizing blood vessels. Representative images of OVCAR5 cells attached to the peritoneal wall harvested at different time points. Column 1, control; column 2, endostatin-treated mice; column 3, angiostatin-treated mice. Scale bars, 0.1 mm. Blue arrows, tumor nodules. B, cumulative data of peritoneal seeding of ovarian cancer cells. Number of tumor cells attached to the peritoneum was determined by morphometric analysis. Five mice were used for each data point. Control-treated (○), endostatin-treated (●), and angiostatin-treated (▲) mice. Bars, SE. Statistical significance was determined by Student’s t test. **, P < 0.01.
Endostatin treatment inhibits attachment of primary ovarian cancer cells to the peritoneal wall. To determine the clinical relevance of endostatin-mediated prevention of ovarian cancer cell attachment, we then determined the effect of endostatin on primary ovarian cancer cells obtained from a limited number of patients. Three primary tumor samples were obtained from the ascites of stage III serous cystadenocarcinoma patients and were evaluated for ex vivo attachment to mouse peritoneal wall. Data in Fig. 6 show representative images of primary tumor cell attachment to peritoneum. Primary tumor cells from ascites had large cellular aggregates (spheroids) and attached to the peritoneum ranging between 190 and 1,080 tumor cells per field (Fig. 6, column 1). MA160 sample showed the highest level of attachment. Pretreatment with endostatin reduced the peritoneal attachment of this tumor sample by 50% (Fig. 6A, column 3). The other two primary ovarian cancer cells, MA161 and MA162, were inhibited by 60% and 73%, respectively, by endostatin pretreatment (Fig. 6B and C). These results further confirm the unique property of endostatin to inhibit ovarian cancer cell attachment and peritoneal seeding.

**Discussion**

Ovarian cancer cells disseminate inside the peritoneum by three different ways (21). The major pathway by which ovarian cancer cells spread within the peritoneum is through the penetration of ovarian capsule and direct shedding into the peritoneal cavity. Tumor cells are then passively carried by the peritoneal fluid and distributed by visceral movements. Second, tumor cells can invade contiguous organs such as the uterus, fallopian tube, or the pelvic peritoneum. Finally, tumor spread has been noted to occur via the lymphatic system.
Ovarian cancer cells shed into the peritoneum from the primary site first adhere to the ECM components of the peritoneal wall. Tumor cells attaching to a nearby peritoneal vasculature are likely to have a better chance of survival when compared with other cells (22). Hypoxic selection pressure favors survival of tumor cells that are adhered close to host vasculature (cooption). In other model systems, initial vessel cooption has been followed by a transient period of vessel regression leading to significant loss of tumor cells (4). The surviving population of tumor cells was found to produce higher levels of proangiogenic growth factors to induce neovascularization. Both vessel cooption and neovascularization are mediated by interactions between cell surface integrins and ECM components (23). Therefore, disrupting the interaction between integrin-mediated tumor cell attachments to ECM can have profound effects on tumor angiogenesis and metastasis. Present studies provide evidence for the first time that integrin-binding angiostatic proteins such as endostatin can affect tumor metastasis by directly preventing cancer cell attachment.

Previous studies have shown that integrins α5β1, α3β1, and α3β1 participate in ovarian and endometrial cancer cell attachment (24–26). Antibodies directed to integrin subunit had been found to block ovarian cancer cell attachment to matrix components to a varying degree. The extent of inhibition correlated well with the levels of integrin expression. Present studies confirm some of the previous observations that the integrin α5β1 is involved in ovarian cancer cell attachment. In addition to matrix attachment, integrin α5β1 expression on ovarian cancer cells was also found to influence direct binding to human endostatin. Flow cytometric analysis in the present studies clearly identified that the attachment to endostatin-coated wells was directly proportional to the levels of integrin α5β1 expression. Lack of correlation with other integrins confirms that endostatin binding is largely mediated by integrin α5β1. Previous studies have shown that antibodies to integrin α5β1 and siRNA-mediated knockdown of α5β1 were able to block ovarian cancer cell attachment to fibronectin and endostatin-coated wells (12). Although endostatin

Figure 5. Effect of adenovirus-mediated expression of endostatin in situ on peritoneal attachment. A, characterization of endostatin secreted by recombinant adenovirus. Left, SDS-PAGE (silver stain) analysis of purified endostatin from conditioned medium of adenovirus-infected OVCAR5 cells (lane 3). Lane 1, endostatin standard expressed in yeast. Lane 2, molecular weight marker. Right, Western blot of purified endostatin from OVCAR5 cells infected with AdEndo (lane 2) and endostatin expressed in yeast (lane 1). B, peritoneal attachment of OVCAR5 cells transduced with AdEndo or AdLacZ virus. Columns, mean of five animals. C, effect of i.p. expression of endostatin on tumor cell seeding. AdEndo or AdLacZ (1.5 × 10⁹ plaque-forming units) was injected into the peritoneum of athymic mice. After 3 d, CFDA-labeled OVCAR5 cells were injected i.p. Peritoneal seeding was determined 2 d after the injection of tumor cells. Columns, mean of five animals per group; bars, SE. **, P < 0.01. D, representative images of OVCAR5 peritoneal attachment of mice injected with AdEndo or AdLacZ.
inhibits endothelial cell proliferation and migration, it did not affect ovarian cancer cells in a similar way. Furthermore, endothelial cells readily internalized endostatin-integrin complex, whereas the ovarian cancer cells retained the complex on the cell surface. Surface retention is likely to competitively inhibit integrin-mediated cell attachment.

Reducing the levels of integrin $\alpha_5\beta_1$ expression in ovarian cancer cells by siRNA inhibited peritoneal attachment of tumor cells in vivo as well. Endostatin treatment of integrin $\alpha_5\beta_1$ siRNA-transfected cells did not show any further decrease in peritoneal attachment. These results suggest that integrin $\alpha_5\beta_1$ expression on tumor cells is necessary for endostatin-mediated inhibition of tumor cell seeding. Ovarian cancer cells were then injected into the peritoneum, simulating the conditions of tumor cell shedding. In this model system, tumor cell attachment increased with time and reached peak levels by 24 h. Subsequently, there was a decrease in the number of cells attached to the peritoneal wall. Reduced tumor cell attachment on day 2 is most likely due to hypoxic selection pressure leading to cell death. An alternate possibility is that the surviving cells could have migrated to other favorable sites. Current studies cannot rule out either of the two possibilities. Following the drop in the number of tumor

Figure 6. Peritoneal attachment of primary ovarian cancer cells ex vivo. Ovarian cancer cells isolated from the ascites of three patients (MA160, MA161, and MA162) were labeled with CFDA and then treated with endostatin. Peritoneal wall isolated from athymic mice were then exposed to control- and endostatin-treated primary tumor cells. A, MA160. B, MA161. C, MA162. Representative images from control-treated (column 1) and endostatin-treated (column 2) groups. Scale bars, 0.2 mm. Tumor cells attached to the peritoneal wall were determined by morphometric analysis (column 3). Peritoneal wall isolated from five animals per group were used to evaluate attachment of MA160 and MA162 cells. Tissue samples from three animals were used to determine attachment of MA161 cells. Statistical significance was determined by Student’s $t$ test. **, $P < 0.01$. 

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cell attachment on day 2, there was a significant increase in the number of tumor cells seeding the peritoneum by day 7, which indicates an expansion of seeded tumor cells. Endostatin treatment inhibited both the initial attachment and subsequent expansion of tumor cells. Mendoza et al. (27) showed that endostatin treatment can reduce hepatic microvascular retention of B16M melanoma cells, leading to inhibition of metastasis. In this experimental metastasis model, tumor cells were directly injected into the systemic circulation. Retention of tumor cells inside the lumen of microvasculature can be in part attributed to vessel constriction, blood flow changes, and tumor cell attachment to the luminal side of the endothelial cells. Our studies focused on tumor cell attachment on the peritoneal wall and at the abluminal (outer) side of the vasculature. This model mimics closely the natural history and pattern of ovarian cancer cell dissemination. Another study by Te Velte et al. (28) showed that endostatin treatment can inhibit metastatic seeding of colon cancer cells in the liver. Their data also suggested that in addition to antiangiogenic effects, endostatin could reduce tumor cell adhesion in the liver sinusoids during the very early phases of metastasis.

Interestingly, the early phases of peritoneal seeding are not affected by angiostatin treatment. This is consistent with the fact that angiostatin does not bind and interfere with human ovarian cancer cell attachment to ECM. Angiostatin treatment, however, showed a significant decrease in tumor cell nodules by day 7. These results suggest that angiostatin affects a later step in tumor angiogenesis. Endostatin, on the other hand, blocks both vessel cooption (an earlier step) and neovascularization (a later step). This conclusion is supported by the fact that both angiostatin and endostatin preparations were able to inhibit growth factor–induced angiogenesis of Matrigel plugs (Supplementary Data 2). However, only endostatin was capable of inhibiting ovarian cancer cell attachment to peritoneum. Temporal differences in the action of these two angiogenesis inhibitors also provide a rationale for combination therapies using both molecules. Indeed, our earlier studies showed synergistic inhibition of angiogenesis when mice were treated with a combination of angiostatin and endostatin (17). This strategy was successfully adapted to gene therapy approaches using bicistronic vectors coexpressing both angiostatin and endostatin (29). Endostatin-mediated inhibition of peritoneal attachment seems to be dictated by integrin α5β1 expression because a melanoma cell line, WM35, with low levels of integrin α5β1 was not affected (Supplementary Data 1). Although endostatin did not inhibit WM35 attachment, it was capable of inhibiting WM35-induced angiogenesis in a Matrigel plug assay (Supplementary Data 2). Present studies suggest that endostatin binding to the “seed” (tumor cells) prevents its attachment to the “soil”, the peritoneal wall, and diaphragm. Endostatin may also indirectly affect the peritoneal seeding by modulating the local milieu. Integrin α5β1 plays multiple roles in tumor microenvironment. For example, urokinase plasminogen activator receptor system is modulated by α5β1 integrin, which, in turn, has been shown to affect matrix metalloproteinases (30). Such an indirect effect can certainly affect tumor cell invasion.

In addition to established tumor cell lines, peritoneal attachment of primary ovarian cancer cells was also inhibited by endostatin treatment. These results further suggest that i.p. expression of endostatin could be useful in reducing peritoneal dissemination. In conclusion, our studies show that endostatin can inhibit ovarian cancer by independently targeting tumor cells and vascular endothelium. Therefore, ovarian cancer is a suitable target for endostatin-mediated antiangiogenic therapy. Recently concluded phase I/II trials using endostatin infusion showed less than anticipated clinical response in patients with advanced disease. Perhaps, endostatin can be more effective in adjuvant settings with minimal residual disease. Our studies suggest that i.p. expression of endostatin is effective in reducing peritoneal seeding in model systems and, therefore, can be included in clinical trial designs of endostatin treatment following adjuvant chemotherapy.

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Yumi Yokoyama, Gerald Sedgewick and S. Ramakrishnan


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