Functional Heterogeneity of CD44 Molecules in Ovarian Cancer Cell Lines

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ABSTRACT
We have previously shown that CD44 partly mediates ovarian cancer cell attachment to peritoneal mesothelium through recognition of mesothelial-associated hyaluronate. CD44 is a major receptor for hyaluronate and exists as a standard 90–180-kDa form (CD44H), as well as several higher molecular mass variant forms produced by alternative splicing. To determine whether functional differences exist between CD44H and its variants we have investigated the relationship between CD44 isofrom expression and mesothelial adhesion in 12 ovarian cancer cell lines. Eight lines were CD44 positive (range, 83–94%) and demonstrated significant binding to mesothylumen and hyaluronate, whereas two lines showed reduced CD44 levels (3–13%) and demonstrated decreased binding. Interestingly, two other lines (OVC-3 and SW626) expressed CD44 in the majority of cells (>93%) and yet bound weakly to mesothelygium. Mean linear fluorescence intensity of CD44 expressed by OVC-3 and SW626 cells was approximately one-half that of strongly binding cell lines, suggesting that the ability to adhere may be partly related to CD44 surface density. However, immunoprecipitation and immunoblot analyses revealed that standard CD44H represented only 23–31% of total CD44 in weakly binding cells, with the majority of species being comprised of CD44 variants. Indirect immunofluorescence of OVC-3 and SW626 cells confirmed the presence of CD44 variants containing exons v3, v6, and v9. In contrast, CD44H represented the majority (75–86%) of total CD44 expressed by strongly binding cell lines such as CAOV-3 and UPN36T. Transfection of CD44H cDNA into weakly binding OVC-3 cells restored significant mesothelial binding which was partly blocked by anti-CD44 antibody. These data suggest that the expression of CD44 is necessary but not sufficient for mediating attachment of ovarian cancer cells to mesothellium. Although CD44 variants may constitute the major CD44 species in certain ovarian cancer cell lines, it appears that these CD44 species are not always capable of mediating significant binding to mesothelium or hyaluronate. Rather, an adequate level of CD44H is the critical determinant of binding in this system. The role of CD44 variants in the process of ovarian cancer metastasis will require further investigation.

INTRODUCTION
Ovarian cancer spreads predominantly by intraabdominal seeding of tumor cells within the peritoneal cavity, resulting in diffuse peritoneal implants and ascites (1). By developing an in vitro binding assay to quantitate ovarian cancer cell adhesion to peritoneal mesothelium, we have investigated some of the molecules which might be involved in this process. Although ovarian cancer cells express several molecules with known adhesion function, such as the β-1 integrins and CD44, we have previously reported that only anti-CD44 antibody is capable of blocking approximately 40–50% of ovarian cancer cell binding to mesothelium (2). Consistent with the known affinity of standard CD44 (CD44H) for hyaluronate, we have shown that pretreatment of mesothelial monolayers with hyaluronidase completely abrogates the CD44-mediated component of binding. Thus, ovarian cancer cell adhesion to peritoneal mesothelium occurs in part through CD44 recognition of mesothelial-associated hyaluronate (2).

CD44 is a major receptor for hyaluronate and exists as a standard 90–180-kDa form, also known as CD44H, as well as higher molecular mass variant forms produced through alternative splicing (3–10). Since CD44 is a critical component of ovarian cancer cell implantation, we have been interested in how the expression and function of this molecule may be regulated in this disease. In this regard, we have found that ovarian cancer cells from ascites frequently express diminished levels of CD44 when compared to tumor cells from peritoneal implants (2). This observation suggests that loss of CD44 might be one of the events which induces ovarian cancer cell detachment from the peritoneal mesothelial surface. However, it is possible that regulation of CD44 function may occur at several levels, including decreased CD44 surface expression, lack of CD44 function despite adequate surface expression (10, 11), or the expression of CD44 variant molecules which may not effectively participate in mesothelial binding. For instance, a variant known as CD44E has been described which appears to lack the ability to bind to hyaluronate, suggesting that functional differences may exist between CD44 variants and CD44H (6). Also, in contrast to CD44H, expression of CD44 variants is associated with increased metastases of certain carcinoma cell lines in vivo (9, 13–15), suggesting a possible role for these molecules in the process of lymphatic or vascular homing (16).

The purpose of the current study was to determine the relationship between CD44 surface expression and ovarian cancer cell binding to peritoneal mesothelium, specifically to better understand the mechanisms by which CD44-mediated binding may be regulated. We have unexpectedly identified two ovarian cancer cell lines which fail to bind effectively to mesothelium.

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The heterogeneity of CD44 in ovarian cancer is discussed. Despite expressing high levels of surface CD44, we provide evidence to suggest that this is related to an absolute decrease in surface CD44H, associated with the expression of several CD44 variants which do not appear to be capable of mediating effective mesothelial binding. The possible consequences of this observation for the process of ovarian cancer cell metastasis are discussed.

**MATERIALS AND METHODS**

**Antibodies.** Murine mAb 515 (IgG1, clone 515, a gift from Dr. Geoffrey Kansas, Dana-Farber Cancer Institute, Boston, MA) reacts with all human CD44 molecules and was used for phenotyping, cell adhesion assays, and immunoprecipitation as outlined below (17). As previously described, this antibody is capable of blocking 40–50% of ovarian cancer cell binding to mesothelium and greater than 90% of binding to hyaluronate-coated plastic (2). Additional murine anti-CD44 mAbs used for immunoblotting and flow cytometry were BBA10 (IgG2a, pan CD44), BBA11 (IgG2b, CD44 variant exon 3), and BBA 13 (IgG1, variant exon 6) each purchased from R and D Systems, Minneapolis, MN. Murine mAb 11.24 recognizes CD44v9 and was a gift from Dr. Ursula Günther (Basel Institute for Immunology, Basel, Switzerland). Clone MD144 (D144) is a hybridoma obtained from the ATCC which secretes an IgG1 murine mAb which is nonreactive with the cells used in this study and serves as a negative control.

**Source of Cells.** The CAOV-3, SKOV-3, and SW626 ovarian epithelial carcinoma cell lines used in this study were obtained from the ATCC (Rockville, MD). These cells were cultured in IMDMEM (GIBCO-BRL, Grand Island, NY) supplemented with 20% FCS (HyClone, Logan, UT). The OVCAR-3 line was also obtained from the ATCC, although we noted the gradual evolution of this line’s expression of CD44, as well as adhesion properties, during serial passage. Specifically, three distinct OVCAR-3 strains were developed by serial passage of the parent line over the course of 3–4 months and are referred to as OVC-1, OVC-2, and OVC-3. The parent OVCAR-3 line from the ATCC is referred to as OVC-P in this article. Additional ovarian cancer cell lines developed in our laboratory from either malignant ascites or tissue implants are UPN36T (2), UPN11, UPN13, UPN14, and UPN21. All ovarian cancer cell lines have been passaged for over 1 year in IMDMEM containing 10–20% FCS without the addition of exogenous growth factors, and they express both keratin and vimentin as assessed by immunoperoxidase staining. Ovarian cancer cells and mesothelial cells were obtained from the malignant ascites of ovarian cancer patients undergoing therapeutic paracentesis for the relief of abdominal distention. Tissue procurement was approved by the Institutional Review Board of the Dana-Farber Cancer Institute.

**Isolation of Peritoneal Mesothelium.** Mesothelial cells were purified from malignant ascites of patients with ovarian cancer by an adaptation of the method described by Rheinwald (18) and Wu et al. (19). Briefly, ascites cells were washed twice in HBSS (GIBCO), resuspended at 5 × 10^6/ml in 10% RPMI 1640 medium (GIBCO), and allowed to adhere in plastic flasks overnight. The nonadherent fraction containing cancer cells, lymphocytes, and neutrophils is then removed, and the adherent fraction (enriched for mesothelial cells) is allowed to grow in 20% FCS-IMDMEM with 5 ng/ml EGF (culture grade; Amgen, Thousand Oaks, CA) and hydrocortisone (0.5 μg/ml). Macrophages are diluted out with subsequent passaging of confluent cultures, and fibroblast contamination is usually minimal and can be assessed morphologically. As noted by Rheinwald (18), tumor cell outgrowth under these conditions is distinctly unusual. Mesothelial cells obtained in this way have a typical morphology, comprised of spread out cytoplasm with a ruffled leading edge and a small nucleus. We have also determined that these cells express cytokeratin and vimentin intermediate filaments as reported by others (19), excluding contamination with either fibroblasts or endothelial cells which express vimentin alone.

**Assessment of Ovarian Cancer Cell Binding to Mesothelium.** Mesothelial cells (1.5 × 10^6) were added to flat-bottomed microtiter wells (Nunc, Denmark) in 100 μl of 20% FCS-IMDMEM supplemented with 5 ng/ml EGF and 0.5 μg/ml hydrocortisone in order 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 (GIBCO) to remove EGF and hydrocortisone. Ovarian cancer cell lines (10^5 cells each) were then labeled with 0.10 ml 51Cr (1 mCi/ml, 200 Ci/g; New England Nuclear, Boston, MA) for 1 h at 37°C, followed by washing in HBSS twice. For some experiments, the cells were subsequently treated (30 min at 4°C) with either control or anti-CD44 (clone 515) antibodies (1:100 final dilution) followed by addition of 50–100 × 10^3 cells/well to microtiter wells containing a confluent layer of mesothelial cells in the continued presence of antibody. Microtiter wells were sometimes coated with human umbilical cord hyaluronate (Biozyme Laboratories, San Diego, CA), 5 mg/ml in PBS, 100 μl/well, and allowed to incubate overnight at 37°C prior to performing binding studies. The plates were spun at 800 rpm for 5 min, and binding was allowed to occur for 30 min at 37°C as described previously. 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 adhering to treated microtiter wells (mesothelium or hyaluronate) was calculated as follows: % specific binding = 100 × [mean cpm (treated surface) − mean cpm (control surface)]/ cpm (total).

**Immunoprecipitation.** Cells (7.5 × 10^6) were suspended in 300 μl PBS and surface labeled with 1 mCi Na^2251 (17 Ci/mg; New England Nuclear) and 1 unit lactoperoxidase (specific activity, 301 units/mg; Calbiochem, La Jolla, CA) for 4 min at 4°C in the presence of 10 μl H_2O_2 (1:1000 dilution in PBS; Sigma) added at the beginning and at 2 min into the incubation period. After washing three times in PBS, the cells were suspended in 1 ml lysis buffer for 30 min at 4°C (1% NP40, 50 mM Tris, 150 mM NaCl) containing 100 mM phenyl-
methylsulfonyl fluoride, 0.135 trypsin inhibitory units of aprotinin, and 40 μM leupeptin (Sigma). Following lysis, the solubilized fraction was obtained by centrifugation (14,000 rpm for 30 min) to remove insoluble debris, followed by preclearing with 50 μl of a 1:1 slurry of lysis buffer and protein A-Sepharose beads (type CL-4B; Pharmacia, Piscataway, NJ) precoated with polyclonal rabbit anti-mouse immunoglobulin (RaM immunoglobulin; Dako). After centrifugation, the pre-cleared supernatant was incubated with either an isotype-identical irrelevant antibody (D144) or with anti-CD44 (515) antibody and RaM-coated protein A-Sepharose beads for 15 h at 4°C followed by pelleting and washing three times in lysis buffer. The immunoprecipitate was boiled for 5 min at 100°C in nonreducing conditions and analyzed by SDS-PAGE followed by autoradiography.

**Immunoblotting.** Lysates (250 μg/lane) were resolved by one-dimensional SDS-PAGE under reducing conditions as described previously (20) followed by transfer onto a 0.2-μm nitrocellulose filter (Schleicher and Schuell, Keene, NH) in transfer buffer at 0.1 amperc overnight at 4°C. After transfer, residual binding sites were blocked by incubating the membrane in Tris-buffered saline containing 1% gelatin (Bio-Rad Laboratories, Melville, NY) for 1 h at RT. The blots were then washed in TBST and incubated for at least 4 h at RT with the primary murine antibody (either anti-CD44 BBA10 or anti-CD44v6 BBA13, 2 μg/ml in TBST). The blots were then washed four times in TBST followed by incubation in 1 μg/ml of rabbit anti-mouse immunoglobulin (Dako) for 1–2 h at RT. After washing, the blot was incubated with a 1:2000 dilution of antirabbit IgG conjugated to alkaline phosphatase (Promega, Madison, WI) in TBST for 2 h at RT. After three additional washes in TBST, the blot was placed in a buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2, 330 μg/ml nitro blue tetrazolium, and 150 μg/ml 5-bromo-4-chloro-3-indoyl phosphate for 10–30 min. The enzymatic color reaction was stopped by rinsing the filters in deionized water.

**Indirect Immunofluorescence Analysis.** CD44 expression by ovarian cancer cell lines was quantified by flow cytometric analysis as described previously (21). Cells (0.5 × 10⁶/sample) were treated with the appropriate concentration of antibody for 30 min at 4°C followed by washing twice and labeling with FITC-conjugated goat anti-mouse Ig (Tago, Burlingame, CA) for 30 min at 4°C. After two additional washes, the cells were analyzed using a Coulter Epics C flow cytometer (Coulter Electronics, Hialeah, FL). Percentage of reactivity was determined by subtracting background fluorescence observed with a nonreactive, isotype-identical control antibody (D144). An assessment of CD44 density was obtained by first determining the mean channel number of fluorescence intensity (X axis) by flow cytometry. Although this value is a measure of MFI, it is expressed on a log scale (channel number range 1–256, spanning 3 logs) and therefore requires linear conversion before direct comparison between cell types can be made. For this purpose, commercially available beads with known amounts of fluorescence were obtained from Flow Cytometry Standards Corporation (Research Triangle, NC) and were used to generate a standard curve of MFI versus linear fluorescence. The MFI of CD44 expression was converted to a linear quantity by using this standard curve, thereby allowing comparison of relative CD44 densities between ovarian cancer cell lines.

**PCR.** CD44 transcripts were detected by reverse transcription-PCR. Total cellular RNA was isolated by cell lysis in guanidinium isothiocyanate GIBCO-BRL followed by addition of water-saturated phenol and precipitation with 70% isopropanol. The precipitate was resuspended in guanidinium isothiocyanate and subjected to a second isopropanol precipitation. Reverse transcription was performed with 1 μg RNA first incubated at 70°C for 5 min with 0.1 μg oligo(dT) (Collaborative Biomedical Products, Bedford, MA) in a total volume of 3 μl followed by the addition of 1 mM dNTP (Pharmacia, Piscataway, NJ), 2 units/μl RNase inhibitor (Promega, Madison, WI), 5 mM DT (GIBCO-BRL), and 200 units superscript reverse transcriptase (GIBCO-BRL). After incubating for 1 h at 42°C, the enzymes were inactivated by heating to 65°C for 10 min. PCR amplification of CD44 cDNA transcripts was then performed using two primers which flank the insertion site of alternatively spliced exons. Therefore, these primers allow for amplification of both standard CD44H transcripts (482 base pairs) as well as alternatively spliced CD44 variant transcripts. The primer sequences (5'-3' orientation) are GACCATATT-GCTTCAATCCTTCAGC and GATGCAAGATGATGCAGC-CATTCTGGGAAT (22). The reverse transcription product (5 μl) was incubated in the presence of both primers (200 nm), as well as 125 μM dNTP, in standard buffer solution for a total of 100 μl. Ampli Taq DNA polymerase (2.5 units/100 μl; Perkin Elmer/Cetus, Norwalk, CT) was added, and PCR was performed for 35 cycles using a DNA thermal cycler 480 (Perkin Elmer/ Cetus). The PCR cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 64°C, and amplification for 1 min at 72°C. Transcripts were electrophoresed in a 1.7% agarose gel and transferred to Hybond-N+ nylon membranes (Amersham Corp., United Kingdom) for subsequent hybridization with a 32P-labeled probe which recognizes an internal portion of the reaction product which is shared by all CD44 transcripts (CCT-GAAGAGATTGTACATCATGTCACAGAC; Ref. 22). For some experiments involving cDNA transfection, PCR amplification was conducted using genomic DNA. For these experiments, oligonucleotides specific for the bcl-2 gene were also used in the PCR reaction as controls for the presence of DNA. The bcl-2 primer sequences (5'-3' orientation) are CAGCCCTT-GAAACATFGATGG and CTAAAGGTCAAACCACATA, yielding an expected transcript size of 563 base pairs (23). The blots were hybridized, washed, and analyzed by autoradiography following standard procedures.

**Generation of Stable Transfectants Expressing CD44H.** The CDNA clone encoding CD44H is contained within a CDM8 vector and is a gift from Dr. Brian Seed (24). CD44-positive OVC-3 cells (which predominantly express CD44 variants) were split and replenished with fresh media 24 h prior to transfection followed by trypsinization, washing in IMDMEM, and resuspension of 2 × 10⁶ cells in 800 μl IMDMEM. The cells were then treated with 20 μg CD44H cDNA-containing plasmid and 2 μg pSV2neo plasmid (a gift from Dr. Michel Streuli, Dana-Farber Cancer Institute), or with 2 μg pSV2neo plasmid alone, incubated at RT for 10 min, and then electroporated (250V/960 mF) using a Bio-Rad Gene Pulser (Richmond, CA). After incubation on ice for 10 min, the cells were trans-
Heterogeneity of CD44 in Ovarian Cancer

The binding of ovarian cancer cell lines to peritoneal mesothelium and to hyaluronate. Cells from each line were chromium labeled and allowed to bind to either mesothelium or to hyaluronate-coated microtiter wells for 30 min at 37°C as described. Specific binding is expressed as the percentage of total cpm added to each well, after subtraction of background binding to plastic alone (usually <1%). For each cell line, results are expressed as mean ± SE from three separate experiments. The cell lines which exhibit weak binding to mesothelium and hyaluronate are OVC-P, OVC-1, SW626, and OVC-3.

RESULTS

Relationship between CD44 Expression and Ovarian Cancer Cell Binding to Peritoneal Mesothelium. The binding of 12 ovarian cancer cell lines to peritoneal mesothelium was determined in an attempt to correlate adhesion with CD44 expression. As shown in Fig. 1, eight of these lines exhibited significant binding to peritoneal mesothelium (range, 29–39% specific binding), whereas four lines showed minimal binding (range, 4–(7%) (P < 0.001). The lines which bound weakly to mesothelium were OVC-P, OVC-1, OVC-3, and SW626. Interestingly, OVC-2 exhibited significant binding, despite being derived from the original OVC-P cell line. In addition, a positive correlation was observed for mesothelial and hyaluronate binding (Fig. 1), suggesting that an alteration in CD44 expression or function might be partly responsible for the decreased binding of certain ovarian cancer cell lines to mesothelium. We therefore assessed CD44 expression in both strongly and weakly binding cell lines by flow cytometry, with the results shown in Fig. 2. CD44 expression was observed in the majority of cells from all strongly binding cell lines, including CAOV-3, SKOV-3, UPN36T, and OVC-2. In contrast, CD44 expression was significantly reduced in the weakly binding lines OVC-P and OVC-1. Interestingly, significant CD44 expression was observed in OVC-3 and SW626 cells, despite the fact that these lines bound weakly to either mesothelium or to hyaluronate. These observations suggest that CD44 may be necessary, but not always sufficient, for mediating binding of ovarian cancer cells to peritoneal mesothelium.

Biochemical Characterization of CD44 in Strongly and Weakly Binding Ovarian Cancer Cell Lines. The data in Fig. 2 suggest that the inability of OVC-3 and SW626 cells to effectively bind to peritoneal mesothelium cannot simply be due to a lack of CD44 expression. Therefore, to compare the types of CD44 molecules expressed in strongly and weakly binding lines, we performed immunoprecipitation of 125I-labeled cells, with the results of a typical experiment shown in Fig. 3. Two different patterns of CD44 molecules were observed, depending on the ability of ovarian cancer cells to adhere to peritoneal mesothelium. The strongly binding cell lines CAOV-3 and UPN36T were found to predominantly express a 90–180-kDa species consistent with CD44H. The associated band at 180 kDa is characteristic of CD44H in nonreducing gels (2, 25) and most likely represents a disulfide-linked homodimer of the 90-kDa species, since we have observed this band to disappear under reducing conditions (Fig. 4). There was also a faint band at approximately 110–120 kDa of uncertain identity. In contrast, the OVC-3 line expressed two predominant bands at 130–150 kDa and at ~230 kDa. As noted above, these bands are consistent with the expected molecular weights of certain CD44 variant proteins such as CD44E (130–180 kDa; Ref. 6). Faint bands were observed at 90–95 kDa and 180 kDa which were consistent with CD44H. Likewise, the SW626 line also expressed two predominant higher molecular mass species, con-
Fig. 3  Immunoprecipitation of CD44 species from strongly and weakly binding ovarian cancer cell lines. CD44 was immunoprecipitated after ¹²⁵I surface labeling of strongly binding (CAOV-3 and UPN36T) and weakly binding (OVC-3 and SW626) lines, with subsequent SDS-PAGE under nonreducing conditions and autoradiography as described. A typical pattern of CD44H, comprised of both a 90- and 180-kDa species, is observed for the strongly binding cell lines CAOV-3 and UPN36T. In contrast, the pattern in the weakly binding lines OVC-3 and SW626 is characterized by a substantial decrease in the 90-kDa species associated with the appearance of new bands in the 130-, 160-180-, and 220-230-kDa molecular mass ranges.

Fig. 4  Immunoblot analysis of CD44 expression in strongly binding cell lines. Lysates were prepared from five additional strongly binding cell lines shown in Fig. 1 followed by SDS-PAGE under reducing conditions and immunoblotting with anti-CD44 antibody (BBA10) as described. All lines were observed to predominantly express the 90-kDa protein characteristic of CD44H.

Quantitation of CD44H Expression in Ovarian Cancer Cell Lines. These data suggest that strongly binding cell lines predominantly express CD44H, whereas weakly binding lines appear to express a predominance of CD44 variant molecules and a correspondingly lower relative amount of CD44H. To more precisely compare the amounts of CD44H expressed by strongly and weakly binding cell lines we first obtained an assessment of total CD44 surface density by flow cytometry (Fig. 2). The mean channel number (log scale) of CD44 expression (Fig. 2) into linear units as described in the text. Data are an average of three experiments, with the mean total CD44 density expressed as arbitrary units.

As shown in Table 1, weakly binding cell lines such as OVC-3 and SW626 expressed approximately 2-fold less CD44 than did strongly binding lines such as CAOV-3 and UPN36T. We next determined the relative

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total CD44 density*</th>
<th>90- + 180-kDa fractionb (densitometry)</th>
<th>90- + 180-kDa densityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAOV-3</td>
<td>2.4 (2.0-2.5)</td>
<td>0.86</td>
<td>2.06</td>
</tr>
<tr>
<td>UPN36T</td>
<td>2.6 (2.5-2.7)</td>
<td>0.75</td>
<td>1.95</td>
</tr>
<tr>
<td>OVC-3</td>
<td>1.4 (1.1-1.9)</td>
<td>0.23</td>
<td>0.32</td>
</tr>
<tr>
<td>SW626</td>
<td>1.0 (0.9-1.2)</td>
<td>0.31</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* Determined by converting the mean channel fluorescence (log scale) of CD44 expression (Fig. 2) into linear units as described in the text. Data are an average of three experiments, with the mean total CD44 density expressed as arbitrary units.

b Determined by performing densitometry of the autoradiogram shown in Fig. 3. The absorbance of bands at ~90 and 180 kDa were combined to derive an estimate of the amount of CD44H present. Data are expressed as the fraction of total CD44 comprised of CD44H.

The data suggest that the weakly binding cell lines OVC-3 and SW626 express a predominance of CD44 variants and are relatively deficient in CD44H. In contrast, binding cell lines such as CAOV-3 and UPN36T appear to predominantly express CD44H. To further study the association between CD44H expression and the binding phenotype we have determined the pattern of CD44 expression in five additional strongly binding lines by immunoblot analysis. As shown in Fig. 4, each of these cell lines predominately expressed the 90-kDa species typical of CD44H under reducing conditions.
amount of CD44H expressed by each cell line by performing densitometry of the autoradiogram shown in Fig. 3. For this analysis, we have combined the absorbance values obtained for both the 90-kDa as well as the 180-kDa species, since these are the expected forms of CD44H observed under nonreducing conditions. Similar values were obtained by measuring the absorbance of the 90-kDa band from gels run under reducing conditions (data not shown). As shown in Table 1, CD44H constituted 23% and 31% of all CD44 expressed by OVC-3 and SW626 cells, respectively. Corresponding values for CAOV-3 and UPN36T cells were 86% and 75%, respectively. The density of CD44H expressed by each cell is the product of the total CD44 density and the CD44H fraction (Table 1, third column). As shown in Table 1, the CD44H density for OVC-3 and SW626 cells is 0.32 and 0.31 arbitrary units, respectively. In contrast, corresponding values for CAOV-3 and SW626 cells are 2.06 and 1.95, respectively. Thus, weakly binding ovarian cancer cells express ~6-fold lower amounts of CD44H than do strongly binding cells.

Identification of CD44 Variants Expressed by Ovarian Cancer Cell Lines. To determine whether the higher molecular mass species observed in OVC-3 and SW626 cells were CD44 variants, as opposed to differentially glycosylated forms of CD44H, we first performed reverse transcription-PCR in order to detect the presence of variant-specific transcripts. Fig. 5 shows the results of reverse transcription-PCR analysis as assessed by hybridization with a 32P-labeled internal probe which was not used in the original amplification reaction (22). The strongly binding cell lines expressed the expected transcript characteristic of CD44H (482 base pairs), as well as two higher molecular weight bands at 620 and 680 base pairs which are consistent with CD44 variant transcripts. In contrast, the weakly binding cell lines OVC-3 and SW626 expressed a more heterogeneous pattern of CD44 variant transcripts comprised of additional bands at approximately 880, 1050, 1180, 1500, and 1600–1700 base pairs.

To correlate the results of reverse transcription-PCR with protein expression we performed flow cytometry with antibodies specific for CD44 variant epitopes v3, v6, and v9, with the results summarized in Table 2. Expression of CD44v6 and CD44v9 was noted in both strongly and weakly binding cell lines, whereas expression of CD44v3 was largely restricted to SW626 cells. These results suggest that variant expression may be a common feature of many ovarian cancer cell lines, and that the presence of CD44 variants predicts for decreased adhesion only when associated with a concomitant decrease in CD44H expression.

Transfection of the cDNA for CD44H Reconstitutes Adhesion of OVC-3 Cells to Peritoneal Mesothelium. To further investigate the functional relationship between CD44H expression and binding, CD44H transfectants of the OVC-3 cell line were generated as described followed by assessment of adhesion to mesothelium or hyaluronate. The phenotype of four representative transfected OVC-3 clones is shown in Fig. 6A. As noted above, the poorly binding OVC-3 line expresses CD44, most of which is comprised of CD44 variants (Figs. 2 and 3). Therefore, we could not simply rely on the surface phenotype of G418-resistant clones to accurately assess the success of transfection. As expected, both the control clones (pSV2neo-transfected) and the CD44H-transfected clones were CD44 positive (Fig. 6A). To determine the success of transfection we performed PCR analysis of genomic DNA, as opposed to reverse transcribed mRNA, in order to detect the presence of transfected CD44H cDNA. Because the regions recognized by our CD44-specific oligonucleotide probes are separated by greater than 10

### Table 2  Expression of CD44 variants in selected ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CD44 (total)</th>
<th>CD44v3</th>
<th>CD44v6</th>
<th>CD44v9</th>
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<tbody>
<tr>
<td>CAOV-3</td>
<td>91</td>
<td>9</td>
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<td>53</td>
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<td>SKOV-3</td>
<td>94</td>
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<td>OVC-2</td>
<td>89</td>
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<td>OVC-3</td>
<td>86</td>
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<td>SW626</td>
<td>90</td>
<td>87</td>
<td>62</td>
<td>77</td>
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</table>

* Cell lines as previously described in the legend to Fig. 1. OVC-2 and OVC-3 refer to distinct strains of the OVCAR-3 parent line developed in our laboratory. CAOV-3, SKOV-3, and OVC-2 are strongly binding lines, whereas OVC-3 and SW626 cells bind poorly to mesothelium.

* Data are expressed as mean specific percentage of positivity (after subtraction of background fluorescence) as determined by flow cytometry from two experiments (for CD44 total and CD44v3) or from four experiments (for CD44v6 and CD44v9). SEM was <20%, for all values. Source of antibodies is described in “Materials and Methods.” CD44 (total) refers to reactivity with antibody 515, which recognizes all forms of CD44.

* CD44v3, CD44 variant exon 3; CD44v9, CD44 variant exon 9.
A

CD44 expression

CD44

<table>
<thead>
<tr>
<th>pSV2 neo (clone ND5)</th>
<th>pSV2 neo (clone NE5)</th>
<th>CD44H (clone HE5)</th>
<th>CD44H (clone HF6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>CD44</td>
<td>CD44H</td>
<td>CD44H</td>
</tr>
<tr>
<td>% 88.26</td>
<td>88.95</td>
<td>85.86</td>
<td>93.17</td>
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<td>M 170.73</td>
<td>157.22</td>
<td>171.07</td>
<td>179.92</td>
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</tbody>
</table>

B

transfectants

ND5  NE5  HE5  HF6

CD44H

482 bp

BCL-2

C

pSV2neo (ND5)
pSV2neo (HE5)

205-          

116-          

80-          

Fig. 6  Analysis of CD44H expression in OVC-3 transfectants. OVC-3 cells were transfected with the CDM8 plasmid containing CD44H cDNA as described in the text. A, immunophenotype of four cloned OVC-3 transfectants. Background fluorescence was <5% for each clone. Because OVC-3 cells are CD44 positive due to the predominant expression of CD44 variants (Figs. 2 and 3), flow cytometry could not be used to assess the effectiveness of transfection. B, PCR analysis of genomic DNA. Note that the data shown in these experiments are derived from PCR amplification of genomic DNA (as opposed to cDNA obtained from reverse transcription as shown in Fig. 5). Therefore, only cells transfected with the CD44H cDNA should show the 482-base pair species characteristic of this transcript (clones HE5 and HF6). bcl-2 Transcripts are observed in all clones as a control for the success of DNA isolation and PCR amplification. C, immunoblot analysis of CD44 species expressed by two OVC-3 transfectants. See legend to Fig. 4 for details. Compared to the pSV2neo-only transfected clone (ND5), the clone transfected with CD44H cDNA (HE5) overexpresses the 90-kDa protein characteristic of CD44H.

DISCUSSION

Although the CD44 molecule is partly responsible for the attachment of ovarian cancer cells to peritoneal mesothelium in vitro, the mechanisms by which this binding is regulated are unknown. Therefore, the purpose of this study was to better understand the relationship between CD44 expression by ovarian cancer cells and their ability to adhere to peritoneal mesothelium and hyaluronate. Three different patterns of CD44 expression were identified in this report which provide insight into the regulation of CD44 function in ovarian cancer. The first pattern is demonstrated by strongly binding cell lines such as CAOV-3 and UPN36T, which predominantly express the 90–180-kDa species characteristic of CD44H (Figs. 3 and 4). These data are consistent with the known ability of CD44H to mediate adhe-
Heterogeneity of CD44 in Ovarian Cancer

CD44 in ovarian cancer is heterogeneous and partly blocked by anti-CD44 antibody. Binding of CD44H transfectants is enhanced compared to neo-only controls. Clones HE5 and HF6 were transfected with CD44H cDNA. ND5

Legend to Fig. 1. Clones followed by assessment of mesothelial binding as described in the legend to Fig. 1. Clones ND5 and NE5 were transfected with pSV2neo alone. Clones HE5 and HF6 were transfected with CD44H cDNA. Binding of CD44H transfectants is enhanced compared to neo-only clones and is partly blocked by anti-CD44 antibody (515). Similar results were obtained for binding to hyaluronate (data not shown).

Fig. 7 OVC-3 transfectants expressing CD44H exhibit strong binding to mesothelium. OVC-3 cells and transfectants were chromium labeled followed by assessment of mesothelial binding as described in the legend to Fig. 1. Clones ND5 and NE5 were transfected with pSV2neo alone. Clones HE5 and HF6 were transfected with CD44H cDNA. Binding of CD44H transfectants is enhanced compared to neo-only clones and is partly blocked by anti-CD44 antibody (515). Similar results were obtained for binding to hyaluronate (data not shown).

We have interpreted pattern 3 to suggest that a quantitative and/or qualitative difference may exist in the CD44 molecules expressed by weakly versus strongly binding ovarian cancer cells. In this regard, we found that weakly binding lines such as OVC-3 and SW626 predominantly expressed CD44 variants associated with significantly lower amounts of CD44H as assessed by flow cytometry and immunoprecipitation studies (Fig. 3 and Table 1). The functional importance of this observation is demonstrated by the fact that overexpression of the CD44H isoform in OVC-3 transfectants restored their ability to bind to peritoneal mesothelium in a CD44-dependent fashion (Fig. 7). The observation that certain ovarian cancer cell lines bind poorly to mesothelium, despite expressing significant amounts of CD44 variants, suggests that the molecules may not be capable of mediating binding to mesothelial-associated hyaluronate. Nevertheless, the expression of CD44 variants does not by itself confer an inability to bind since epitopes for several CD44 variants were easily detected by flow cytometry in strongly binding lines as well (Table 2). The fact that variant expression may occur to some degree in strongly binding cell lines is also supported by the frequent presence of a minor band at ~110 kDa in immunoprecipitation and immunoblot analyses (Figs. 3 and 4), as well as by the presence of transcripts in the 620–680-base pair range as detected by reverse transcription-PCR (Fig. 5). Taken together, these data suggest that CD44 variant expression may be a feature of many ovarian cancer cell lines, but that the regulation of adhesion is more dependent on the absolute level of surface CD44H.

The fact that a binding phenotype can be reconstituted in OVC-3 cells after transfection of CD44H cDNA demonstrates the importance of this specific isoform in ovarian cancer cell adhesion, and also argues against an antagonistic role of CD44 variants in hyaluronate recognition. We chose OVC-3 cells for these experiments because this cell line was relatively deficient in CD44H expression (Fig. 3 and Table 1) and already expressed high levels of other CD44 variant isoforms, including CD44→v9 (Table 2). Since antibodies specific for standard CD44H do not currently exist, it was not possible to assess the level of CD44H expression in OVC-3 transfectants by using surface immunophenotyping techniques. Instead, we established that each clone contained the expected sequence for transfected CD44H cDNA as assessed by PCR amplification, without reverse transcription, as shown in Fig. 6B. Amplification of this species by PCR proves that transfection of the CD44 cDNA was successful in these clones, since it is not otherwise possible to amplify this sequence from genomic DNA or in the absence of reverse transcription. To determine whether the CD44H protein was expressed we performed Western blot analysis which showed dramatic up-regulation of CD44H protein in the CD44H transfectants, as opposed to neo-only controls (Fig. 6C). Although this analysis does not formally prove that the protein is surface expressed, this is likely to be the case for several reasons. The cDNA construct used in these experiments contains a transmembrane domain and has been successfully used to obtain high-level surface expression by other investigators (6), as well as ourselves during transfection experiments with Namalwa cells. Furthermore, it is important to note that the predicted molecular weight of the CD44H protein core, before posttranslational processing, is 37 kDa. Therefore, the molecular weight of the species expressed by OVC-3 transfectants (85–90 kDa) suggests that the protein has already undergone glycosylation in the Golgi apparatus and is therefore ready to be surface expressed. Finally, although clonal selection during the transfection procedure could possibly explain the binding differences observed between neo-only and CD44H transfectants (Fig. 7), the fact that partial blocking was observed in the presence of anti-CD44 neutralizing antibody demonstrates the specificity of this effect for surface-expressed CD44.

The observation that anti-CD44 antibody was able to only partially inhibit binding of OVC-3 transfectants to mesothelium (Fig. 7) is in agreement with our previous experience, in which the binding of CAOV-3 and SKOV-3 cells was blocked by approximately 40–50% in the presence of this antibody (2). The anti-CD44 monoclonal antibody used in these experiments (clone 515) is capable of almost complete inhibition of ovarian cancer cell binding to hyaluronate-coated plastic, demonstrating the excellent neutralizing ability of this reagent for hyaluronate recognition (2). The inability to obtain complete inhibition of OVC-3 transfectant binding in the presence of this antibody suggests that CD44 may partly mediate adhesion through an
Functional heterogeneity of CD44 molecules in ovarian cancer cell lines.

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