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CD44H Plays an Important Role in Peritoneal Dissemination of Scirrhous Gastric Cancer Cells

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The role of the adhesion molecule CD44H in the peritoneal adhesion and invasion of cancer cells was assessed using cell lines with low and high peritoneal seeding ability, OCUM-2M (2M) and OCUM-2MD3 (2MD3), respectively. The *in vitro* binding ability to peritoneal components (mesothelial cells, fibronectin and type I collagen) and invasive ability of 2MD3 cells were higher than those of 2M cells. The expression level of CD44H on 2MD3 cells was higher than that on 2M cells as determined by western blot analysis and flow cytometry. The adhesiveness of 2MD3 cells to hyaluronic acid, which is expressed on the surfaces of mesothelial cells, was greater than that of 2M cells. The binding ability of 2MD3 cells to mesothelial cells was inhibited in the presence of anti-CD44H monoclonal antibody, but that of 2M cells was not. These results suggested that the 2MD3 cell binding to mesothelial cells is regulated by the CD44-hyaluronic acid dependent system. The *in vitro* binding to submesothelial components and the invasiveness of 2MD3 cells were also inhibited in the presence of anti-CD44H antibody. The *in vivo* inoculation of 2MD3 cells treated with an anti-CD44H antibody resulted in a significant prolongation of survival time as compared with control mice that were inoculated with 2MD3 cells alone. In conclusion, CD44H was associated with attachment not only to hyaluronic acid on mesothelial cells, but also to peritoneal stromal components. Thus, CD44H may play an important role in cancer cell binding and invasion in the peritoneal dissemination of scirrhous gastric cancer cells.

Key words: Peritoneal dissemination — Scirrhous gastric cancer — Mesothelial cell — Adhesion — CD44H

Human scirrhous gastric carcinoma (diffusely infiltrating carcinoma or Borrmann's type IV carcinoma) is characterized by carcinoma cell infiltration and proliferation with extensive fibrosis in the stroma.[1)] The prognosis of patients with scirrhous gastric carcinoma depends on the existence of peritoneal implantation.[2)] Such metastasis is a multistep process involving the detachment of malignant cells from the primary tumor, their migration into the peritoneal cavity, their attachment to the peritoneum, and finally their proliferation to form secondary tumor foci. We know from clinical studies that cancer cells floating free in the abdominal cavity do not always develop peritoneal dissemination. Thus, their attachment to the peritoneum is considered to be an important step. However, the binding mechanism of scirrhous gastric cancer cells to the peritoneum is not well understood.

Several investigators have studied the process of tumor cell adhesion after intraperitoneal inoculation in experimental animals,[3–5)] and have found that cancer cells do not adhere to the mesothelial cells, but rather to the submesothelial connective tissue after the exfoliation of the mesothelial cells. However, the adhesion of cancer cells to mesothelial cells during peritoneal seeding can not be ignored, because the direct attachment of some cancer cells to mesothelial cells has been reported.[6, 7)] The peritoneum is composed of a superficial monolayer of mesothelial cells and submesothelial connective tissue. Since peritoneal mesothelial cells express some extracellular matrix components on their cell surface, such as hyaluronic acid and collagen,[8, 9)] the binding of cancer cells to the mesothelial cells might be mediated by adhesion molecules expressed on the cancer cells.

The cell surface proteoglycan CD44 is expressed by a wide variety of cell types, including epithelial cells and hematopoietic cells such as lymphocytes.[10)] Several CD44 isoforms have already been described.[11)] These isoforms are generated by various alternative splicing events and post-translational modifications. In particular, CD44H has been shown to recognize both the major cell-surface receptor for hyaluronate and the extracellular matrix. CD44H also has been shown to play a role in migration of tumor cells, such as melanoma cells.[12)]

The cell line OCUM-2MD3,[13)] which was established in our department from a scirrhous gastric tumor, readily develops peritoneal metastases with bloody ascites after peritoneal inoculation. The purpose of this study was to elucidate the mechanism of peritoneal dissemination by examining peritoneal binding and invasive ability using

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this cell line, and to assess the role of the adhesion molecule CD44H in the peritoneal dissemination.

MATERIALS AND METHODS

Cells and cell culture The human scirrhous gastric cancer cell line OCUM-2M (2M)[14] was established in our department from a primary tumor. The OCUM-2 MD3 (2MD3) cell line, which has a high potential to form peritoneal dissemination was established from 2M cells using orthotopic tissue implantation as follows. Briefly, a xenografted tumor of 2M cells was transplanted into the gastric wall of a 4-week-old BALB/C *nu/nu*, female nude mouse (CLEA Japan Inc., Osaka). Nine weeks after the implantation, several nodules were observed in the peritoneum, and the 2MD3 cell line was established by cell culture of these nodules. While no peritoneal implantation was observed after the peritoneal inoculation of the 5×10^7 2M cells, peritoneal implantation with bloody ascites developed after peritoneal inoculation of 1×10^7 2MD3 cells in all treated mice. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Human mesothelial cells were obtained from the surgically resected greater omentum or the mesentery from surgical patients. These specimens were washed with phosphate-buffered saline (PBS) and then incubated at 37°C for 20 min in 0.05% trypsin-EDTA (Gibco, Grand Island, NY) to isolate mesothelial cells. The suspension was centrifuged and the pelleted cells were suspended in DMEM with 10% FBS, and incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air. These cells were confirmed to be mesothelial cells by their expression of cytokeratin and vimentin. This immunochemical staining excluded the presence of contamination with either fibroblasts or endothelial cells, which express vimentin alone. The mesothelial cells formed a monolayer with a paving stone sheet appearance. When the mesothelial cells reached to confluence, the monolayer was used for *in vitro* assays.

Flow cytometry The expression of CD44 isoform in the cell lines was determined by flow cytometric analysis. The cells were prepared as single-cell suspensions. Aliquots of 1.0×10^6 cells were treated individually in 1 ml of FACS buffer (phosphate-buffered saline with 0.1% sodium azide and 1% bovine serum albumin) with 0.1 μg/ml of monoclonal antibodies specific for CD44V3, CD44V4/5, CD44V6 (R & D Systems, UK) or CD44V7/8 (Bender MedSystems, Vienna, Austria) ,CD44H (R & D Systems) for 60 min at 4°C, followed by washing twice and labeling with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago, Burlingame, CA) for 30 min at 4°C. After two additional washes, the cells were analyzed using a flow cytometer (Becton Dickinson Labware, Mountainview, CA).

Western blotting 2M and 2MD3 cells were washed three times with phosphate-buffered saline and disrupted with 10 mmol/liter Tris-HCl (pH 7.8) containing 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), 0.15 mol/liter NaCl, 1 mmol/liter EDTA, and 2 mmol/liter phenylmethylsulfonyl fluoride for 30 min at 4°C. Cells were pelleted by centrifugation at 100,000*g* for 30 min and boiled for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. PAGE in the presence of SDS was performed according to the method of Laemmli.[15] Protein concentrations were measured by a Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard. Twenty micrograms of protein was run on 7.5% SDS-polyacrylamide gel and transferred to PVDF membrane. After the electrophoretic transfer, the membrane was blocked overnight at 4°C with 5% BSA, then washed once with Tris-buffered saline containing 0.05% Tween 20 (TBST) (5 min) and incubated with anti-CD44H antibody (1 : 1000 in 0.05% TBST and 0.2% BSA). After incubation with the primary antibody, the PVDF membrane was washed three times with TBST (5 min each) and incubated with biotinated rabbit anti-mouse IgG antibody for 2 h. It was further washed with PBS, then incubated with the avidin-biotin peroxidase complex for 40 min and again washed. The reactive bands were visualized by immersing the membrane in 0.03% 3,3′-diaminobenzidine solution in 20 m*M* Tris-HCl, pH 7.4, containing 0.005% H_2O_2.

Adhesion assay In order to assess the binding of 2M and 2MD3 cells to mesothelial cells, 4×10^5 cancer cells labeled with 10 μCi/ml [^{3}H]thymidine (28 Ci/mmol; Amersham) for 24 h at 37°C in serum-free medium, were seeded onto confluent mesothelial cells in 96-well microtiter plates (Costar, Cambridge, MA). They were allowed to adhere to each well for 30 min at 37°C, and then gently washed twice in PBS. The adhesion of cancer cells was quantified by measurement of the [^{3}H]thymidine content of the adherent cells using a liquid scintillation counter. Prior to the experiments, the kinetics of binding of 2M and 2MD3 cells to the mesothelial cells were investigated. The peak adhesion of these cancer cells to the peritoneal cells was observed after 30 min (data not shown).

The presence of the adhesion molecules, ICAM-1 (intercellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1) and β_1-integrin, and hyaluronic acid on mesothelial cells has been reported.[7,16] We purchased blocking antibodies against ICAM-1 (Immunotech S. A., France),[17] VCAM-1 (Becton Dickinson Labware)[18] and β_1-integrin (Chemicon International Inc., Temecula, CA),[19] which can block cell binding

mediated by these molecules. To investigate the role of these adhesion molecules and hyaluronic acid in the binding of cancer cells to the mesothelial cells, cancer cells were incubated with mesothelial cells that had been treated with 10 μg/ml of a monoclonal antibody against ICAM-1, VCAM-1 or β_1-integrin prior to seeding. Some mesothelial cells were treated with 5 mg/ml of hyaluronidase (Cosmo Bio. Co., Tokyo) for 1 h at 37°C before the seeding of the cancer cells on 96-well microtiter plates. To assess the *in vitro* binding to hyaluronic acid, 96-well microtiter plates were coated with 5 mg/ml of hyaluronic acid (Biozyme Laboratories Ltd., South Wales, UK) and the plates were left at 4°C overnight and at 37°C for 1 h. The plates then were washed twice with serum-free medium before the cells were seeded. [^{3}H]Thymidine-labeled cancer cells were allowed to attach to each well coated with hyaluronic acid for 30 min at 37°C. The binding of the cancer cells was quantified as described above.

The binding of the cancer cells to extracellular matrix (ECM) components (Matrigel; Collaborative Research Co., Bedford, MA; an extract of the basement membrane from an Engelbreth-Alamo Swarm mouse sarcoma[20]), fibronectin (Mallinckrodt Specialty Chemicals Co., St. Louis, MO), and type I collagen (Advance Biofactures Co., Lynbrook, NY) was investigated. Ninety-six-well microtiter plates were coated with Matrigel (8 μg/well), fibronectin (4 μg/well) or type I collagen (8 μg/well) and the binding of the cancer cells to each substrate was assessed in the same way.

Each adhesion assay was performed in the presence or absence of 10 μg/ml anti-CD44H monoclonal antibody. A mouse IgG1 standard (10 μg/ml; TAGO, Camarillo, CA) was used as a control antibody.

The percentage of cells adhering to the microtiter wells treated with each substrate was calculated as follows:

$$\% \text{ specific binding} = 100 \times (\text{radioactivity of treated surface} - \text{control surface}) / \text{total surface}$$

Control surface means the nonspecific binding of cancer cells to albumin, and total surface means total cancer cells, 4.0×10^5 cells, seeded on the microtiter plates. For each group, the assay was performed in triplicate.

Invasion assay The invasive ability of tumor cells was assayed by the method of Albini *et al.*[21] with some modifications. Briefly, a Transwell cell culture chamber (Millipore Co., Bedford, MA) equipped with a microporous membrane (pore size, 12 μm) was used for the invasion assay. Each chamber was placed into a 24-well cluster plate with 1 ml of DMEM, and the microporous membranes were coated with Matrigel (100 μg/filter) to form a matrix barrier. Then 2.0×10^4 mesothelial cells in 200 μl of DMEM with 10% FBS were seeded onto the Matrigel-coated filters. After culturing for 24 h, the mesothelial cells reached confluence. The 2MD3 cells were then resuspended to a final concentration of 2.0×10^4 cells/ml in DMEM with 10% FBS. The tumor cell suspension (200 μl) was added onto Matrigel with a monolayer of mesothelial cells in the upper compartment of the chamber in the presence or absence of 5 μg of CD44H monoclonal antibody for 5 days at 37°C in a 5% CO_2 atmosphere. This assay was used as a model to study cancer cell invasion into the peritoneal cavity. The filters were fixed with ethanol and stained with hematoxylin. A mouse IgG_1 standard (5 μg/ml; TAGO) was used as a control antibody. The tumor cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells which had invaded through the Matrigel and the filter to the lower surface were counted manually under a microscope at a magnification of ×200. Four randomly chosen fields were counted for each assay. The mean of the four fields was calculated as the sample value. Each sample was assayed in triplicate, and the assays were repeated twice.

Peritoneal implantation models in nude mice A peritoneal inoculation of 1×10^7 2MD3 cells into nude mice resulted in peritoneal implantation with 100% tumorigenicity. The survival time of six nude mice, given a peritoneal inoculation of 1×10^7 2MD3 cells that had been treated with 100 μg of CD44H antibody, was determined by following the animals until they succumbed to the tumor burden. We used 1×10^7 2MD3 cells treated with standard IgG as the control. The animals were autopsied at the time of death to verify peritoneal implantation. The percent survival was calculated as a function of time, and the survival times of these two group were compared.

Statistical analysis The data were analyzed statistically using Student's t test. A P value of less than 0.05 was considered statistically significant. The cumulative survival rate was determined using Kaplan-Meier survival curves, and the Cox-Mantel test was used to compare survival between the two groups. Two-tailed P-values less than 0.05 were considered to be significant.

RESULTS

Role of adhesion molecules in the binding of 2M and 2MD3 cells to mesothelial cells Cancer cells adherent to mesothelial cells are shown in Fig. 1. The adhesiveness of 2MD3 cells was about 3 times higher than that of 2M cells, being consistent with the greater potential of 2MD3 to form peritoneal implants. Mesothelial cells express the adhesion molecules ICAM-1, VCAM-1 and β_1-integrin, as well as hyaluronic acid, on their surface. In order to assess the participation of these adhesion molecules and hyaluronic acid in mesothelial cell binding, the binding of cancer cells to mesothelial cells which had been pre-

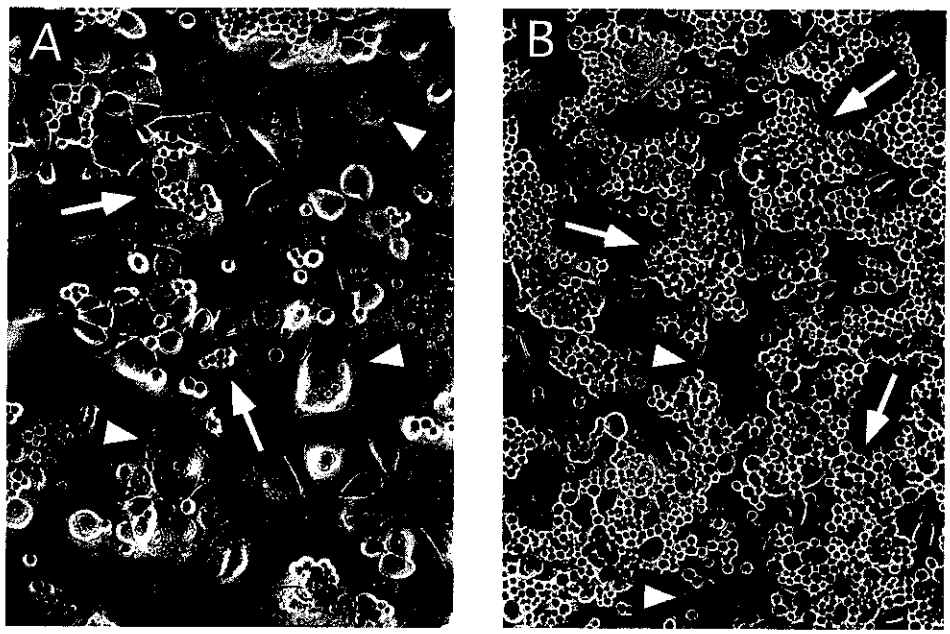

Fig. 1. Scirrhous gastric cancer cells adherent to the mesothelial cells. A, A few 2M cells (arrows) adhered to the mesothelial cells (arrowheads). B, Many 2MD3 cells (arrows) adhered to the mesothelial cells (arrowheads). The number of 2MD3 cells adherent to the mesothelial cells was greater than that of 2M cells. ×150.

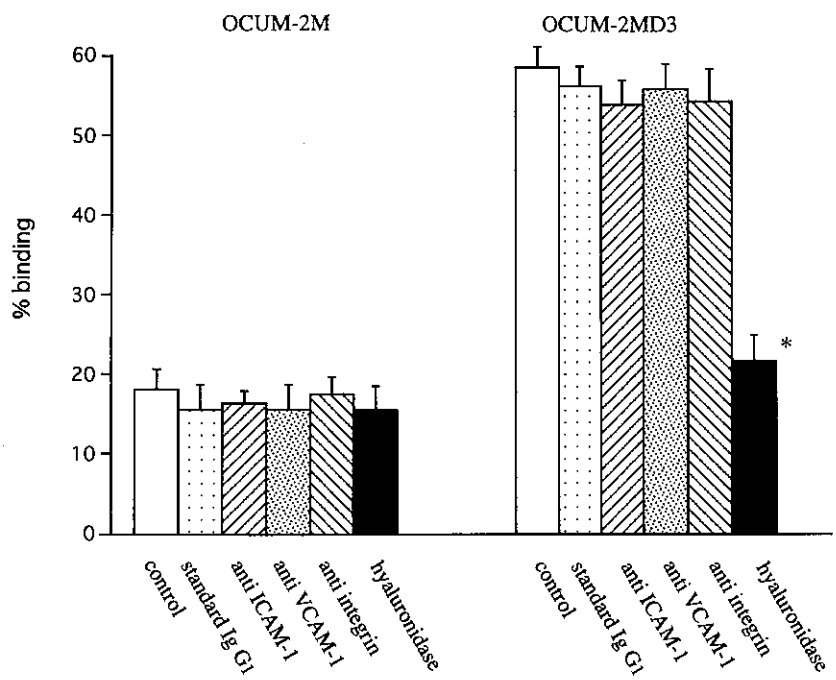

Fig. 2. Binding ability of cancer cells to mesothelial cells. 2M and 2MD3 cells were incubated with mesothelial cells treated with a variety of neutralizing anti-adhesion molecule antibodies (10 μg/ml), i.e., anti-ICAM-1, anti-VCAM-1, anti-β_1-integrin, and hyaluronidase (5 mg/ml) prior to the adhesion assay. A significant inhibition of the adhesion of 2MD3 cells was observed only when the mesothelial cells were pretreated with hyaluronidase. The data are expressed as the means (columns) and standard deviation (bars) of three independent experiments. The significance of the differences was determined using Student's t test. * $P<0.01$ for % binding of the control vs. the % binding of the cancer cells to the mesothelial cells treated with hyaluronidase.

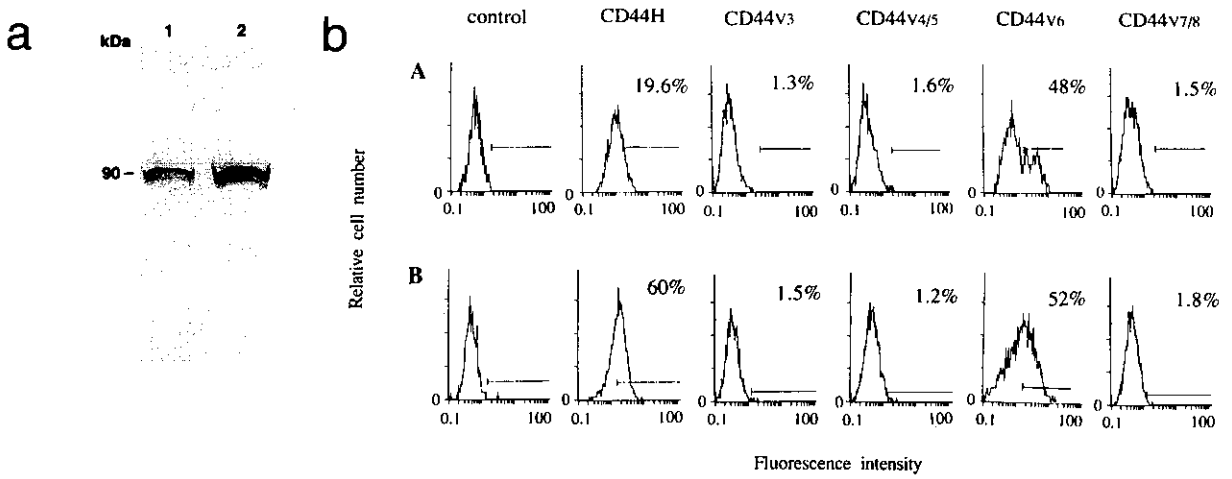

Fig. 3. a, Western blot analysis of CD44H. Proteins purified from scirrhous gastric cancer cells were run on a 10% SDS-PAGE gel, blotted onto nitrocellulose membranes and stained with CD44H antibody, followed by peroxidase-conjugated rabbit anti-mouse IgG and DAB. Lane 1, 2M cells; lane 2, 2MD3 cells. b, Cell surface expression of the variant CD44 isoform on the two scirrhous gastric cell lines. The expression of the variant CD44 isoform in the two cell lines was determined by flow cytometric analysis. CD44V3, CD44V4/5 and CD44V7/8 were poorly expressed on both OCUM-2M and OCUM-2MD3 cells. There was no difference between the expression levels of CD44V6 in the two cell lines. CD44H expression was increased in OCUM-2MD3 cells compared to OCUM-2M cells.

treated with an antibody against ICAM-1, VCAM-1 and β_1-integrin, or with hyaluronidase, was investigated. A significant inhibition of cancer cell adhesion was demonstrated after treatment with hyaluronidase, but not with antibody against ICAM-1, VCAM-1 or β_1-integrin (Fig. 2).

To assess the role of CD44H, the adhesion molecule for hyaluronic acid, in the binding of 2M and D3 cells to mesothelial cells, the expression of CD44H in these two cell lines was investigated. A band of 90 kDa was detected in the two cell lines by western blotting using a monoclonal antibody against CD44H (Fig. 3a). The expression level of CD44H on 2MD3 cells was higher than that on 2M cells. Furthermore, the expression of CD44 variants was investigated using flow cytometry. CD44V3, CD44V4/5, and CD44V7/8 were not expressed in the two cell lines. Similar expression of CD44V6 was observed in both cell lines (48% and 52%). There was no difference in the expression levels of variant CD44 isoforms in the scirrhous gastric cancer cell lines, except for CD44H (19.6% and 60%) (Fig. 3b).

In order to determine whether the CD44H expressed by these scirrhous gastric cancer cell lines has affinity for hyaluronic acid, their binding ability to hyaluronic acid was investigated. The binding of 2MD3 cells to hyaluronic acid was 4 times higher than that of 2M cells, and was inhibited significantly in the presence of anti-CD44H antibody, which did not affect the binding of 2M cells (Fig. 4a). We performed adhesion assay with antibodies against variant forms CD44V3, CD44V4/5, CD44V6 and CD44V7/8 in addition to CD44H. No antibody other than that against CD44H inhibited the binding of 2MD3 to hyaluronic acid. Furthermore, western blot analysis using CD44H antibody detected only a 90 kD band, and no other molecular band. Though it may be surprising that anti-CD44H antibody did not recognize the variant forms, this antibody is thought to have high specificity for the CD44H molecule.

Furthermore, the *in vitro* binding of 2MD3 cells to confluent mesothelial cells was significantly inhibited by 60% in the presence of anti-CD44H monoclonal antibody (Fig. 4b).

Binding and invasion of 2M and 2MD3 cells into the ECM The number of 2MD3 cells adherent to the various ECM components was significantly greater than that of the 2M cells: about 7 times greater to Matrigel, 3 times greater to fibronectin, and 3 times greater to type I collagen. The binding of 2MD3 cells to Matrigel, fibronectin and type I collagen was inhibited 25%, 75% and 50%, respectively, in the presence of anti-CD44H antibody (Fig. 4c).

Numerous 2MD3 cells invaded through the mesothelial cells and Matrigel to the lower surface of the filters, whereas only a few 2M cells did so. The number of 2MD3 cells that invaded was about 8 times greater than that of 2M cells. The invasion by 2MD3 of Matrigel covered with mesothelial cells was inhibited 65% in the presence of anti-CD44H antibody (Fig. 5).

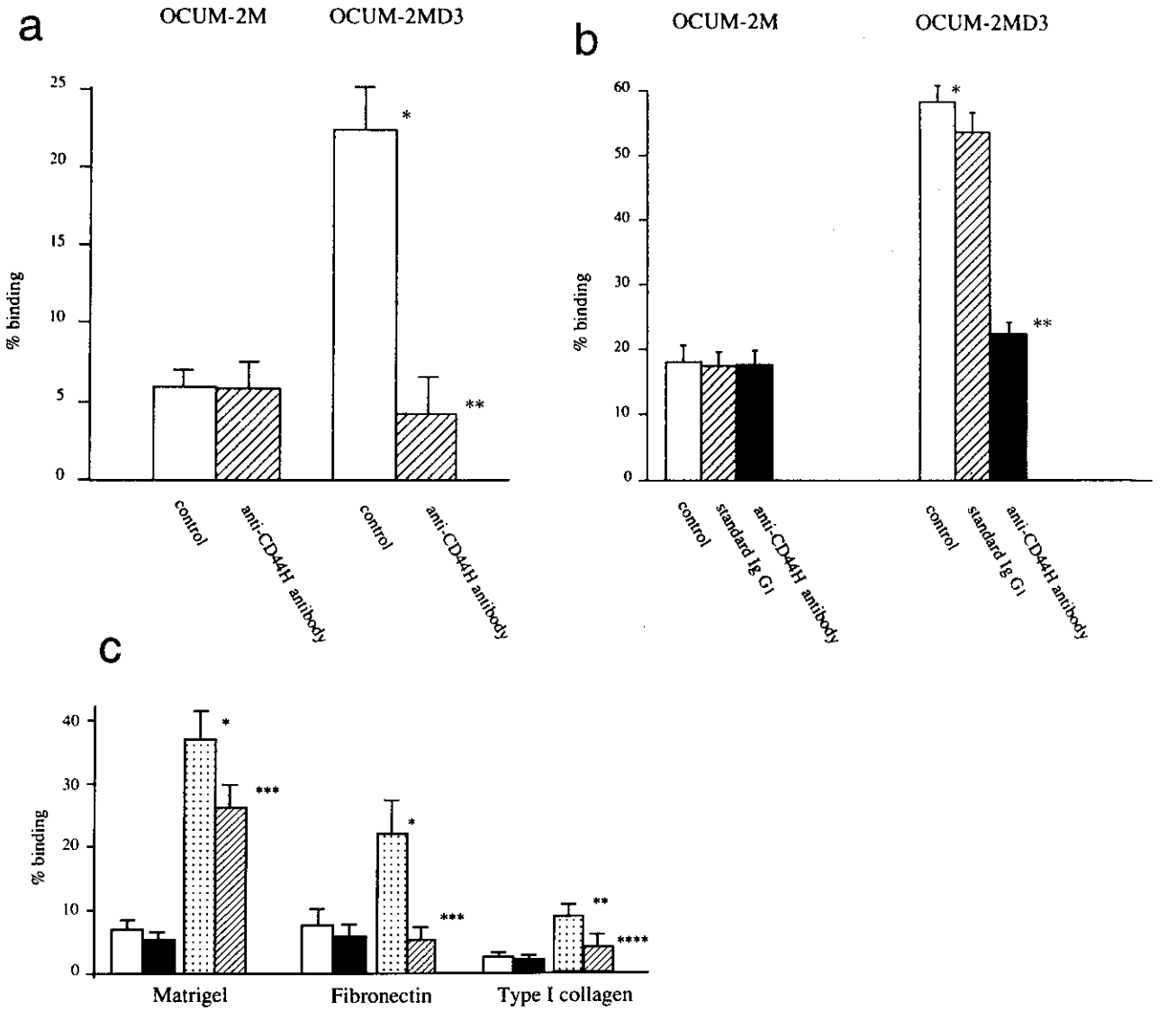

Fig. 4. a, Binding ability to hyaluronic acid. The binding of cancer cells labeled with [^{3}H]thymidine to hyaluronic acid was estimated. The adhesiveness of 2MD3 cells to hyaluronic acid was significantly higher that of 2M cells. The binding of 2MD3 cells to hyaluronic acid was significantly inhibited by anti-CD44H antibody (10 μg/ml), which did not affect the binding of 2M cells. Data are expressed as the means (columns) and standard deviation (bars) of three independent experiments. The significance of the differences was determined using Student's t test. * $P<0.05$ for % binding of control 2M vs. % binding of control 2MD3. ** $P<0.05$ for % binding of control 2MD3 vs. % binding in the presence of anti-CD44H antibody. b, Binding ability to mesothelial cells and the role of adhesion molecules in this binding. The adhesiveness of 2MD3 cells was significantly higher than that of 2M cells in the adhesion assay. The binding of 2MD3 cells was significantly inhibited by the addition of an anti-CD44H monoclonal antibody in the adhesion assay. Data are expressed as the means (columns) and standard deviation (bars) of three independent experiments. The significance of differences was determined using Student's t test. * $P<0.01$ for % binding of control 2M vs. % binding of control 2MD3. ** $P<0.01$ for % binding of control vs. % binding in the presence of anti-CD44H antibody. c, Effect of anti-CD44H antibody on adhesion to ECM. From the amount of radioactivity bound, the number of cancer cells labeled with [^{3}H]thymidine which had adhered to individual extracellular matrix components (Matrigel, fibronectin and type I collagen) was estimated. The binding of 2MD3 cells (▨) to Matrigel, fibronectin and type I collagen was significantly higher than that of 2M cells (□). The binding of 2MD3 cells (▨) was inhibited by the addition of anti-CD44H monoclonal antibody. However, the adhesion of the 2M cells was not altered by this antibody (■). Data are expressed as the means (columns)±SD (bars). The significance of the differences was determined using Student's t test. * $P<0.01$ for % binding of control 2M vs. % binding of control 2MD3. ** $P<0.05$ for % binding of control 2M vs. % binding of control 2 MD3. *** $P<0.01$ for % binding of control 2MD3 vs. % binding in the presence of anti-CD44H antibody. **** $P<0.05$ for % binding of control 2MD3 vs. % binding in the presence of anti-CD44H antibody.

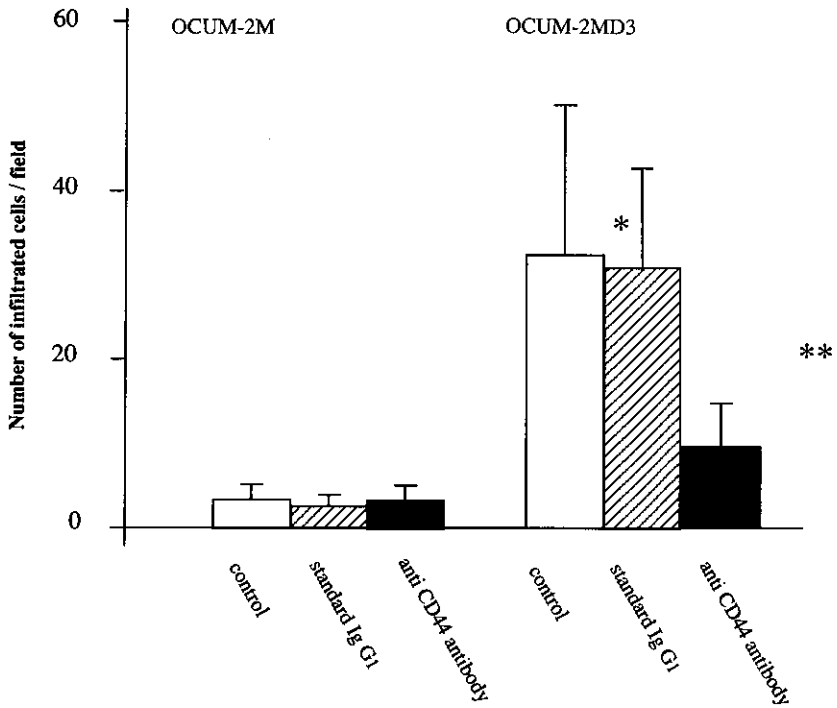

Fig. 5. Invasion ability using a 12 μm porous Transwell cell culture chamber. The 2MD3 cells invaded through Matrigel covered with mesothelial cells and a filter to the lower surface of the microfilter. They were stained with hematoxylin and counted manually under a microscope. Transwell cell culture chambers equipped with filters (12 μm pore size) were used in the invasion assay. The number of 2MD3 cells which invaded was significantly greater than that of 2M cells. The invasion of 2MD3 cells was significantly inhibited by the addition of anti-CD44H monoclonal antibody (dotted columns). Data are expressed as the mean (columns) and standard deviation (bars) of three independent experiments. The significance of the differences was determined using Student's t test. * $P<0.01$ for invasion on control 2M vs. invasion of control 2MD3. ** $P<0.01$ for invasion of control 2MD3 vs. invasion in the presence of anti-CD44H antibody.

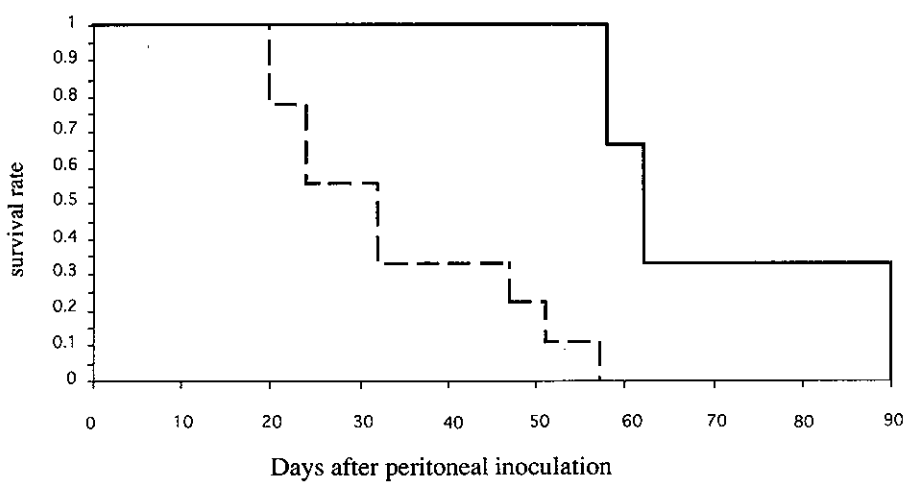

Fig. 6. Survival curves of mice given peritoneal inoculations of 2MD3 cells treated with or without anti-CD44H monoclonal antibody. Solid line; mice given 2MD3 inoculations and anti-CD44 monoclonal antibody ($n=6$). Dotted line; mice given 2MD3 cells alone ($n=9$). The mice were housed under identical conditions, and the date of death of each mouse was recorded. All the mice died of peritoneal implantation. The difference in survival times between the two groups of mice was statistically significant ($P<0.05$).

Inhibitory effect of anti-CD44H antibody on the experimental peritoneal implantation model The inoculation of 2MD3 cells treated with anti-CD44H antibody resulted in a significant prolongation of survival time as compared with control mice (Fig. 6).

DISCUSSION

Binding of cancer cells to the peritoneum might be mediated by adhesion molecules expressed on the cancer cells in peritoneal dissemination. We have already analyzed the role of integrin expression of 2M and 2MD3 cells. Expression of $\alpha_2\beta_1$-integrin and $\alpha_3\beta_1$-integrin on 2MD3 cell (73.3%, 86.6%) was significantly increased in comparison with that on 2M cells (47.8%, 34.3%) (data not shown). Furthermore, binding of 2MD3 cells to peritoneal stromal components such as laminin, fibronectin and type I collagen, was significantly inhibited by anti $\alpha_2\beta_1$-integrin and $\alpha_3\beta_1$-integrin antibody (data not shown). These results suggested that $\alpha_2\beta_1$-integrin and $\alpha_3\beta_1$-integrin might play an important role in the peritoneal dissemination of 2MD3 cells. On the other hand, hyaluronic acid as the ligand for adhesion molecule CD44H is contained in the peritoneum, and CD44H may play an important role in the dissemination of scirrhous gastric cancer. Therefore, we investigated the role of CD44, including variant forms, in this study.

2MD3 cells, which have high peritoneal seeding potential, were more adhesive to a monolayer of peritoneal mesothelial cells as compared with 2M cells, which have low peritoneal seeding potential. Mesothelial cells express hyaluronic acid, integrin, ICAM-1, and VCAM-1 on their cell surface. The binding between 2MD3 cells and mesothelial cells was significantly suppressed when the mesothelial cells were pretreated with hyaluronidase, but was not affected by the use of neutralizing antibodies against β_1-integrin, ICAM-1 or VCAM-1. The ability of 2M cells to adhere to mesothelial cells was not affected by any of these treatments. 2MD3 cells expressed a higher level of CD44H on the cell surface than 2M cells as determined by western blot analysis and flow cytometry. Differences in the hyaluronic acid-binding ability of the two cell lines can be accounted for by quantitative differences in the level of CD44H expressed on the cell surface. Adhesion ability of 2MD3 cells to mesothelial cells and hyaluronic acid was significantly suppressed when the 2MD3 cells were treated with anti-CD44H antibodies. These findings indicate that both hyaluronic acid expressed on mesothelial cells and CD44H expressed on 2MD3 cells are closely associated with the adhesion process in peritoneal dissemination.

Expression of CD44 in KATO III gastric cancer cells, derived from signet ring cell carcinoma, was investigated using western blot analysis, and an 80 kD band was detected.[22] Not all scirrhous gastric cancer may express CD44H, but we certainly demonstrated the expression of CD44H in 2M cells and 2MD3 cells derived from scirrhous gastric cancer. Several CD44 isoforms have already been described.[23–25] These isoforms are generated by various alternative splicing events and post-translational modifications. Recently, the expression of CD44 splice variants has been found in various types of human malignancies, and is thought to be implicated in tumor progression and metastasis.[26–29] The two cell lines were evaluated for CD44 variants using flow cytometry. CD44V3, CD44V4/5, and CD44V7/8 were not expressed in the two cell lines. There was no difference in the expression of CD44V6. CD44 variants may not be related to peritoneal dissemination after the peritoneal inoculation of 2MD3 cells.

We next investigated the adhesive and invasive abilities of these cancer cells in relation to submesothelial stromal components. The submesothelial stroma is mainly composed of fibronectin, type I collagen and laminin as determined by immunohistochemistry (data not shown). The 2MD3 cells were more adhesive to Matrigel, fibronectin, and type I collagen than were the 2M cells. The invasion ability of 2MD3 cells was also greater than that of 2M cells. CD44 has been shown to interact with ECM components such as fibronectin[30] and type I collagen.[31] The adhesive and invasive abilities of 2MD3 cells were significantly inhibited by treating the cancer cells with anti-CD44H antibodies. CD44 may mediate the invasion of the cancer cells into the peritoneum by facilitating their attachment to mesothelial cells and submesothelial tissue.

Although the role of CD44H in the binding between ovarian cancer cells and mesothelial cells in peritoneal dissemination has already been reported,[7] we showed here that CD44H on 2MD3 cells was associated with attachment not only to hyaluronic acid on mesothelial cells, but also to peritoneal stromal components. Although high expression of CD44 in gastric cancers has been found,[23] the role of CD44 in peritoneal dissemination has not previously been reported. We demonstrated the role of CD44H in peritoneal dissemination for the first time in the present study. In our *in vivo* model of peritoneal dissemination by 2MD3 cells, treatment with anti-CD44H antibodies prolonged the survival time of nude mice compared with untreated control mice. These results suggest that CD44H may be involved *in vivo* in the peritoneal implantation of disseminated cancer cells in the abdominal cavity, and suggest the possibility of therapeutic intervention to prevent peritoneal dissemination by targeting the adhesion molecule CD44H. However, since cancerous peritonitis could not completely be prevented with anti-CD44H antibodies, other factors may also play a role in peritoneal metastases.

In conclusion, CD44H on cancer cells was associated with the attachment of the cancer cells to hyaluronic acid on mesothelial cells and with invasion into peritoneal stromal components. CD44H may play an important role in the peritoneal dissemination of scirrhous cancer cells.

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