Binding of Ovarian Cancer Antigen CA125/MUC16 to Mesothelin Mediates Cell Adhesion*

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Mesothelin is a glycosylphosphatidylinositol-linked cell surface molecule expressed in the mesothelial lining of the body cavities and in many tumor cells. Based on the finding that a soluble form of mesothelin specifically binds to ovarian carcinoma cell line OVCAR-3, we isolated cDNAs encoding a mesothelin-binding protein by expression cloning. The polypeptides encoded by the two cloned cDNA fragments matched to portions of CA125, an ovarian cancer antigen and a giant mucin-like glycoprotein present at the surface of tumor cells. By flow cytometric analysis and immunoprecipitation, we demonstrate that CA125 binds to mesothelin in a specific manner. Binding of CA125 to membrane-bound mesothelin mediates heterotypic cell adhesion as anti-mesothelin antibody blocks binding of OVCAR-3 cells expressing CA125 to an endothelial-like cell line expressing mesothelin. Finally, we show that CA125 and mesothelin are co-expressed in advanced grade ovarian adenocarcinoma. Taken together, our data indicate that mesothelin is a novel CA125-binding protein and that CA125 might contribute to the metastasis of ovarian cancer to the peritoneum by initiating cell attachment to the mesothelial epithelium via binding to mesothelin.

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CA125 is a tumor antigen originally defined by the monoclonal antibody OC125 (1) that is routinely used for diagnosis of ovarian cancer and to monitor the recurrence after therapy (2, 3). CA125 is expressed on the cell surface, and, in addition, soluble proteolytic fragments are also released into the extracellular space. The primary structure of CA125 was established recently, indicating that it is a type I transmembrane protein with a short intracellular and a giant extracellular domain, the latter with 22,097 amino acid residues. The extracellular part is composed of an amino-terminal part spanning 12,070 residues (4), followed by more than 60 tandem repeats of a 156-amino acid motif and a 229-residue linker to the transmembrane domain (5). Both the amino-terminal part and the repeat domains are rich in serine and threonine residues and are highly glycosylated. The carbohydrate content was estimated to be 24–28%, with O-linked and N-linked glycans (6). Because highly O-glycosylated repeats are the landmark of the mucin family of glycoproteins, CA125 was also named MUC16 (7). The mucin-like repeats contain homology to the so-called SEA module, a domain that was reported to be susceptible to proteolytic cleavage and subsequent self-association (8). An additional potential proteolytic cleavage site in CA125 was reported to be located immediately membrane-proximal (5).

Although the structure of CA125 has been elucidated, a functional role for this molecule in the physiological context or in cancer remains unknown. However, a number of publications have pointed out several properties of CA125 that may be of relevance for its biological function. First, because of its expression in embryonic membranes and adult derivatives of the fetal periderm, CA125 has been suggested to play role as a lubricant, preventing adhesion of membranes (9). Anti-adhesive properties have also been assigned to branched O-glycans on leucosialin/CD43 and other mucins (10). In accordance with these findings, CA125 protein coated on plastic culture dishes interferes with the attachment of a variety of cell lines to the plastic dishes (11). Second, close analysis of the glycans present on CA125 revealed the presence of several glycan structures that have been implicated in immune suppression (6), raising the possibility that CA125 might help protect the embryo from maternal immune rejection and play an immunomodulatory role in ovarian cancer. Furthermore, mucins can bind to various sugar-binding molecules, such as selectins (12, 13) and galectins (14). In fact, galectin-1 was recently shown to bind to CA125 specifically (15). Finally, in an in vitro Matrigel invasion assay, CA125 from human peritoneal fluid was shown to enhance the invasiveness of a benign endometriotic cell line, EEC 145, but it did not affect the invasiveness of a variety of non-endometrioid cell lines (16), raising the possibility that CA125 plays a role in endometriosis. However, this study did not address the question of how soluble CA125 might bind to EEC145 cells to exert this bioactivity.

Taken together, recent evidence suggests that CA125 may exert a number of different functions in parallel. Some of these functions may be mediated by specific molecular interactions, others by the physical properties of this huge, glycosylated molecule. Interestingly, several other mucins have been implicated in invasion and metastasis of cancer, partly because of similar functions. For example, MUC1 induces T cell apoptosis (17) and increases invasiveness (18), MUC18 has been implicated in tumor angiogenesis (19), MUC2 enhances colon cancer metastasis to the liver (20), although it appears to inhibit initial neoplasia as MUC2-deficient mice develop colorectal cancer (21), MUC8 is up-regulated in metastatic medulloblas-
mesothelin is partly identified by the antibody CAK-1 on mesothelial cells, mesotheliomas, and ovarian cancers (24, 25). It is a secreted protein anchored at the cell membrane by glycosylphosphatidylinositol (GPI) linkage. The amino-terminal 31-kDa fragment of the 69-kDa protein is shed by proteolytic cleavage, leaving a membrane-bound 40-kDa peptide. Both fragments contain N-glycosylation sites. The soluble human amino-terminal 31-kDa fragment was reported to have megakaryocyte potentiating activity in a mouse bone marrow colony assay (26) and was named megakaryocyte potentiating factor (MPF). However, there is no report in the literature to our awareness that demonstrates megakaryocyte potentiating activity either with human MPF on human cells or with mouse MPF on mouse cells. A soluble splice variant of the 40-kDa carboxyl-terminal fragment called “soluble mesothelin/MPF-related” was found in sera of patients with ovarian carcinoma (27). This slightly longer splice variant lacks the hydrophobic GPI anchor motif as the result of a shift in the reading frame.

In addition to mesothelioma and ovarian cancer, mesothelin has recently been found to be overexpressed in cancers of the pancreas (28), stomach (29), lung (30), and endometrium (31). At least in pancreatic cancer, expression of mesothelin is partly the result of hypomethylation of its promoter region (32). In amurine cell line, mesothelin has been shown to be a target gene of the Wnt/β-catenin pathway (33). Expression in the mouse embryo was reported to be high on days 7 and 17 of gestation, but absent on day 11 and low on day 15, suggesting developmental regulation (34). Mouse and human mesothelin sequences are 59% identical over the entire length of the protein. There is no obvious sequence motif except for a signal peptide on the amino terminus and a GPI anchor motif at the carboxyl terminus (25, 26). Mesothelin-deficient mice were reported to be healthy and fertile. They show normal platelet counts and no obvious phenotype (34). Accordingly, the biological function of mesothelin is still uncertain.

To get more insight into the biological function of mesothelin/MPF, we have here succeeded in isolating its receptor/binding protein. We show that mesothelin binds to CA125 and that this interaction mediates cell adhesion. Moreover, we demonstrated that CA125 and mesothelin are co-expressed in advanced stages of ovarian adenocarcinoma. Taken together, our data suggest a role for CA125 and mesothelin in metastases formation of ovarian carcinoma.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Media—**NIH/OVCAR-3 (OVCAR-3) cells were maintained in RPMI medium 1640 (RPMI) supplemented with 15% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 μg/ml bovine insulin, and antibiotics. LO is a murine endothelial-like cell line derived from the mouse AGM region (35). LO cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 1 mM sodium pyruvate, and antibiotics. Human lymphoma cell lines K562 and U937 cells were maintained in RPMI with 10% FBS and antibiotics. Human umbilical cord vascular endothelial cells (HUVEC) were maintained in endothelial cell basal medium-2 (modified MCDB 131), purchased from Cambrex and supplemented as recommended by the manufacturer. Hydrolyzed cells were maintained in RPMI with 10% FBS, 50 μM β-mercaptoethanol, 85 μg/ml hypoxanthine, 24 mg/liter thymidine, and hybridoma supplement (Sigma). Cells were incubated in a 5% CO₂ culture incubator at 37 °C. Inhibition of O-glycosylation in OVCAR-3 cells was carried out by adding 5 mM benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside to the culture medium over night.

**Antibodies and EST Clones—**Anti-murine mesothelin monoclonal antibodies were generated in Wistar rats (Nihon SLC) immunized with 10⁵ LO cells as described (35). Flow cytometric screening against mesothelin-transfected COS7 cells identified a monoclonal antibody against mesothelin. This antibody, named B35, was produced in nude mice and purified with a protein G-Sepharose column (Hitrap, Amersham Biosciences) according to the instructions from the manufacturer. B35 was radiolabeled with 1 µCi/ml of Na251Cl (PerkinsElmer Life Sciences). Protein G-Sepharose immobilized with 5B2 antibody, followed by anti-mouse antibody conjugated to 1 mg/ml in phosphate-buffered saline (PBS) without preservatives.

**Flow Cytometry and Fluorescence-activated Cell Sorting—**Cells were harvested from culture dishes with enzyme-free cell dissociation buffer (Invitrogen) and 10⁶ cells were incubated on ice for 30 min with 0.5 μg of monoclonal antibody or isotype control in 50 µl of PBS, followed by washing and labeling with fluorescein- and/or phycoerythrin-conjugated cross-species adsorbed secondary antibodies (Chemicon and Cedar Lane Laboratories, respectively). To assess blocking activity of antibody against CA125 or mesothelin in conditioned culture supernatant from FLAG/tagged soluble mesothelin- or mock-transfected COS7 cells and washed, if applicable. To assess blocking activity of antibody against ligand binding, a 0.2 volume conditioned medium of COS7 transfected with the soluble FLAG-tagged mesothelin cDNA was preincubated with 0.8 volume of hybridoma antibody or fresh culture medium as a negative control, then used for cell incubation for 30 min, followed by incubation with M2 and fluorescence-labeled antibody consequently. Analyses were carried out using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cell sorting was carried out with a FACSVantage cell sorter (Becton Dickinson Immunocytometry Systems).

**Library Construction, Expression Cloning, and Sequencing—**Messenger RNA was extracted from one confluent 10-cm culture dish of OVCAR-3 cells using the FastTrack 2.0 kit (Invitrogen, Tokyo, Japan) according to the instructions from the manufacturer. Five μg of poly(A)⁺ RNA was used for constructing a cDNA library primed by a random hexamer primer using the Time Saver kit (Amersham Biosciences). Expression cloning of cDNA encoding a binding partner of mesothelin was carried out by using COS7 cells as previously described (37), except that fluorescence-activated cell sorting was employed instead of plate plating. The DNA sequences of the cDNAs were determined by using a dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an automated DNA sequencer (Applied Biosystems, Foster City, CA).

**Expression of Mesothelin in COS7 Cells and Immunoblotting—**Twenty μg of expression vector containing truncated CA125 and/or mesothelin coding cDNA was electroporated into COS7 cells (10⁷ cells/ml of cell lysate were put aside as controls. Three μg of OVCAR-3 cDNA was added to the remaining lysate, followed by the addition of protein G-Sepharose (Amersham Biosciences). Proteins in the immune complexes as well as control lysates (15 μl or ~0.2 mg of total protein) were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blot was incubated with 5B2 antibody, followed by anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, United Kingdom). The immunocomplex was detected on Hyperfilm (Amersham Biosciences) using an enhanced Luminol oxidation reagent (PerkinElmer Life Sciences).

**Heterotypic Cell Adhesion Assay—**The heterotypic adhesion assay...
was carried out in triplicates using a protocol adapted from Cannistra et al. (38). LO cells were grown to confluence in 6-well culture plates. Thirty minutes prior to the addition of OVCAR-3 cells, LO medium was exchanged for OVCAR-3 medium supplemented with the appropriate amount of blocking or control antibody.

OVCAR-3 cells were dissociated from culture dishes with enzyme-free cell dissociation solution and added on top of the LO cells at 10^6 cells/well. Culture plates were spun at 800 rpm for 3 min to bring OVCAR-3 cells in contact with LO cells, then incubated at 37 °C for 60 min to adhere. Non-adherent cells were removed by repeated washing in PBS with strong finger tapping to agitate the wells. All bound cells were then dissociated with enzyme-free cell dissociation solution, counted, and analyzed by flow cytometry. MUC1 antibody was used to identify OVCAR-3 cells, B35 antibody to identify LO cells.

Alternatively, mouse embryonic diaphragm cells were used instead of LO cells. Diaphragms were dissected from embryos of C57BL/6J mice at day 14.5 post coitus. Cells were dispersed with 0.05% (w/v) trypsin and 0.5 mM EDTA in PBS at 37 °C for 10 min and by repeated pipetting, and then filtered through a 40-µm mesh to obtain a single cell suspension. Cells were cultured in 6-well plates, one embryo equivalent per plate, in the same medium as LO cells but without any growth factors.

On day 3 of culture, when culture had reached confluence, the adhesion of OVCAR cells to the cultured diaphragm cells was assessed exactly as described above. To block binding, 6 µg/ml antibody B35 was used.

Cell binding was likewise tested in the inverse configuration. Detached LO cells or cultured embryonic diaphragm cells were added on top of confluent OVCAR-3 cells, attached by centrifugation in LO medium with or without 6 µg/ml antibody B35, washed with PBS, and analyzed by flow cytometry as above. To test whether divalent ions are required for the binding, non-attached cells were washed out with PBS supplemented with 1 mM EDTA or EGTA.

Immunohistochemistry and Measurement of CA125 Serum Concentration—This study was approved by the local ethics committee, and informed consent was obtained from each subject. Samples were collected from patients undergoing surgery for ovarian tumor. Serum concentration of CA125 in each patient was determined before operation by chemiluminescent enzyme immunoassay with the Lumipulse CA125 II kit (Fuji Rebio) according to the instructions from the manufacturer. Of the 18 patients in this study, 9 had serous papillary adenocarcinomas, 3 had mucinous cystadenocarcinomas, 2 had clear cell carcinomas, and 1 had mucinous adenoma. Type and stage of tumors were determined according to FIGO (39). All specimens were fixed with 10% formalin for 5 h immediately after resection and were embedded in paraffin.

Immunostaining was performed with some modifications as previously described (40). Four-micrometer sections were processed for immunohistochemical procedures based on the Labeled Polymer method (Dako). Briefly, sections were treated with 3% hydrogen peroxide in absolute methanol for 30 min to quench endogenous peroxidase activity. After antigen retrieval with Target Retrieval Solution (Dako), sections were preincubated in 0.01 M PBS containing 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h. After antigen retrieval, sections were incubated in primary antibodies containing 0.5% bovine serum albumin at room temperature for 2 h. Primary antibodies 5B2 and M11 were both used at dilution 1:20. After washes with 0.1 M PBS, the sections were incubated in goat anti-mouse immunoglobulins conjugated with peroxidase-labeled dextran polymer (EnVision+, Dako) at room temperature for 30 min. After washes with 0.1 M PBS, the horseradish peroxidase reaction was developed in 0.1 M Tris-buffered saline, pH 7.4, containing 0.05% 3,3’-diaminobenzidine tetrahydrochloride (Sigma), 0.02% nickel sulfate, and 0.01% hydrogen peroxide. Methyl green was used for counterstaining.
CA125 and Mesothelin Mediate Cell Adhesion

TABLE I

| Cell line | Antibody binding | Mesothelin binding | Reported expression |
|-----------|------------------|--------------------|---------------------|
| OVCAR-3   | + +              | + +                | CD43 (58)           |
| K562      | + +              | -                  | CD68 (59)           |
| U937      | +                | -                  | CD43 (58), MUC17 (60)|
| Caco-2    | +                | -                  | MUC18 (61)          |
| HUVEC     | -                | -                  |                     |

Fig. 4. Heterotypic cell adhesion mediated by mesothelin. OVCAR-3 cells were added to a monolayer of LO cells. After washing, OVCAR-3 cells bound to LO cells were counted as described under "Experimental Procedures." Addition of B35 anti-mesothelin antibody (solid squares) blocks OVCAR-3 cell binding to mesothelin expressing LO cells in a dose-dependent manner, whereas isotype control antibody (open circles) does not affect cell binding.

Statistical Analysis—SAGEmap gene expression data were analyzed by pairwise linear regression and analysis of variance using the spreadsheet software Excel (Microsoft, Redmond, WA). Fisher's exact test was used to test the association of tissue mesothelin expression with histological grade. Data from multiple experiments are expressed as mean (± standard deviation).

RESULTS

Identification of CA125 as a Mesothelin Counter-receptor—To identify a counter-receptor of mesothelin, an expression construct of murine mesothelin fused to the FLAG peptide at the COOH terminus, mMes-f, was electroporated into COS7 cells. A double band of 40 and 46 kDa could be detected in the culture supernatant by Western blotting with M2 antibody that recognized the FLAG epitope (data not shown). Considering that the theoretical molecular mass of the epitope-tagged carboxyl-terminal fragment of mesothelin is 38.6 kDa, these two bands are in agreement with two differentially glycosylated forms of mesothelin as has been observed previously (25), being released into the supernatant because the fusion with the FLAG tag had destroyed the GPI linkage motif. Using culture supernatant with the recombinant mMes-f protein, we searched for cell lines that bind mesothelin by flow cytometry and found that OVCAR-3, an ovarian cancer line, showed strong specific binding (Fig. 1). The binding could be blocked significantly with B35, a monoclonal antibody specific for murine mesothelin that we had previously identified from a panel of monoclonal antibodies against LO cells.

To isolate a cDNA for the mesothelin-interacting protein, we constructed a cDNA expression library of OVCAR-3 cells. The cDNA library was transfected into COS7 cells by spheroplast fusion, and COS7 cells expressing a mesothelin-binding protein were enriched by flow cytometric cell sorting.

Plasmid DNA recovered from the sorted COS7 cells was subjected to a second cycle of selection. After four cycles of selection, two cDNA fragments of 1.1 and 1.6 kb were enriched. Sequencing analyses of the cloned cDNA fragments revealed that these two clones represented overlapping fragments of the same gene, corresponding in sequence to bases 63444–64531 and 63354–64956 of CA125 (GenBank™ accession AF414442). As illustrated in Fig. 2, the cloned cDNA fragments comprise 2.5 and 3.5 units of the mucinous repeats, respectively. The 23 carboxyl-terminal amino acids of the longer clone are part of a short non-repetitive sequence interspersed between the last two repeats.

As expected, flow cytometric analysis revealed that COS7 cells transfected with either cloned cDNA but not mock-transfected COS7 cells could bind mMes-f protein, but not to an irrelevant FLAG-tagged protein (data not shown). Although our CA125 cDNA clones contain neither a signal peptide nor a putative transmembrane domain coding region, their protein products were evidently present on the cell surface of transfected COS7 cells.

Binding Specificity—To test whether human mesothelin binds to CA125 in the same way as we had observed for mouse mesothelin, we constructed an expression construct of the carboxyl-terminal fragment of human mesothelin, termed f-hMes, in which an amino-terminal FLAG tag is fused to human mesothelin glutamic acid 298, just below the proteolytic cleavage site. The f-hMes expression vector was transfected into COS7 cells, and the recombinant protein could be detected at 42 and 48 kDa in cell lysates (Fig. 3B), indicating differential glycosylation as the calculated molecular mass of the recombinant protein plus GPI anchor is 40 kDa. A small amount of the recombinant protein could also be detected in the culture supernatant (data not shown). The conditioned supernatant was incubated with OVCAR-3 cells. Specific binding was observed by flow cytometry using M2 antibody as shown in Fig. 3A.

To create a CA125 expression construct extending our 1.6-kb cDNA clone to the 3' end, we identified an 1.9-kb EST clone in GenBank™ that covers the 3' end of the CA125 open reading frame and overlaps with our 1.6-kb clone. We obtained the EST clone from the I.M.A.G.E. Consortium and fused it with a FLAG epitope tag and our 1.6-kb clone on the 5' side. The resulting recombinant protein, termed f-MucTM, includes the 1100 carboxyl-terminal amino acid residues of CA125 (Fig. 2).

Lysates of COS7 cells transfected with the f-MucTM and f-hMes expression constructs were immunoprecipitated with OC125 antibody and subjected to Western analysis using the mesothelin-specific antibody 5B2. As controls, COS7 cells transfected with either construct or mock-transfected COS7 cells were used. The results, shown in Fig. 3B, indicate that mesothelin can be precipitated from transfected COS7 cells by antibody OC125 only in the presence of CA125 protein. These
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Shown are patient numbers along with serum CA125 concentration and clinical stage for each of the tissue samples investigated. Histological grade is indicated for all of the serous papillary adenocarcinomas. Results of immunohistochemical analysis of CA125 and mesothelin expression in tumor tissues are indicated, and five samples from patients 4876, 4595, 4079, 2711, and 4193 are shown in Fig. 5.

| Patient no. | Clinical stage | Histological grade | Serum CA125 | Tissue CA125 | Tissue mesothelin |
|-------------|----------------|--------------------|-------------|--------------|------------------|
| 4876        | IIIc           | 2                  | 23,000      | +            | +                |
| 4595        | IIIc           | 1                  | 483         | +            | -                |
| 4079        | IV             | 2                  | 3662        | +            | +                |
| 4424        | IIIc           | 1                  | 448         | +            | -                |
| 2711        | Ia             | 3                  | 1000        | +            | +                |
| 4193        | IIIc           | 3                  | 31          | +            | +                |
| 3169        | Ia             | 3                  | 103         | -            | -                |
| 4083        | IIIc           | 3                  | 3232        | +            | +                |

Mucinous adenocarcinoma

1042        IIIc | 107 | - | - 
4766        Ia   | 90  | - | - 

Clear cell adenocarcinoma

823         IIIc | 6425 | + | - 
6849        Ic   | 31   | + | - 

Endometrioid adenocarcinoma

4465        Ic   | 502  | + | - 

Serous cystic tumor (borderline malignancy)

5492        Ia   | 272  | + | - 
1351        Ia   | 29   | + | - 

Mucinous cystic tumor (borderline malignancy)

Serous cystadenoma (benign)

6022        262 | - | - 
5013        182 | - | - 
3531        <35  | - | - 

Results confirm our finding from the flow cytometric analyses that human mesothelin binds to CA125.

Next, we investigated whether mesothelin binding is specific to CA125 or shared by other glycoproteins of the mucin family. We tested several cell lines, which are known to express various mucin molecules, for binding to mesothelin as well as for expression of CA125 and MUC1. The results, summarized in Table I, show that mesothelin does not bind to mucins in general, indicating that binding to CA125 is specific.

CA125 and Mesothelin Mediate Heterotypic Cell Adhesion—CA125 is presented on the surface of ovarian cancer cells. Mesothelin is expressed by the cells which form the mesothelial lining of the peritoneal cavity, a preferred site of metastases formation of ovarian cancer. To test whether binding of CA125 and mesothelin might play a role in heterotypic cell adhesion, a process that is crucial to initiate metastases, we developed a cell adhesion assay. LO cells grow as adherent cells that express mesothelin abundantly, whereas OVCAR-3 cells are also adherent cells that express CA125. OVCAR-3 cells were detached from culture dishes, and 10⁶ cells were added on top of confluent LO cells. Cells were then brought in close contact by gentle centrifugation. After brief incubation and extensive washing, adherent OVCAR cells were counted by flow cytometry. Under the conditions used, 8.9 (±0.8%) of input OVCAR-3 cells were found attached. Washing with EDTA or EGTA did not reduce cell adhesion at all, indicating that the binding is independent of Ca²⁺, but in the presence of antibody B35, only 3.3 (±0.7%) of input LO cells were found attached. When cultured embryonic diaphragm cells were attached to confluent OVCAR-3 cells, the mesothelin expressing diaphragm cells were found to attach three times more efficiently to OVCAR-3 cells than the mesothelin negative cells, even though in the presence of antibody B35, mesothelin positive and negative cells attached to OVCAR-3 cells equally well. Taken together, these results suggest that both CA125 and mesothelin are present on the apical side of cells and mediate apical-to-apical cell binding.

CA125 and Mesothelin Are Co-expressed in Advanced Ovarian Carcinomas—In a first approach to investigate the molecular interaction between CA125 and mesothelin in the pathological context of cancer, we investigated the expression of both antigens in consecutive paraffin sections from biopsies of ovarian tumors.

As summarized in Table II, samples were obtained from 18 different patients with various subtypes of ovarian adenocarcinoma and adenoma. Immunohistochemistry revealed CA125 staining in 13 of 15 of the malignant samples, including two borderline malignancies, but not in the benign adenomas. In contrast, mesothelin was detected in five of the eight serous papillary adenocarcinomas, but not in other tumor types including the borderline malignancies. Both of two grade 1 serous papillary adenocarcinomas were mesothelin-negative, whereas five of the six serous papillary adenocarcinomas with grade 2 or higher stained positive for mesothelin, the outlier being a stage Ia tumor (Table II, patient 3169). Five representative consecutive sections of serous papillary adenocarcinomas are shown

Table II
Summary of patient data

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in Fig. 5 to illustrate different patterns of co-expression of CA125 and mesothelin. Some samples (e.g. Fig. 5 patients 2711 and 4079) clearly show mesothelin staining in a more restricted part of the tumor than CA125, a pattern that would be expected if tumors acquire mesothelin expression at a later stage of malignant transformation than CA125 expression.

The CA125 concentration in the patients’ serum was determined prior to surgery and was elevated (≥35 IU/ml) in all but three patients, two of them with stage I and only one with an advanced (stage IIIc, grade 3) tumor. Notably, that patient expressed CA125 and mesothelin in the tumor tissue in an overlapping fashion (Fig. 5, patient 4193). This observation suggests that mesothelin could be responsible for trapping CA125 in the tumor tissue in this patient.

In addition, we analyzed co-expression of CA125 and mesothelin in libraries of the SAGEmap project (41). An unambiguous Unigene ID (Ha.155981 and Ha.98502) and SAGE (serial analysis of gene expression) tag (CCCCCTGCAG and CCTGATCTGC) could be identified for mesothelin and CA125, respectively. Of the 154 libraries in SAGEmap (as of July 21, 2003), 20 contained two or more tags for mesothelin. Seven of the 20 mesothelin-positive libraries contained two or more tags for CA125. Of 123 mesothelin-negative libraries, only one contained two and none more than two CA125 tags. (Eleven libraries were not classified as either mesothelin-positive or -negative because they contained exactly one mesothelin tag. None of those 11 contained more than one CA125 tag either.) Table III lists the 20 libraries containing two or more tags of either mesothelin or CA125 plus the human reference RNA library.

High expression of both mesothelin and CA125 was observed in a mesothelioma of the peritoneum, three ovarian serous adenocarcinomas, a pool of ovarian carcinoma cell lines, and a pancreas epithelium ductal adenocarcinoma.

To confirm the observed co-expression of mesothelin and CA125 in SAGEmap libraries, we statistically compared the expression patterns of the respective tags with a number of SAGE tags of other cancer markers and genes, which might have a similar regulation of expression. First, we analyzed the expression of galectin-1 (tag GCCCCCAATA) because it is the only other protein that is known to bind specifically to CA125 (15), and the expression of MUC1 (tag CCTGGGAAGT), a mucin family member implicated in cancer progression, also known as CA15–3 (17, 18, 42, 43). Pairwise regression analyses over expression levels in all 154 SAGE libraries showed that CA125 and mesothelin have a Pearson correlation coefficient of \( r = 0.43 \) at a high significance \( (p = 3 \times 10^{-5}) \), whereas MUC1 expression is less strongly correlated with CA125 expression \( (r = 0.25, p = 0.002) \) and galectin-1 expression shows no significant correlation with CA125 expression \( (p > 0.05) \). Even though galectin-1 was found in most CA125-positive SAGE libraries, it was expressed at similar levels in CA125-negative libraries in an almost ubiquitous manner. Mesothelin expression showed no significant correlation with either MUC1 or galectin-1 expression. Furthermore, neither CA125 nor mesothelin expression was significantly correlated to CEA/CEACAM5, another mucin and well known marker of colon cancer (44), or to Claudin 4, S100A4, stratifin/14 core domain 2), which was recently reported to be a specific marker of ovarian cancer (45) (correlation \( r = 0.44 \) for WFDC2 and \( r = 0.31 \) for WFDC2 and mesothelin). These results demonstrate that CA125 and mesothelin show a remarkably similar expression pattern even when compared with other genes that are also known as cancer markers.

**DISCUSSION**

CA125 is one of the most commonly used diagnostic antigens of ovarian cancer, although its biochemical nature has long
been elusive. Only very recently, its primary structure was elucidated, demonstrating that CA125 shows several unusual features. First, it is an extremely large glycoprotein consisting of more than 22,000 amino acid residues (4). Second, the extracellular domain contains more than 60 tandem repeats of a 156-amino acid motif. These repetitive sequences as well as the remainder of the extracellular part of the protein are rich in serine and threonine residues, resulting in the addition of O-linked sugar chains (5). Third, CA125 is secreted via the endoplasmic reticulum (ER) and Golgi apparatus by a signal-peptide independent mechanism (15). Although the function of CA125 still remains unknown, CA125 is expected to interact with other molecules as O’Brien et al. (46) co-immunoprecipitated CA125 with two unidentified proteins of –35 and 45 kDa from peritoneal fluid of ovarian cancer patients. In the present study, we demonstrate the binding of CA125 to mesothelin, a glycoprotein which is present in peritoneal fluid of ovarian cancer patients (27) and has a molecular mass of 42–45 kDa because of proteolytic cleavage and glycosylation (27).

Mesothelin is a glycoprotein on the cell surface and has been known as a tumor antigen of mesotheliomas as well as ovarian and pancreatic adenocarcinomas (24, 28). In this study, we isolated two cDNA fragments encoding a mesothelin-binding protein, and both cDNA sequences match portions of CA125. The binding of mesothelin to CA125 was demonstrated by flow cytometry as well as immunoprecipitation experiments. As the cloned cDNA fragments encode only a few units of the repeating motif of CA125, this repeating motif must be responsible for the binding to mesothelin. As CA125 is reported to contain more than 60 units of this motif, mesothelin binding is most likely highly multivalent.

Just like the clones we isolated, full-length CA125 lacks a signal peptide (4). A truncated carboxyl-terminal fragment of CA125, which includes the region encoded by our cloned cDNA fragments, has been reported to be secreted via the ER-Golgi pathway, implying a signal peptide-independent mechanism of CA125 protein insertion into the ER (15). Provided both CA125 fragments we cloned are secreted by the same mechanism as full-length CA125, the putative minimal recognition motif for ER insertion should be fully contained in our 1.1-kb fragment, i.e. between tyrosine 21081 and threonine 21444. It is possible that the putative recognition motif for ER insertion exists in other repeats as well. However, the fact that we pulled out two overlapping clones covering this small portion of CA125 strongly suggests that this sequence mediates both protein secretion and binding to mesothelin. Because the CA125 cDNA fragments were cloned by binding of mesothelin to COS7 cells transfected with the cDNA library, the CA125 peptides encoded by two independently cloned cDNAs must be attached to the cell surface. However, neither of the cloned fragments contained a putative transmembrane domain. Although the mechanism of their binding to the cell surface is unclear, it is possible that CA125 fragments were retained on the cell surface through binding to galectin-1 that is expressed in COS7 cells (data not shown). Inhibition of O-glycosylation of OVCAR-3 reduced the binding of mesothelin but not CA-125-specific antibody M11 to the cells, indicating that glycosylation of CA125 is important for binding to mesothelin (data not shown).

We also found that the anti-mesothelin antibody B35 blocked the attachment of OVCAR-3 cells expressing CA125 to mesothelin-positive LO cells, indicating that binding between CA125 and mesothelin mediates cell-cell adhesion. Likewise, binding of OVCAR-3 cells to a heterogeneous primary cell culture containing mesothelial cells from the diaphragm could be partially blocked by the same antibody, demonstrating that heterologous cell adhesion through mesothelin is independent of the cellular background and therefore specific. Because of the specificity of our antibody, these cell binding experiments had to be carried out in a mouse-human cross-species system. We have therefore in addition demonstrated biochemically that human mesothelin binds to human CA125. Taken together, these results argue that the molecular binding of CA125 to mesothelin is involved in cellular adhesion. The apical-to-apical

### Table III

**Summary of mesothelin and CA125 expression in SAGE libraries**

| SAGE library       | Tags per million | Library origin                        |
|--------------------|------------------|---------------------------------------|
| G189               | 1683             | Gastrointestinal tract carcinoma      |
| Meso 12            | 1563             | Mesothelioma of peritoneum            |
| OVT 8              | 1365             | Ovary serous adenocarcinoma           |
| OVT 5              | 641              | Ovary carcinoma cell lines, pooled    |
| OVT 6              | 541              | Ovary serous adenocarcinoma           |
| OVT 7              | 509              | Ovary serous adenocarcinoma           |
| Panc 96 6252       | 363              | Pancreas epithelium ductal adenocarcinoma |
| SW3837             | 212              | Adenocarcinoma colon                  |
| Fce                | 202              | Fuchs’ corneal endothelium            |
| HOSE4              | 185              | Normal ovary epithelium, short term culture |
| Normal lung        | 157              |                                       |
| X101               | 156              | Gastric cancer xenograft              |
| OC14               | 114              | Ovarian carcinoma                     |
| CAPAN1             | 105              | Pancreas adenocarcinoma cell line     |
| CAPAN2             | 78               | Pancreas adenocarcinoma cell line     |
| IDC4               | 76               | Breast, invasive ductal carcinoma, high grade |
| G234               | 75               | Stomach adenocarcinoma                |
| HCT116             | 66               | Adenocarcinoma colon cell line        |
| Panc 91 16113      | 58               | Pancreas epithelium ductal adenocarcinoma |
| HS766T             | 49               | Pancreas adenocarcinoma cell line     |
| ZR75 1 untreated   | 19               | Breast cancer cell line               |
| Universal reference human RNA | 0 | Pooled: brain, cervix, testis, mammary gland, liver, B-cell, carcinoma, adenocarcinoma, liposarcoma, leukemia, melanoma, glioblastoma multiiforme, embryo, macrophage, lymphocyte |
Cannistra’s group (38) devised an in vitro binding assay for ovarian cancer cells using confluent monolayers of normal peritoneal mesothelial cells. Using monoclonal antibodies raised against ovarian cancer cells, they reported that CD44H and integrin $\beta_1$, expressed on ovarian cancer cells, bind to hyaluronic acid and fibronectin, respectively, expressed on mesothelial cells (38, 53). However, they also observed residual specific cell binding that could not be blocked by the ovarian cancer-directed antibodies they used (53). As CA125 and mesothelin, expressed on ovarian cancer cells and on mesothelial cells, respectively, can interact with each other and mediate heterotypic cell attachment in vitro, the residual binding might be a result of binding between CA125 and mesothelin. Consequently, mesothelin might be an additional promising drug target to prevent metastasis in ovarian cancer patients at risk.

Contrary to the broad expression of CA125 in ovarian tumors from borderline malignancies to adenocarcinomas, tissue staining patterns of mesothelin suggest that mesothelin expression occurs at an advanced stage of malignant progression of the tumors. However, the association between advanced histological grade ($\geq 2$) and mesothelin expression in serous papillary adenocarcinomas observed in this study was statistically not significant ($p = 0.11$) because of the small number of samples. Although we observed mesothelin expression only in the serous papillary subtype of ovarian adenocarcinomas, Frixen et al. have reported mesothelin expression in endometrioid and undifferentiated ovarian carcinomas in a large scale tissue microarray study (31). Because that study investigated neither CA125 expression nor tumor stage and grade, it remains to be determined, with a statistically amenable number of cases, whether or not mesothelin expression is indicative of more advanced malignancy of ovarian carcinomas than CA125.

This should add a biological basis to recent proposals for the combined test for CA125 and mesothelin in patient sera (27).

An unresolved clinical issue is the poor correlation between CA125 found in cancer tissue and CA125 concentration in patient serum. The number of patients whose tumor tissue stains positive for CA125 is ~90% in serous ovarian adenocarcinoma, as well as in endometrioid endometrial carcinoma. However, the numbers of patients whose serum CA125 levels are elevated are only 80 and 21% for ovarian cancer and endometrioid endometrial carcinoma, respectively (54, 55). It has been reported that basement membranes surrounding the tissues in which the tumors arise, as well as peritumoral barriers, hinder such high molecular weight proteins as CA125 from entering the circulation (56). However, this retention mechanism alone cannot fully explain the lack of serum CA125 in some patients with advanced stage disease where the basement membrane of the tissue of origin has ruptured, and it has thus been proposed that an unidentified mechanism for CA125 tissue retention must exist, being active with differential efficiency in different gynecological cancers (55). In our study, there is only one advanced stage ovarian cancer patient with normal serum CA125 but positive CA125 tissue staining. In this patient (patient 4193 in Fig. 5 and Table II), CA125 staining is precisely co-localized with mesothelin staining, raising the possibility that mesothelin may be able to trap CA125 in the tissue when it is overexpressed in the same location. If this finding can be confirmed in a larger study of ovarian cancer samples from patients with normal CA125 serum levels, it will give a second biological rationale to combined CA125 and mesothelin serum testing.

The SAGE data base analysis revealed co-expression of mesothelin and CA125 genes in a peritoneal mesothelioma, three ovarian, and one pancreatic adenocarcinoma. Protein co-expression was observed by antibody staining in five serous papillary adenocarcinomas. Furthermore, human peritoneal mesothelial cells have been reported to express CA125 (57) as well as mesothelin (24). In contrast, in our cell binding assay, we showed that CA125 on cancer cells mediates cell attachment to mesothelin expressing cells. We did not investigate how different levels of co-expression of both molecules might modify this cell binding, nor did we investigate what influence soluble fragments of CA125 and mesothelin in the peritoneal fluid might have either on the detachment of tumor cells from the ovarian carcinoma or on their attachment to mesothelial cells. Yet, the fact that OVCAR-3 cells do express some mesothelin (24) and the multivalent nature of the mesothelin binding site on CA125 prompt us to speculate that mesothelin co-expressed with CA125 on ovarian tumor cells may not competitively inhibit but rather promote attachment to mesothelial cells by forming a secondary anchor which retains CA125 molecules even after proteolytic cleavage.

In conclusion, the binding between CA125 and mesothelin described herein may be of importance in metastasis formation of ovarian cancer, and it gives a rational basis to combined CA125 and mesothelin serum testing. The clinical relevance of our findings needs to be confirmed in animal models and with larger clinical data sets.

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