In Vivo Inhibition of CD44 Limits Intra-Abdominal Spread of a Human Ovarian Cancer Xenograft in Nude Mice: A Novel Role for CD44 in the Process of Peritoneal Implantation

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Abstract

Ovarian cancer cells frequently metastasize by implanting onto the peritoneal mesothelial lining of the abdominal cavity. Data obtained from in vitro adhesion studies have suggested a possible role for the CD44 molecule in this process. The purpose of the present study was to determine the in vivo role of CD44 in ovarian cancer metastasis by using a nude mouse xenograft model of peritoneal implantation. Three groups of 10 athymic female nude mice each received an i.p. inoculum of 10 x 10^6 cells from a CD44-positive human ovarian cancer cell line (36M2) in the presence of either anti-D144 antibody (Ab; nonreactive IgG1), anti-DF3 Ab (reactive IgG1 Ab that does not inhibit in vitro binding), or neutralizing anti-CD44 Ab (IgG2a). The number of peritoneal and diaphragmatic metastases that demonstrate an important role for the CD44 molecule in the implantation of human ovarian cancer cells within the murine peritoneal cavity.

Materials and Methods

Source of Reagents and Antibodies. Monoclonal antibodies used in the characterization of ovarian cancer cell lines and for i.p. treatment are anti-D144 (nonreactive IgG1; Ref. 2), anti-DF3 (reactive IgG1, a kind gift of Dr. Donald Kufe, Dana-Farber Cancer Institute, Boston, MA), and anti-CD44 (reactive IgG1, clone 515, a kind gift of Dr. Geoffrey Kansas, Northwestern University, Chicago, IL). As expected, both anti-D144 and anti-DF3 are incapable of inhibiting ovarian cancer cell binding to mesothelium in vitro over a concentration range of 1–50 μg/ml (data not shown; Refs. 2 and 3). Anti-CD44 antibody clone 515 has been previously shown to neutralize CD44-mediated binding of cells to mesothelium and to hyaluronic acid-coated wells in vitro (2, 3). This antibody does not induce tumor cell clumping of the 36M2 human ovarian cancer cell line (described below) in suspension culture under conditions that prevent plastic adherence. The use of these antibodies in indirect immunofluorescence analysis by flow cytometry has been described previously (3). All antibodies were affinity-purified using Affi-Gel protein A agarose (Bio-Rad Laboratories, Hercules, CA). The BBA10 antibody (IgG2a, pan-CD44; R&D Systems, Minneapolis, MN) was used in immunoblot analysis to characterize the molecular masses of CD44 species present on 36M2 cells and its subclones as described below.

Cell Lines. Human ovarian cancer cell lines used in this study were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 7.5% FCS (Hyclone, Logan, UT) unless otherwise specified. The UPN36T human ovarian cancer cell line was originally derived from a patient...
with papillary serous ovarian cancer by injecting $100 \times 10^6$ cells from malignant ascites into the peritoneal cavity of a female Swiss nu/nu mouse, with subsequent selection of a peritoneal tumor nodule for in vitro propagation. This line has been previously shown to be partly dependent upon the CD44 molecule for its ability to bind peritoneal mesothelium in vitro (2, 3). To obtain a line that resulted in highly efficient implantation in nude mice, the original UPN36T line was passaged a second time by injecting $100 \times 10^6$ cells i.p. into a Tac-Cr(NCcr)-nu/BR female athymic nude mouse 24 h after irradiation of the animal with 300 R. After 5 weeks, the animal was sacrificed, and a peritoneal tumor nodule was aseptically removed, dissociated with Dnase/collagenase, and expanded in vitro in 20% FCS/Lifescove's modified DMEM (Sigma) containing 5 ng/ml recombinant human EGF (culture grade; Angen, Thousand Oaks, CA). After subcloning, the line was referred to as 36M2 (M, mouse- selected; 2, second passage). Like the parent UPN36T line, 36M2 cells strongly express both CD44 and DF3 molecules as assessed by flow cytometry (94 and 83% specific reactivity, respectively; data not shown). In pilot studies we determined that the mesothelial binding properties of 36M2 cells were similar to those of parent UPN36T cells and that neither anti-D144, anti-DF3, nor anti-CD44 antibodies were capable of inhibiting cell growth in vitro at a concentration of $10\mu g$ of antibody/ml for 5 days in culture (data not shown). The SW626 ovarian cancer cell line was used as a positive control for the presence of CD44 splice variants in immunoblot analysis (3) and was purchased from the American Type Culture Collection (Rockville, MD).

### In Vivo Assessment of Ovarian Cancer Cell Implantation.

To determine the effects of anti-CD44 antibody on ovarian cancer cell implantation in vivo, athymic female nude mice [Tac-Cr(NCcr)-nu/BR] were preirradiated (300 R), followed 24 h later by i.p. inoculation with $10 \times 10^6$ 36M2 cells/mouse in the presence of either anti-D144, anti-DF3, or anti-CD44 antibody. For each mouse, the cells were initially incubated in the appropriate antibody at a concentration of 45 $\mu g$ of antibody/0.5 ml of PBS for 30 min at 4°C to ensure adequate antibody coating before i.p. injection. After resuspension, the cells were injected i.p. in the continued presence of antibody (0.5 ml). We have previously determined in pilot studies that maximum inhibition of CD44-mediated binding in vitro occurs at anti-CD44 antibody concentrations of $\approx 1.0 \mu g/ml$. Therefore, a dose of 45 $\mu g$/i.p. injection was used to achieve a final in vivo concentration of 1.5 $\mu g$/ml, assuming an average nude mouse weight of 30 g and therefore a possible maximum volume of distribution of 30 ml. Thereafter, antibody treatment was repeated for a total of 10 i.p. doses/mouse (45 $\mu g$ of antibody in 0.5 ml) equally spaced over a 20-day duration. After 5 weeks, the mice were sacrificed, and nodules on the peritoneal mesothelial surfaces of the abdominal cavity and the underside of the diaphragm were quantified under low-power magnification using a micrometer (Manostat). For some experiments, UPN36T cells were injected s.c. as indicated to exclude the possibility of a direct antiproliferative effect of antibody on the growth of CD44-positive tumor cells in vivo.

### In Vitro Assessment of Ovarian Cancer Cell Binding to Mesothelium.

The in vitro binding characteristics of 36M2 ovarian cancer cells to mesothelium were characterized using a previously described $^{51}$Cr-labeled binding assay (2). Mesothelial cells ($1.5 \times 10^4$) obtained from ascitic fluid were added to flat-bottom microtiter wells (Nunc, Roskilde, Denmark) in 100 $\mu l$ of 20% FCS/Lifescove's modified DMEM supplemented with 5 mg/ml EGF and 0.5 $\mu g/ml$ hydrocortisone to permit cell growth to confluency (2–3 days). On the day of the binding assay, mesothelial monolayers were washed twice in 1% FCS/MEM (Life Technologies, Inc.) to remove EGF and hydrocortisone. Ovarian cancer cells ($2.5 \times 10^6$) were then labeled with 0.10 ml of $^{51}$Cr (1 mCi/ml, 200 Ci/g; DuPont New England Nuclear, Boston, MA) for 1 h at 37°C, followed by washing twice in HBSS. Cells were subsequently treated (30 min at 4°C) with either control or anti-CD44 (clone 515) antibodies (10 $\mu g$/ml), followed by the addition of 50–100 $\times 10^3$ cells/well to microtiter wells containing a confluent layer of mesothelial cells in the continued presence of antibody. After the addition of cells, the plates were spun at 800 rpm for 5 min, and binding was allowed to occur for 30 min at 37°C. After incubation, the nonadherent cells were removed by three washes with 1% FCS/MEM, followed by lysis of bound cells with 0.1% NP40. The radioactivity of each lysate was measured in a gamma counter. The mean cpm for each treatment group was determined for quadruplicate wells. The percentage of cells specifically bound was calculated as follows: % specific binding = $[\text{mean cpm (mesothelial monolayer)} - \text{mean cpm (plastic)}]/\text{cpm (total)}$.

### Immunoblotting.

Lysates (50 $\mu g$/lane) were resolved by one-dimensional SDS-PAGE under reducing conditions, followed by transfer onto a 0.45-$\mu m$ polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) in transfer buffer at 0.2 amper for 2 h. After transfer, residual binding sites were blocked by incubating the membrane in TBS containing 10% nonfat dry milk for 1 h at RT. The blots were then incubated with a pan-CD44 antibody (BBA10; 2 $\mu g/ml$) in TBS containing 5% nonfat dry milk for 16 h at 4°C. The blots were then washed 3 times for 10 min in TBST, followed by incubation with sheep anti-mouse immunoglobulin conjugated to horseshad peroxidase (Amersham, Arlington Heights, IL) in TBST containing 5% nonfat dry milk for 1 h at RT. After 3 washes for 10 min in TBST, the blots were developed using the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's protocol and exposed to X-ray film (Eastman Kodak).

### Immunohistochemistry Analysis.

In some experiments, the APAAP technique was used to assess the bioavailability of murine antibody to s.c. tumor nodules by detecting the presence of murine monoclonal antibody (2). Briefly, s.c. tumor was processed 1 h after i.p. injection of either anti-D144 or anti-CD44 antibody by snap-freezing in isopentane, followed by cryostat sectioning (6–8 $\mu m$), and fixation in acetone for 10 min at RT. Fixed slides from cryostat sections were then treated for 30 min at RT with 50 $\mu l$ of rabbit anti-mouse immunoglobulins (1:25 dilution in TBS; DAKO) and incubated for 30 min at RT, followed by washing. APAAP complexes (50 $\mu l$ of a 1:50 dilution in TBS; DAKO) were added and incubated for 30 min at RT, followed by washing. The alkaline phosphatase substrate was freshly prepared and consisted of 2 mg of naphthol AS-MX phosphate, free acid, 0.2 ml of dimethylformamide, 9.8 ml of 0.1 m Tris buffer (pH 8.2), 1 ml levamisole, and 10 $mg$ of Fast-Red TR salt (Sigma). Each slide was flooded with substrate and incubated for 20 min at RT. After washing, the slides were counterstained with hematoxylin and mounted with Glycergel (DAKO).

### Statistical Analysis.

Data are expressed as mean ± SE when appropriate. Significance levels for comparison of binding between cell lines were determined using the two-sided Student's t test for unpaired samples.

### Results and Discussion.

To determine the effects of anti-CD44 antibody on ovarian cancer cell implantation in vivo, athymic female nude mice [Tac-Cr(NCcr)-nu/BR] were preirradiated with 300 R, followed 24 h later by i.p. inoculation with $10 \times 10^6$ 36M2 cells/mouse in the presence of either nonreactive control antibody (anti-D144), reactive control antibody (anti-DF3), or anti-CD44 antibody as stated above. Anti-D144 antibody was a control for the presence of mouse IgG1 antibody, and anti-DF3 was an isotype-identical control for the possibility that cell coating with murine antibody might induce a cytotoxic response in vivo through ADCC. Thereafter, antibody treatment was repeated for a total of 10 i.p. doses/mouse (45 $\mu g$ of antibody in 0.5 ml of each dose) equally spaced over a 20-day period. Each treatment group was comprised of 10 mice, with each mouse receiving a total of 450 $\mu g$ of the appropriate antibody (45 $\mu g \times 10$ doses) by the completion of therapy.

Five weeks after i.p. tumor cell inoculation, the mice were sacrificed, and nodules on the peritoneal mesothelial surfaces of the abdominal cavity and the underside of the diaphragm were quantified under low-power magnification. All mice were alive and healthy at the time of sacrifice, with no differences observed in the mean weight of mice in each treatment group (mean weight, 23.7 ± 0.5 g; n = 30/group). As shown in Figs. 1 and 2, there were equivalent total numbers of tumor implants observed in the anti-D144 and anti-DF3 antibody-treated groups (103 ± 17 nodules/mouse versus 120 ± 20 nodules/mouse, respectively, mean ± SE; n = 10/group; P > 0.2). Treatment with either D144 or DF3 did not affect implantation or growth of peritoneal tumor compared with untreated animals (data not shown). In contrast, there was a significant decrease in the total number of nodules observed in the anti-CD44 antibody treatment group compared to either of the control groups (35 ± 4 nodules/mouse; n = 10;
Three groups of 10 athymic female nude mice each were preirradiated with 300 R and treatment group). Peritoneal nodules present on all serosal surfaces, excluding dia-

implants were quantitated under low-power magnification. Peritoneal implantation of 36M2 cells is expressed as mean ± SE nodules in the indicated locations (n = 10/treatment group). Peritoneum, nodules present on all serosal surfaces, excluding diaphragm. Total, nodules present on peritoneal and diaphragmatic surfaces.

P < 0.002). There were no differences in mean diameter of nodules observed in the anti-D144, anti-DF3, or anti-CD44 treatment groups (0.58 ± 0.02, 0.6 ± 0.02, and 0.58 ± 0.05 mm, respectively; P > 0.2). The location of tumor nodules (diaphragmatic versus other peritoneal surfaces) was also assessed to determine whether differences exist in the distribution of implants between the three treatment groups. As shown in Fig. 1, the ability of anti-CD44 antibody to inhibit implantation seemed to be partly dependent upon tumor location, with a mean of 46% inhibition observed for the diaphragmatic implants (P = 0.1) versus 73% inhibition of implants involving other peritoneal surfaces (P < 0.002).

To determine whether the reduction in the number of tumor nodules observed in anti-CD44-treated animals was due to selection of cells with diminished proliferative capacity, tumor cell implants were excised in each of the three treatment groups and expanded in vitro, with subsequent determination of the growth rate in culture over a 5-day period. No difference in proliferative rate was observed between 36M2 subclones derived from the anti-D144, anti-DF3, or anti-CD44 antibody treatment groups (data not shown), an observation consistent with the fact that the mean size of tumor implants was identical in the three groups of animals. In addition, CD44 was expressed by 93, 94, and 89% of cells in 36M2 subclones derived from anti-D144, anti-DF3, or anti-CD44 antibody-treated mice, respectively, as assessed by flow cytometry. Immunoblot analysis revealed that the predominant CD44 species expressed by 36M2 subclones had a molecular mass of 90 kDa, consistent with CD44H in vivo. To further exclude a direct cytotoxic effect of anti-CD44 antibody in vivo, we determined whether antibody treatment was capable of inhibiting the growth of ovarian cancer cells grown s.c., as opposed to i.p., in nude mice. Two groups of 10 preirradiated mice each received a s.c. injection of 0.5 × 10⁶ UPN36T ovarian cancer cells, which strongly express CD44H, into the right flank and were allowed to form a palpable tumor nodule measuring ~5 mm² (occurring after a mean of 18 days). The mice were then treated with 40 μg of either anti-D144 or anti-CD44 antibodies i.p. in 0.5 ml of PBS for a total of 10 doses equally spaced over a 3-week period during which tumor measurements were made. As shown in Fig. 4, there was no difference in the growth rate of s.c. tumor nodules in either treatment group, suggesting that the presence of anti-CD44 antibody by itself did not mediate an antiproliferative or cytotoxic effect in this system. To ensure that antibody delivery via the i.p. route was bioavailable to the tumor cells in these experiments, tumor nodules were excised 1 h after i.p. antibody injection, and the presence of tumor-associated anti-

A possible role for CD44H in mediating ovarian cancer metastasis was first suggested by its ability to promote in vitro attachment of tumor cells to peritoneal mesothelial monolayers through the recognition of mesothelial-associated hyaluronic acid (2). Because adhesion of tumor cells to the peritoneal mesothelium is a critical early step in ovarian cancer cell metastasis, we were interested in determining the physiological relevance of these observations for the process of peri-

The ability to significantly reduce the number of implants in mice treated with anti-CD44 antibody most likely represents a specific effect on implantation for several reasons: (a) treatment with a reactive isotype-identical antibody (anti-DF3) exerted no effect on tumor implant formation, thus excluding an important role for ADCC in this phenomenon (Figs. 1 and 2). The absence of ADCC is also consistent with the use of IgG1 isotype antibodies in these studies; (b) the lack of effect of anti-CD44 antibody on the growth of CD44-positive tumor nodules grown s.c., as opposed to i.p., again argues against a major role for either ADCC or natural killer cell-mediated cytotoxicity (Fig. 4); and (c) none of the antibodies used in this study inhibited the growth of ovarian cancer cells in vitro, reducing the likelihood of a direct antiproliferative effect of anti-CD44 antibody on tumor nodule formation in vivo. This conclusion is also supported by the observation that the size of tumor nodules present in anti-CD44 antibody-treated mice was equivalent to those present in control animals, suggesting that once implantation occurred, the growth of

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Fig. 2. Representative appearance of i.p. tumor implants in mice from each treatment group. A, anti-D144 antibody-treated mouse, demonstrating grossly visible 36M2 tumor implants. B, anti-DF3 antibody-treated mouse. C, anti-CD44 antibody-treated mouse, demonstrating reduction in the extent of tumor implants.

Fig. 3. Characterization of 36M2 subclones derived from residual implants in treated mice. Implants were excised and expanded in vitro as described in the text to determine whether differences existed in CD44 expression and function between 36M2 cells derived from the three treatment groups. 36M2 (original) refers to the line used at the time of the initial i.p. inoculation. 36M2 (D144), 36M2 (DF3), and 36M2 (CD44) refer to subclones later isolated from anti-D144, anti-DF3, and anti-CD44 antibody-treated mice, respectively. A, immunoblot developed with anti-CD44 antibody, showing predominant expression of a 90-kDa species characteristic of CD44H in 36M2 cells and the presence of higher molecular weight CD44 species characteristic of splice variants in SW626 as described previously. B, in vitro binding of 36M2 subclones to confluent layers of mesothelial cells, revealing equivalent amounts of CD44-dependent binding in cells derived from each of the three treatment groups.

ovarian cancer cells proceeded normally despite the presence of anti-CD44 antibody.

We have previously shown that it is not possible to completely inhibit ovarian cancer cell adhesion to mesothelium in the presence of anti-CD44 antibody, suggesting that other adhesion molecules may be involved in the implantation process (2, 3). We therefore considered the possibility that treatment with anti-CD44 antibody might select for tumor implants that are relatively deficient in CD44 expression and that bind to mesothelium through a CD44-independent mechanism. However, 36M2 subclones obtained from each treatment group showed equivalent levels of CD44H expression and of CD44-dependent adhesion to mesothelium in vitro (Fig. 3). These data suggest that the presence of tumor implants in mice treated with anti-CD44 anti-
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body reflects incomplete inhibition of CD44 rather than selection of cells with diminished CD44 expression or function. The fact that anti-CD44 antibody was less efficient at inhibiting the formation of diaphragmatic implants compared to other peritoneal sites suggests that the effectiveness of neutralizing antibody may be dependent upon the distribution of this reagent within the peritoneal cavity.

Previous investigators have suggested that CD44 may be involved in the hematogenous spread of malignant cells (5–7). For instance, CD44H-expressing transfectants of Namalwa cells exhibit an increased rate of metastasis when the cells are injected i.v., and this effect is inhibited in the presence of soluble CD44H-immunoglobulin fusion protein (5, 6). Interestingly, certain splice variants of the CD44 molecule, such as CD44E, do not seem to enhance hematogenous metastasis, suggesting that the standard 90-kDa CD44H protein is a major determinant of malignant potential in at least certain animal tumor models (5). Nevertheless, other studies have suggested that expression of certain forms of CD44 splice variants, such as those containing exon 6, may increase the likelihood of distant metastasis (8–10). Also, CD44 splice variant expression may be associated with a worse prognosis in certain forms of human malignancy such as lymphoma (9) and colorectal cancer (11), although this is not a universal phenomenon (12, 13). Taken together, these data suggest that both standard CD44H and its splice variants may contribute to the process of distant metastasis, although the mechanism by which these molecules mediate hematogenous or lymphatic spread is not fully understood. It is important to note that ovarian cancer is distinct from many other epithelial malignancies in that it typically remains confined to the peritoneal cavity and does not usually spread via the hematogenous route. Thus, in contrast to previous reports, the data presented in this paper suggest a novel and distinct role for the CD44 molecule in the metastatic process, specifically by mediating an early step (implantation) in ovarian cancer spread.

In summary, our data suggest that the CD44 molecule plays an important role in human ovarian cancer metastasis and that i.p. administration of agents that inhibit CD44 function may represent a potentially useful strategy for the treatment of patients with this disease. Because most patients present with disseminated tumor at the time of diagnosis, it is possible that this approach might be most beneficial as an adjunct to surgery and chemotherapy in an attempt to prevent or delay recurrence of peritoneal implants. Although not the goal of the present study, it will be important to determine which dose and schedule of anti-CD44 antibody treatment will produce optimal inhibition of implantation and whether anti-CD44 antibody treatment can affect the survival of mice with pre-established intra-abdominal tumor.

References

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