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CD44H Participates in the Intrahepatic Growth of Murine Colon 26 Adenocarcinoma Cells

Takahiko Ogoshi, Seiichi Ishii[1], Takayuki Mizoi, Nobuhiko Harada, Wataru Sato, Kazuya Saito, Hitoshi Ogawa, Keiko Inabe, Ken-ichi Shiiba and Seiki Matsuno

First Department of Surgery, Tohoku University School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574

The purpose of this study was to determine if CD44, a metastasis-associated cell adhesion molecule, is involved in the hepatic colonization by murine colon 26 adenocarcinoma cells. Indirect membrane immunofluorescence and FACS analysis showed strong expressions of CD44 and integrin β_1 on colon 26 cells. Injection of 1×10^5 colon 26 cells into the superior mesenteric vein of syngeneic BALB/c mice produced macroscopic hepatic nodules in 92% (22/24) of the mice 14 days after inoculation. When colon 26 cells were pretreated with an anti-CD44 monoclonal antibody (mAb), IM7, only 30% (3/10) of the mice produced minute nodules in the liver on day 14 ($P<0.001$), though IM7 did not inhibit growth of the cells *in vitro*. Pretreatment of colon 26 cells with an anti-integrin β_1 mAb did not significantly block the hepatic metastasis. Histologically, microcolonies of tumor cells were detected in all of the livers on day 14 including the IM7-pretreatment mice that were free of gross nodules. However, percentages of tumor-occupied areas in the liver were consistently lower in IM7-pretreatment mice than in control mice (0.82% vs. 5.0% on day 14; $P<0.005$). Reverse transcription-polymerase chain reaction (RT-PCR) amplification of mRNA revealed that colon 26 cells and splenocytes only expressed the hematopoietic isoform of CD44 (CD44H), which had no insertion of variant exons, while normal colonocytes expressed possible variant isoforms. These data suggest that malignant transformation of murine colonic epithelium altered the expression pattern of CD44 isoforms and that CD44H participates in the intrahepatic growth of colon 26 cells.

Key words: Colorectal cancer — Colon 26 — CD44 — Hepatic metastasis

Colorectal carcinoma is one of the most common malignancies in humans, and often metastasizes to the liver. Only 20 to 30% of hepatic metastases from colorectal cancer can be treated surgically, while the others are unresectable and resistant to anti-cancer agents.[1, 2] Thus, a better understanding of the mechanism of hepatic implantation by colorectal cancer is required to overcome metastatic liver diseases.

Free carcinoma cells released from the primary sites may reach the liver through portal blood flow. Studies on experimental metastases have revealed that not all cancer cells can grow in the liver but only a minor proportion, or sometimes none, of the cells can persist and survive in the hepatic microenvironment.[3, 4] Multiple cell adhesion molecules and growth factors are involved in the successful implantation and proliferation of cancer cells in the liver.[5–7]

CD44 is a membrane glycoprotein which was first described by Trowbridge *et al.*[8] as the lymphocyte homing receptor that mediates the recruitment of lymphocytes to the high endothelial venules of specific lymphoid organs.[9, 10] The hematopoietic isoform of CD44 (CD44H) was then proved to be the cellular hyaluronate receptor.[11, 12] Expression of CD44 is not restricted to hematopoietic cells but is broadly observed on cells of diverse origin, including normal and malignant epithelia.[13, 14] Günthert *et al.* and Rudy *et al.* showed that surface expression of a variant isoform of CD44 determined the metastatic potential of a rat pancreatic carcinoma cell line to the lung.[15, 16] Involvement of CD44 in malignant progression and metastasis formation of human malignancies has been suggested in experimental and clinical studies.[17–20] However, CD44 has not been directly proved to be a responsible molecule for hepatic metastasis of colorectal cancer.

In this study we determined if CD44 participates in the hepatic metastasis of colorectal carcinoma cells using a CD44-positive and highly metastatic murine adenocarcinoma cell line, colon 26. The CD44 isoforms expressed in colon 26 cells and normal colonocytes were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) amplification of mRNA. Then involvement of CD44 in the hepatic colonization of colon 26 cells was examined by testing the ability of an anti-CD44 monoclonal antibody (mAb) to block the production of colonies in the liver.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Animals Six-week-old male BALB/c CrSlc mice weighing 20–25 g were obtained from Funabashi Farms, Inc. (Tokyo) and were kept under pathogen-free conditions. Tumor cell injection was performed under general anesthesia with intraperitoneal administration of pentobarbital. All animal experiments were approved by the Animal Care Use Committee of Tohoku University, Sendai.

Monoclonal antibodies Monoclonal antibodies used were a rat anti-mouse CD44 IgG2b (IM7), a rat anti-integrin β_1 IgG2a (9EG7) and a rat non-specific IgG2b (R35-38). The three antibodies were purchased from Pharmingen, Inc. (San Diego, CA). A goat anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) was obtained from CALTAG Laboratories (San Francisco, CA) and was used as the secondary antibody.

Preparation of carcinoma cells, mouse splenocytes and normal colonocytes A murine adenocarcinoma cell line colon 26, which had been established from a chemically induced colon carcinoma in a BALB/c mouse[21] was kindly supplied by the Department of Surgery I, Okayama University, Okayama. The cells were maintained in RPMI 1640 medium (Gibco Laboratories Life Technology, Inc., Grand Island, NY) with 10% fetal bovine serum (FBS; Sigma Chemical Company, St. Louis, MO), 1% L-glutamine (Gibco), 100 units/ml of penicillin G (Gibco) and 100 μg/ml of streptomycin (Gibco). Colon 26 cells semi-confluent in tissue culture flasks were recovered by treatment with trypsin (Gibco) when the cells were used in animal experiments. The trypsinized cells were washed twice with phosphate-buffered saline (PBS; Gibco) and suspended at a concentration of 1×10^6 cells/ml in PBS. Viability of the suspended cells was determined by trypan blue exclusion and was at least 95% in each experiment.

Murine splenocytes were recovered from the spleen. Murine normal colonic epithelial cells (colonocytes) were isolated from colonic mucosa by means of the EDTA-chelation technique.[22]

Experimental hepatic metastasis Experimental hepatic metastasis was established following the methods described by Tominaga *et al*.[23] Briefly, the murine abdomen was entered through a small upper midline incision under general anesthesia. The superior mesenteric vein was gently exposed and 1×10^5 colon 26 cells in 0.1 ml of PBS were carefully injected into the vein through a 31-gauge needle (Hamilton Company, Reno, NV). Hemorrhage from the punctured vein was stopped with a dental cotton roll (Hakujuji, Inc., Tokyo). Groups of 3 to 20 mice were killed at 1 min, 30 min, 24 h, 72 h, 5 days, 7 days and 14 days after inoculation of colon 26 cells and gross findings of the liver, lungs and peritoneal nodules were quickly checked. Then the specimens were fixed with ice-cooled 10% formalin in PBS or periodate-lysine 4% paraformaldehyde (PLP) for routine histology and immunohistochemistry, respectively.

Indirect membrane immunofluorescence and FACS analysis Surface expression of CD44 and integrin β_1 on colon 26 cells was confirmed by indirect membrane immunofluorescence and FACS. Colon 26 cells semi-confluent on a tissue culture flask were trypsinized and washed twice with PBS. The cells were suspended at 1×10^6 cells/ml in PBS containing 5 μg/ml of either IM7 for CD44, 9EG7 for integrin β_1 or R35-38 as a non-specific control antibody. The cell suspensions were incubated for 30 min at 4°C, then washed twice and resuspended at 1×10^6 cells/ml in PBS. The cell suspensions were incubated again with a goat anti-rat IgG antibody conjugated to FITC at a concentration of 10 μg/ml for 30 min at 4°C. The cells were washed three times and re-suspended in PBS. Surface expression of cell adhesion molecules was then examined with a fluorescence microscope. Intensity of the expression of CD44 and integrin β_1 on colon 26 cells was evaluated using the FACScan (Becton Dickinson, Mountain View, CA).

RT-PCR amplification of mRNA Total RNA was extracted from colon 26 cells, murine splenocytes and normal murine colonocytes with phenol solution ("ISOGEN"; Nippon Gene Inc., Tokyo) according to the manufacturer's protocol. cDNAs were synthesized by RT of mRNA using "Superscript" (Gibco) with random hexamers. The cDNA synthesis was started with 2 μg of total RNA in each sample and 1 μl aliquots of RT products were used for subsequent PCR. The cDNAs were then amplified by PCR for 30 cycles with the *Taq* DNA polymerase ("Ex Taq"; Takara Biomedicals, Tokyo) on a thermal cycler (Stratagene, La Jolla, CA). The cycle parameters were 94°C for 1 min, 63°C for 1 min and 72°C for 2 min followed by 72°C for 10 min for the final elongation. The PCR primers used were CD44 sense: ACCCCAGAAGGCTACATTTTGC, CD44 anti-sense: CTCATAGGACCAGAAGTTGTGG, β actin sense: CAACTGGGACGACATGGAGAAGA and β actin anti-sense: CAATAGTGATGACTTGGCCGTC.[24, 25] The CD44 primers bracketed the insertion site on mouse CD44 cDNA and would generate a product of 220 base pairs (bp) for CD44H that has no insertion of variant exons to 1,462 bp if all splice exons were present.[25] The β actin primers were expected to generate a single band at approximately 550 bp.[24] The PCR products were applied on a 3% agarose gel and were electrophoresed. The gel was then stained with ethidium bromide and illuminated on a UV table.

Pretreatment of colon 26 cells with monoclonal antibodies or hyaluronidase Colon 26 cells suspended at 2×10^6 cells/ml in PBS were incubated with 5 μg/ml of either IM7 for CD44, 9EG7 for integrin β_1 or PBS alone for 30 min at 4°C. The cells were washed twice with PBS

and were resuspended at 1×10^6 cells/ml in PBS. The cell suspensions were kept at 4°C and quickly used in animal experiments. A suspension of 1×10^5 cells in 0.1 ml of PBS was injected into the superior mesenteric vein of each mouse under general anesthesia. Production of hepatic colonies in the three groups was compared on days 3, 5, 7 and 14 after inoculation of the colon 26 cells. To check if treatment with IM7 had any effects on the growth of colon 26 cells, the antibody-treated cells and PBS-treated cells were cultured *in vitro* in the presence of 10% serum of BALB/c mice plus 10% FBS or 20% FBS in RPMI1640.

In another experiment, colon 26 cells were treated with 1,000 U/ml of testicular hyaluronidase (Sigma) in RPMI1640 for 1 h at 37°C. The cells were used in the metastasis formation assay in the same manner as described above.

Histological studies The murine livers, except for some small pieces taken for immunohistochemistry, were fixed with 10% formalin in PBS at 4°C in eight pieces from each mouse. The fixed specimens were paraffin-embedded and then sliced at 3 μm for hematoxylin and eosin (H & E) staining. The area of the liver in each section was measured under a microscope with a grid micrometer at magnifications of 100× to 400×. The area of tumors in each section was also evaluated in the same manner and the percentage of tumor-occupied area in each liver section was calculated.

For immunohistochemistry, small pieces of the murine livers were PLP-fixed and frozen-embedded with OCT compound (Miles, Inc., Elkhart, IN). Then cryostat sections were stained for CD44 by means of the ordinary streptavidin-biotin complex (SABC) method using a biotin-conjugated IM7 mAb and a biotin-streptavidin-peroxidase kit (Histofine kit; Nichirei, Tokyo). Counterstaining of the sections were performed with methyl green. Endogenous peroxidase activity was checked by directly immersing the frozen sections without any antibodies in 3,3′-diaminobenzidine tetrahydrochloride (DAB)/H_2O_2 solution.

Histochemical staining of polysaccharides and acidic mucopolysaccharides in the murine liver with metastases was performed by using the periodic acid Schiff (PAS) reaction and alcian blue staining in the usual manner. The nuclei were weakly stained with hematoxylin and kernechtrot for the PAS reaction and the alcian blue staining, respectively.

Statistical analysis The significance of differences among means of groups was tested by one-way analysis of variance (ANOVA). When ANOVA showed that means within an experiment were significantly different from one another, the significance of the differences between individual group means was tested using either the Fisher PSLD or Dunnett's *t* test at a significance level of 1%. All calculations were performed on a Macintosh 7500/100 microcomputer using StatView 4.11 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Colon 26 cells produced multiple hepatic metastasis Injection of 1×10^5 colon 26 cells via the superior mesenteric vein produced macroscopic tumors on the surface of the liver in 38% (3/8) and 92% (22/24) of mice on days 7 and 14, respectively (Table I, Fig. 1a). All mice given 1×10^5 colon 26 cells produced some peritoneal nodules (data not shown) as well as multiple liver colonies on day 14, while none of the mice developed metastases in the lung (data not shown).

Colon 26 cells expressed CD44 as well as integrin β_1 Surface expression of CD44 on colon 26 cells was examined by FACS with the IM7 mAb. CD44 was intensely positive on colon 26 cells while an isotype-matched control antibody, R35-38, was negative (Fig. 2a). More than 90% of colon 26 cells were positive for CD44 as confirmed by indirect membrane immunofluorescence under

Table I. Production of Macroscopic and Microscopic Hepatic Metastases by Injection of Either 1×10^4 or 1×10^5 Colon 26 Cells into the Superior Mesenteric Vein of BALB/c Mice

Numbers of cells injected		Numbers of mice with hepatic nodules on the following days (%)				
		day 1	day 3	day 5	day 7	day 14
1×10^4	macroscopic:	ND	ND	0/5 (0)	1/5 (20)	ND
	microscopic:	ND	ND	1/5 (20)	3/5 (60)	ND
1×10^5	macroscopic:	0/3 (0)	0/6 (0)	0/6 (0)	3/8 (38)	22/24 (92)
	microscopic:	0/3 (0)	3/6 (50)	4/6 (67)	7/8 (88)	24/24 (100)

Microscopic metastases in the livers were confirmed by histology with H & E staining. Eight sections were prepared from each liver and the sections were checked at magnifications of 100× to 400×. When no cancer colony was detected in the eight sections the livers were assessed as negative for microscopic metastasis.
ND: not done.

Fig. 1. Gross appearance of murine livers 14 days after injection of 1×10^5 colon 26 cells. a: Livers of control mice that had been given colon 26 cells pretreated with PBS alone. b: Livers of the mice given IM7-pretreated colon 26 cells. Most of the control mice developed multiple nodules on the surfaces of the livers, while 70% of the IM7-pretreatment mice were grossly negative for hepatic tumors on day 14 (cf. Table II).

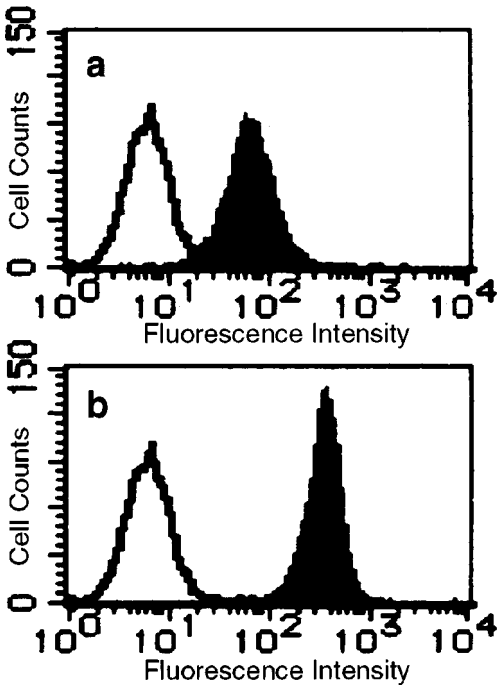

Fig. 2. Surface expressions of CD44 and integrin β_1 on colon 26 cells determined by FACS. Open histograms indicate control cells stained with the non-specific IgG, R35-38; dark histograms indicate the cells stained with IM7 for CD44 (a) or with 9EG7 for integrin β_1 (b). Both CD44 and integrin β_1 are positive on colon 26 cells, though integrin β_1 is more intensely expressed than CD44.

a fluorescence microscope (data not shown). To obtain a reactive control antibody for colon 26 cells, a panel of cell adhesion molecules, including integrins β_1 and β_4, ICAM-1 and LFA-1α chain, was stained with specific antibodies. As a result, only integrin β_1 was proved positive on colon 26 cells (Fig. 2b) and others were negative (data not shown). Integrin β_1 was expressed more intensely than CD44 on the tumor cells (Fig. 2, a and b).

Colon 26 cells only expressed CD44H whereas murine colonocytes expressed variant isoforms The CD44 isoforms expressed in colon 26 cells, murine normal colonocytes and splenocytes were examined by RT-PCR amplification of mRNA. Amplification of the CD44 cDNA in colon 26 cells and splenocytes produced a single band at 220 bp, which was consistent with the expected size of CD44H cDNA (Fig. 3, lanes e and g). The intensity of the band in the case of colon 26 cells was stronger than that for splenocytes, suggesting higher expression of CD44H mRNA in colon 26 cells. In contrast, amplification of the CD44 cDNA in murine normal colonocytes produced at least five bands ranging from around 800 bp to 1,450 bp, with relatively weak intensity (Fig. 3, lane f). Amplification of β actin cDNA generated a single strong band at approximately 550 bp in all three types of cells (Fig. 3, lanes b, c and d).

Pretreatment of colon 26 with IM7 inhibited production of hepatic tumors To test if CD44 was involved in the development of hepatic metastasis, colon 26 cells were pretreated with the IM7 mAb and the cells were inoculated into the superior mesenteric veins of syngeneic mice. Ninety-two percent (22/24) of the mice that had been injected with colon 26 cells pretreated with PBS

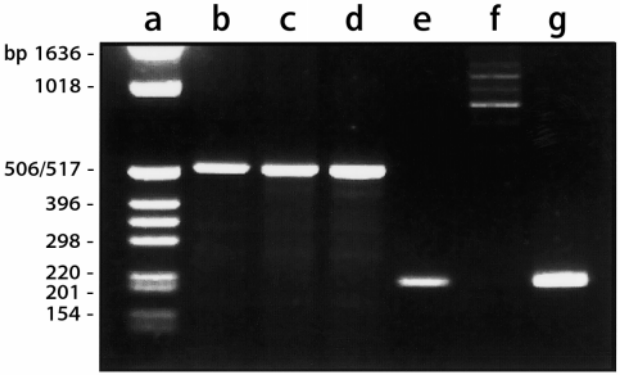

Fig. 3. RT-PCR amplification of mouse mRNA. The mRNA applied was extracted from splenocytes (lanes b and e), normal colonocytes (lanes c and f) and colon 26 cells (lanes d and g). a: DNA size markers; b, c and d: amplification of β-actin cDNA; e, f and g: amplification of CD44 cDNA.

alone produced multiple liver tumors on day 14 (Fig. 1a, Table II). In contrast, only 30% (3/10) of the mice given IM7-pretreated cancer cells developed minute nodules

while the remaining 70% of the IM7-pretreatment group was entirely free of gross tumors on the surface of the liver (Fig. 1b, Table II; $P<0.001$). Pretreatment of colon 26 cells with an anti-integrin β_1 mAb, 9EG7, did not significantly block the production of hepatic tumors (Table II).

Microscopic findings of colon 26 cells implanted in the liver Early phases of hepatic colonization by colon 26 cells were examined histologically with H & E staining. Single isolated colon 26 cells were detected in the lumen of the portal venules at 1 min after injection (Fig. 4a). During the next 48 h, colon 26 cells could not be identified in the hepatic microvasculature on the basis of simple morphology. At 72 h, microcolonies of tumor cells were observed in half of the mice, though no mouse had developed macroscopic hepatic nodules (Fig. 4b, Table I). Colon 26 colonized predominantly the zone 1 sinusoidal areas (Fig. 4b).

CD44 immunohistochemistry of murine livers was performed with the IM7 mAb. Colon 26 cells implanted in the liver were strongly positive for CD44 (Fig. 4c). Endothelial cells of the intrahepatic portal veins and Kupffer cells were also CD44-positive (Fig. 4c).

To examine if the colon 26 cells that proliferated in the liver produced hyaluronate, histochemical staining of polysaccharides and acidic mucopolysaccharides was performed with the PAS reaction and alcian blue stain (Fig. 5). Hepatocytes were positive for PAS staining, while colon 26 cells were almost negative, indicating loss of mucin production and poor differentiation of the tumor cells (Fig. 5b). The alcian blue that stains acidic mucopolysaccharides including hyaluronate did not stain either hepatic cells or colon 26 cells (Fig. 5c). Thus, hyaluronic acid was absent from hepatic cells and colon 26 cells, as judged from the histochemistry.

Table II. Inhibitory Effect of the Anti-CD44 mAb on the Production of Macroscopic Liver Tumors by Colon 26 Cells

Pretreatment of colon 26 cells	Numbers of mice with hepatic tumors on the following days (%)			
	day 3	day 5	day 7	day 14
PBS alone:	0/3	0/3	1/3	22/24[a)] (92)
anti-integrin β_1:	ND	ND	ND	4/6 (67)
anti-CD44:	0/3	0/3	0/3	3/10[a)] (30)

Colon 26 cells were treated either with PBS alone, anti-integrin β_1 or anti-CD44 antibodies prior to injection into the superior mesenteric vein of mice. Monoclonal antibodies used were the IM7 and the 9EG7 antibodies for murine CD44 and integrin β_1, respectively.
a) $P<0.001$ by Fisher's exact probability test: PBS vs. anti-CD44.
ND: not done.

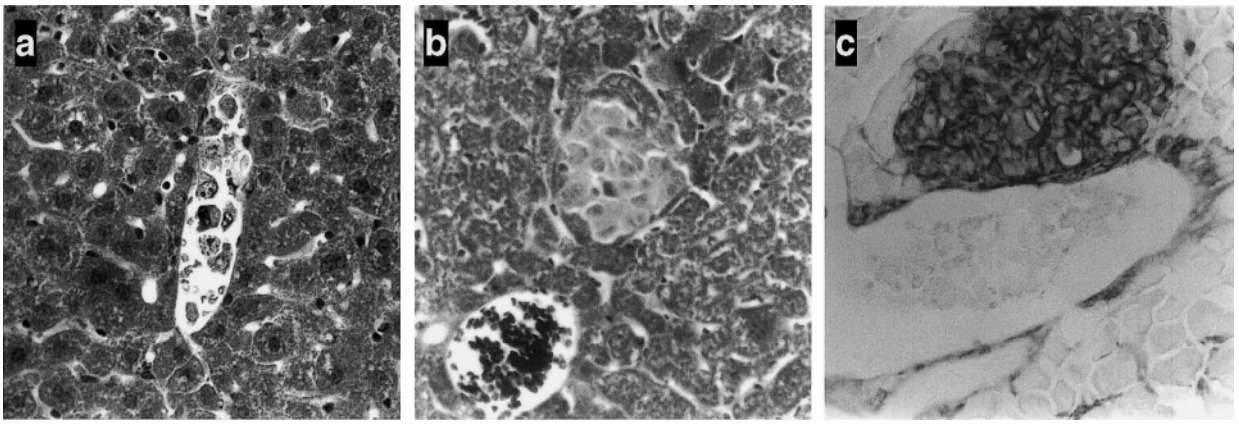

Fig. 4. Histological sections of murine livers after inoculation of colon 26 cells (original 400×). a: A section of the liver at 1 min after inoculation of colon 26 cells (H & E). Isolated carcinoma cells without contact to endothelial cells can be seen in the lumen of a portal venule. b: Growth of colon 26 cells in the liver at 72 h (H & E). Colon 26 cells produced colonies predominantly in the zone 1 sinusoidal areas. c: CD44 immunohistochemistry of the liver stained with the IM7 antibody on day 5. CD44 is strongly expressed on the surface of colon 26 cells proliferating in the hepatic parenchyma. Endothelial cells of intrahepatic portal vein and Kupffer cells are also positive for CD44.

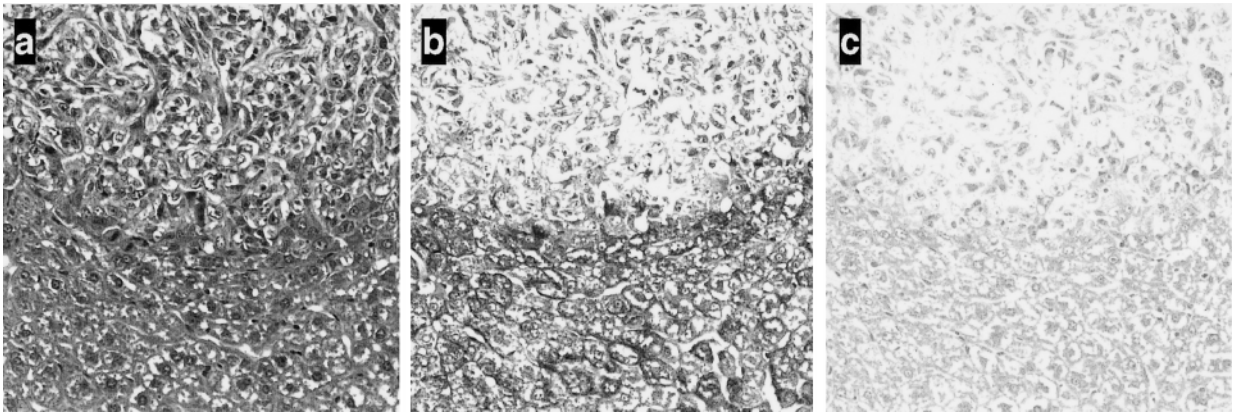

Fig. 5. Histochemical staining of polysaccharides in the murine liver (original 200×). a: H & E; b: PAS staining for polysaccharides i.e. glycogen, mucin, etc.; c: alcian blue staining (pH 2.5) for acidic mucopolysaccharides i.e. hyaluronate, sialomucin, etc. The three sections were obtained in a serial manner from a murine liver that had metastatic nodules of colon 26 cells on day 14. a: The upper half of the frame is occupied with colon 26 cells, while hepatocytes occupy the counterpart. b: Hepatocytes that contain glycogen granules are PAS-positive, while colon 26 cells are almost negative for PAS. c: Both colon 26 cells and hepatic cells are negative for alcian blue. Positive staining with PAS and alcian blue was confirmed in murine normal colonic mucosa (data not shown).

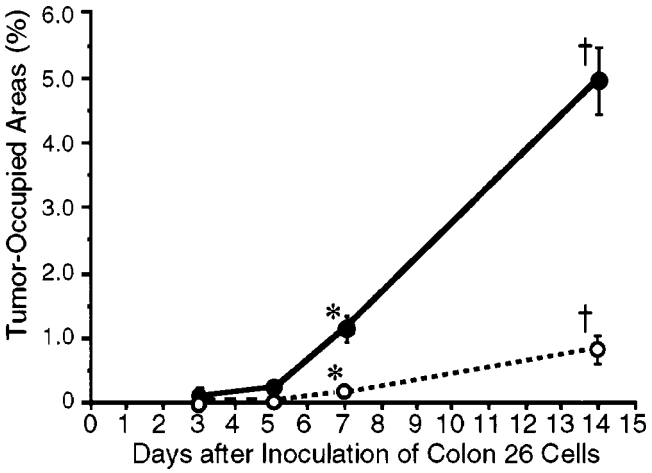

Fig. 6. Percentages of tumor-occupied areas in murine liver determined by histology. Colon 26 cells were treated with either PBS alone (●) or the IM7 antibody for CD44 (○) prior to injection into the superior mesenteric vein of mice. Tumor-occupied areas in each section were obtained with a grid micrometer at magnifications of 100× to 400× as described in "Materials and Methods." ∗ $P<0.05$ (1.1±0.21% vs. 0.13±0.008% on day 7, $n=3$) and † $P<0.005$ (5.0±0.52% vs. 0.82±0.17% on day 14, $n=5$): PBS vs. anti-CD44.

IM7-pretreatment significantly reduced tumor-occupied areas in the liver When the livers of mice in the IM7-pretreatment group were examined histologically, microcolonies of colon 26 cells were observed in all of the livers including those that were free of gross tumors on day 14. However, the percentage of tumor-occupied areas was consistently lower in the IM7-pretreatment group than in the control group throughout the observation period (Fig. 6; 0.82% vs. 5.0% on day 14; $n=5$, $P<0.005$).

Pretreatment with IM7 did not block growth of colon 26 cells *in vitro* Colon 26 cells pretreated with IM7 or PBS alone were cultured *in vitro* in the presence of 10% serum of BALB/c mice plus 10% FBS or 20% FBS. IM7-pretreated cells grew well, as did control cells, and the presence of murine serum in the tissue culture medium did not inhibit proliferation of the antibody-treated cells (data not shown).

DISCUSSION

Colon 26 is a highly metastatic murine colonic adenocarcinoma cell line which is commonly used in experimental models of metastasis to the liver and lung.[21, 23] Intraportal inoculation of 1×10^5 colon 26 cells developed macroscopic hepatic tumors in 38% and 92% of mice on days 7 and 14, respectively (Table I, Fig. 1a). Injection of 1×10^4 colon 26 cells produced hepatic nodules in 20% of mice on day 7 (Table I). These data are similar to the results reported by Tominaga *et al.*[23] To obtain sufficient reproducibility we used injection of 1×10^5 cells for subsequent studies.

Cellular biological studies of colon 26 cells have shown the involvement of cell adhesion molecules in the course of metastasis. Kawakami *et al.* demonstrated that carbohy-

drate chains including Lewis^X antigen were involved in the hepatic implantation of colon 26 cells, by means of immunostaining of cancer cells.[5] Komazawa *et al.* suggested that the Arg-Gly-Asp-Ser (RGDS) structure, which is the cell-binding domain of fibronectin, is important as a ligand of integrins in the adhesion of colon 26 cells to extracellular matrix (ECM) proteins of the lung.[6] In the present study, colon 26 cells were confirmed to be intensely positive for CD44 (Figs. 2a and 4c) and treatment of the cells with an anti-CD44 mAb, IM7, inhibited the development of tumors in the liver (Figs. 1 and 6, Table II), suggesting the involvement of CD44 in hepatic metastasis.

Molecular cloning of CD44 has identified multiple variant isoforms that are generated by insertions of variant exons at a single site in the extracellular domain through alternative exon splicing.[24–26] It has been suggested that expression of variant isoforms of CD44 is associated with metastatic potential of cancer cells.[15, 16] Human normal colonocytes and most colorectal cancer cells express variant isoforms of CD44, including the epithelial isoform.[5, 14, 20] In contrast, colon 26 cells only expressed CD44H but not other isoforms, whereas murine normal colonocytes expressed possible variant isoforms (Fig. 3, lanes f and g). These data suggest that malignant transformation of murine colonic epithelium altered the expression pattern of CD44 isoforms. The exclusive expression of CD44H on colon 26 cells indicates that not the metastatic variant, but the hematopoietic isoform of CD44 is involved in the inhibitory effect of IM7 on metastasis formation.

CD44H has been characterized as the lymphocyte homing receptor as well as the cellular hyaluronate receptor.[9–12] Participation of CD44H in peritoneal implantation of human cancer cells has been described.[27, 28] The IM7 antibody, which is cross-reactive with both mouse and human CD44,[8] recognizes an epitope on the common region of the extracellular domain and thus reacts with all isoforms of CD44.[10] Since the epitope resides close to the hyaluronate-binding domain and IM7 blocks binding of CD44H to hyaluronate,[12, 29] the inhibition of metastasis by treatment of colon 26 cells with IM7 may depend on cell adhesion to hyaluronate. In the liver, however, hyaluronate and laminin are deficient in the space of Disse since hepatocytes and hepatic sinusoids only have an attenuated ECM consisting mostly of fibronectin and some type I collagen with no basement membrane structure, while epithelial and endothelial cells are usually lined by basement membranes with substantial extracellular matrix.[30–32] Production of acidic mucopolysaccharides including hyaluronate by colon 26 cells or hepatic cells was not detectable with alcian blue staining (Fig. 5c). Pretreatment of colon 26 cells with hyaluronidase did not affect either the viability of the cells or the development of hepatic metastasis (data not shown). Thus, it is not likely that the IM7 antibody inhibited attachment of colon 26 cells to hyaluronate to block implantation of the cells in the liver.

Intrahepatic growth of tumor cells is affected not only by direct cell-cell and cell-matrix contacts but also by growth factors released from hepatocytes and other cells in the hepatic microenvironment.[7] Two of the authors of this study, Mizoi and Ishii, have reported that expression of a novel variant of CD44 in a CD44-negative human colon cancer cell line altered the *in vivo* growth of the transformant cells, but did not increase the metastatic potential of the cells to the liver.[33] Similarly, Sleeman *et al.* showed CD44-dependent, but hyaluronate-independent metastatic behavior of a rat pancreatic cancer cell line.[34] Most recently, Weber *et al.* reported that osteopontin, a cytokine which is secreted by T cells, macrophages and other cells and induces cellular chemotaxis, is a ligand of CD44.[35] The authors demonstrated that the IM7 antibody blocked binding of osteopontin to CD44 expressed on a monocyte cell line by 97% and that osteopontin-induced cellular chemotaxis was inhibited by an anti-CD44 monoclonal antibody.[35] Since osteopontin has been shown to have a potential role in metastasis formation,[36] they suggested that osteopontin-induced cell migration may promote metastasis of CD44-positive tumor cells.[35] In our experiments, pretreatment of colon 26 cells with IM7 significantly delayed growth of the tumor cells in the liver (Figs. 1 and 6, Table II), whereas the antibody did not abrogate microscopic implantation. As Ishii *et al.* have shown, the metastatic potential of colorectal cancer cells is associated with the capacity of tumor cells to grow in the hepatic parenchyma after implantation.[3] Thus, the inhibitory effect of IM7 on the metastasis formation of colon 26 cells might be related not to CD44-mediated cell adhesion, but to other CD44-associated factors that could affect tumor cell growth after implantation in the liver.

When cancer cells are treated with antibodies reactive with cell surface antigens, *in vivo* growth of the cells may be inhibited by the antibody-dependent cell-mediated cytotoxicity and/or cytolysis by complements. Such non-specific cytotoxicity, however, did not seem to have affected the viability of the colon 26 cells treated with IM7 because 1) an anti-integrin β_1 mAb, 9EG7, which reacted with colon 26 cells more strongly than IM7, did not significantly block production of hepatic metastasis (Fig. 2 and Table II) and 2) pretreatment of colon 26 cells with IM7 did not inhibit *in vitro* proliferation of the cells even in the presence of murine serum (data not shown). These results suggest that pretreatment of colon 26 cells with IM7 inhibited hepatic metastasis not by non-specific cytotoxicity, but via a CD44-dependent mechanism.

In conclusion, the highly metastatic murine colon 26 adenocarcinoma cells intensely expressed the hematopoi-

etic isoform of CD44, while normal colonocytes expressed variant isoforms, and the growth of colon 26 cells in the liver was significantly inhibited by an anti-CD44 mAb. These results suggest that malignant transformation of murine colonic epithelium altered the expression pattern of CD44 isoforms and that CD44H participates in the intrahepatic growth of colorectal cancer cells after implantation in the liver.

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