

Homotypic Adhesion through Carcinoembryonic Antigen Plays a Role in Hepatic Metastasis Development

Toshiaki Yoshioka,^{1,5} Takashi Masuko,² Hitoshi Kotanagi,¹ Osamu Aizawa,¹ Yuri Saito,¹ Hiroshi Nakazato,³ Kenji Koyama¹ and Yoshiyuki Hashimoto⁴

¹Department of Surgery, Akita University School of Medicine, 1-1-1 Hondo, Akita 010, ²Department of Molecular Cell Biology, Pharmaceutical Institute, Tohoku University, Aramaki, Aoba-ku, Sendai 980, ³Suntory Institute for Biomedical Research, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618 and ⁴Sasaki Institute, 2-2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101

We established a cell line with high metastatic potential to the liver (LS-LM4) after four successive repetitions of splenic injection of liver-metastatic cells in SCID mice. This cell line strongly expressed CEA and showed increased homotypic adhesion as compared with the parent cell line (LS174T). To examine the role of CEA in the increased homotypic adhesion, LS-LM4 cells were treated with anti-CEA antibody and subjected to an *in vitro* adhesion and aggregation assay. Further, to study the role of CEA in the hepatic metastasis of cells with high metastatic potential, LS-LM4 cells were treated with anti-CEA antibody, and the inhibition of hepatic metastasis after splenic injection *in vivo* was examined. There was a 62% decrease in the homotypic adhesion of anti-CEA antibody-treated (100 $\mu\text{g/ml}$) LS-LM4 cells under a Ca^{2+} -free condition as compared with the control ($P<0.01$). Anti-CEA antibody (100 $\mu\text{g/ml}$) inhibited cell aggregation under a Ca^{2+} -free condition ($P<0.05$). Treatment with anti-E-cadherin antibody (60 $\mu\text{g/ml}$) plus anti-CEA antibody (100 $\mu\text{g/ml}$) inhibited cell aggregation more potently than anti-E-cadherin antibody treatment alone in the presence of Ca^{2+} . *In vivo*, there was a 75% decrease in the number of hepatic metastatic nodules in the G125 anti-CEA antibody-treated group as compared with the control group ($P<0.01$). Similarly, there was a 40% decrease in the diameter of metastatic nodules and there was a 90% decrease in total tumor volume of hepatic metastasis in the G125 anti-CEA antibody-treated group as compared with the control ($P<0.01$). These results suggest that increased metastatic potential to the liver is at least partly due to increased homotypic binding mediated by CEA.

Key words: Carcinoembryonic antigen — Homotypic cell adhesion — Hepatic metastasis — E-Cadherin

By means of repeated successive splenic injection or cecal wall injection of cells from hepatic metastasis in nude mice, cancer cells with high metastatic potential to the liver have been obtained.^{1, 2)} It has been shown that matrix metalloproteinases,²⁾ mucin,^{3, 4)} integrin,^{5, 6)} sialyl Le^x (sLe^x),^{7, 8)} platelet aggregating activity⁹⁾ and carcinoembryonic antigen (CEA)^{10–12)} promote hepatic metastasis. In addition, high expression of E-cadherin, a member of the cadherin family (E-, P-, N-cadherin and so on) which functions as a homotypic intercellular adhesion molecule,¹³⁾ favors hepatic metastasis.¹⁴⁾ CEA, which has been well characterized and widely used clinically as a tumor marker, was also recently classified as an adhesion molecule belonging to the Ig superfamily,¹⁵⁾ and reported to function as a homotypic intercellular adhesion molecule.^{16–20)} Cells transfected with cDNA for CEA formed aggregates and this aggregation was completely inhibited by anti-CEA antibody.^{16, 17)} Furthermore, the transfectants developed liver metastases after splenic injection,¹⁸⁾ and the metastasis was inhibited by anti-CEA antibody.^{19, 20)} These results suggest that CEA plays an important role in the process of hepatic metastasis.

In this study we established a cell line with high metastatic potential to the liver by repeated successive splenic injection of cells that metastasized to the liver in SCID mice. This cell line strongly expressed CEA and showed increased homotypic adhesion as compared with the parent cell line. To determine the role of CEA in the increased homotypic adhesion, LS-LM4 cells were treated with anti-CEA antibody and assayed for adhesion and aggregation activity *in vitro*. Further, to determine the role of CEA in the hepatic metastasis of cells with high metastatic potential, LS-LM4 cells were treated with anti-CEA antibody, and the inhibition of hepatic metastasis after splenic injection *in vivo* was examined. In order to rule out a role of E-cadherin in homotypic binding through CEA, we also performed a cell aggregation assay after anti-E-cadherin antibody treatment.

MATERIALS AND METHODS

Cancer cell line and mice Human colon cancer LS174T cells²¹⁾ were used in this study. They were maintained in RPMI1640 medium (Nissui, Tokyo), supplemented with L-glutamine, NaHCO_3 , kanamycin and 10% fetal bovine serum (UBC, Tokyo) at 37°C in a 5% CO_2 atmosphere. Male severe combined immunodeficiency (SCID) mice at

⁵To whom reprint requests should be addressed.

ten weeks old (FOX CHASE C.B17/Icr- Scid, Jcl, Clea Japan, Tokyo) were used in this study. They were maintained in a laminar flow cabinet under pathogen-free conditions. The protocols for the animal experiments described in this paper were approved by the Animal Research Committee, Akita University School of Medicine. All animal experiments adhered to the Guidelines for Animal Experimentation of the University.

Experimental metastasis Metastatic potential to the liver was determined by using the intrasplenic injection assay described by Kozlowski *et al.*¹⁾ Cells were harvested from subconfluent monolayer cultures by 10–15 min treatment with 75 $\mu\text{g/ml}$ Actinase (Kaken Seiyaku, Tokyo) and 0.02% EDTA (Wako Pure Chem., Osaka) under a Ca^{2+} -free condition. After having been washed in phosphate-buffered saline (PBS), cells were suspended in a medium composed of equal amounts of RPMI1640 medium and PBS. A single cell suspension with greater than 90% cell viability was used for the intrasplenic injection. Cell viability was determined by means of the trypan blue exclusion test. Mice were anesthetized with 50 mg/kg of pentobarbital (Abbott Lab., Chicago, IL) given intraperitoneally, and the spleen was exteriorized via a small abdominal incision. Viable cells ($2 \times 10^6/50 \mu\text{l}$ of medium) were injected into the spleen with a 27 gauge needle. The spleen was returned to the abdominal cavity and the wound was closed. Mice were observed every day and moribund mice were killed and autopsied. Hepatic tumor, when present, was resected, minced and dissociated with 1000 pu/ml of Dispase (Godo Shusei, Tokyo) and 500 u/ml of DNase (Takara Shuzo, Tokyo) for 30 min at 37°C. The cells were washed, seeded in tissue culture dishes coated with type I collagen (Koken Co., Tokyo) and kept at 37°C in a 5% CO_2 environment. Cells were passaged and samples were stored at -80°C. Viable cells (2×10^6) within three passages were injected into the spleen of SCID mice. For estimation of metastatic potential, we measured the size and number of macroscopic hepatic tumors, and the tumor volume was calculated by using the formula: Tumor volume = $\sum (\text{diameter of nodule}/2)^3 \times 4\pi/3$. The tumor volume index was calculated by using the formula: Tumor volume index = (tumor volume) / (interval between splenic injection and autopsy).

In vitro growth rate Each cell line was plated in triplicate at a density of 3×10^5 cells/60 mm plastic culture dish (Falcon Plastics, Oxnard, CA). The number of viable cells/dish was determined every 24 h for 4 successive days. A growth curve was constructed for each cell line to permit calculation of the cell doubling time.

Changes in expression of cell surface antigens We used the following 6 antibodies for the analysis of changes in expression levels of cell surface antigens: anti-sialyl Le^a (sLe^a) antibody (2D3, Seikagaku Co., Tokyo), anti-sLe^x antibody (CSLEX1, Becton Dickinson, San Jose, CA),

anti-CEA antibody (4C6, MBL, Nagoya), anti-E-cadherin antibody (SHE78-7 and HECD-1, Takara Shuzo), anti-neural cell adhesion molecule (NCAM) antibody (NCAM16.2, Becton Dickinson), anti-VLA (very late activated antigen) β -1 (CD29) antibody (k20, MBL).

Expression levels of cell surface antigens were analyzed using a flow cytometer (EPICS Elite ESP, Coulter, Miami, FL). Cells (2×10^5) were treated with 0.05% trypsin and 0.02% EDTA under a Ca^{2+} -free condition and incubated with 5 $\mu\text{g/ml}$ of antibody for 30 min at 4°C. For the analysis of E-cadherin expression, cells were treated with 0.01% crystallized trypsin in the presence of 0.5 mM CaCl_2 and 0.01% EDTA at 37°C for 30 min. The cells were washed with Ca^{2+} - and Mg^{2+} -free 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)-buffered (pH 7.4) Hanks balanced salt solution (CMF-HBSS), then incubated with CMF-HBSS containing 1% bovine albumin (Sigma, St. Louis, MO) (1% bovine albumin-CMF-HBSS), 2 mM CaCl_2 and 5 $\mu\text{g/ml}$ of anti-E-cadherin antibody on ice. We used 1% bovine albumin-CMF-HBSS in the presence of 2 mM CaCl_2 continuously for the following experiment on E-cadherin expression. The cells were washed and further incubated with anti-mouse immunoglobulin G (IgG) antibody coupled with FITC (Dako A/S, Tokyo) in the dark for 30 min on ice. They were again washed and sieved through a 40 μm pore mesh, then a total of 1×10^4 cells was analyzed. Cells were defined as positive when their fluorescence intensity was above the cut-off level. This was set at 2% less than the maximum level in the control. Control cells were incubated with anti-mouse IgG antibody. We compared the histogram and percentage of positive cells between cells with high metastatic potential (LS-LM4) and their parent cells (LS174T).

Cell adhesion assay Cells (3×10^5) were treated with 0.1% trypsin and 0.02% EDTA in a Ca^{2+} -free condition for 10–15 min at 37°C. The cells were labeled with 30 μCi of $\text{Na}_2^{51}\text{CrO}_4$ and incubated with 1% bovine albumin-CMF-HBSS. The radio-labeled cells were added to unlabeled monolayer cells with anti-CEA antibody (1, 10, 50 or 100 $\mu\text{g/ml}$) or anti-mouse IgG antibody as a control. Monolayer cells were prepared by seeding 1.2×10^5 cells/well on 96-well microtiter plates 24 h before the experiment. Inhibition of homotypic cell adhesion was assessed using two monoclonal anti-CEA antibodies. G125 antibody, which recognizes the N-domain of CEA, is reported to inhibit homotypic CEA-CEA binding.²²⁾ E117 antibody, which recognizes domain III of CEA, is reported not to inhibit CEA-CEA binding.²²⁾ After incubation at 37°C in a 5% CO_2 environment for 1 h with 1% bovine albumin-CMF-HBSS, the cells were washed three times. Adherent cells were subsequently harvested by trypsin and EDTA treatment, and radioactivity was determined in an Auto-well gamma counter (Aloka, Tokyo). The experiment was done in quadruplicate.

The cytotoxicity of anti-CEA antibodies was examined by the WST-1 method.²³⁾ Cells (5×10^4 /well) were incubated with anti-CEA antibodies at 50, 100 or 500 $\mu\text{g/ml}$ at 37°C for 2 h, then washed and further incubated with 10 μl of WST-1 (Dojindo Lab., Kumamoto) at 37°C for 1 h to 48 h. The absorbance of each well was measured at a wavelength of 450 nm (reference wavelength: 650 nm) on an Immuno Plate Reader (International Reagents Co., Tokyo) by enzyme-linked immunosorbent assay at 1, 2, 4, 12, 24 and 48 h later.

Cell aggregation assay Cadherin- and CEA-mediated cell aggregation was assayed by the method described by Takeichi,²⁴⁾ using LS-LM4 cells. The cells were treated with 0.01% crystallized trypsin in the presence of 0.5 mM CaCl_2 with 0.01% EDTA at 37°C for 30 min and then washed with CMF-HBSS to obtain a single cell suspension. These cells were suspended in CMF-HBSS with 0, 0.4 or 1 mM CaCl_2 and with anti-CEA antibody (G125 or E117) or anti-E-cadherin antibody (SHE78-7, which inhibits homotypic cell binding through E-cadherin)²⁵⁾ at various concentrations (G125, 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$; E117, 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$; SHE78-7, 30 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$). The suspension was placed in a 24-well plate and incubated to allow aggregation for 30 min at 37°C on a gyratory shaker rotating at 80 rpm. The aggregates were photographed using a phase contrast microscope (Olympus, Tokyo). The degree of cell aggregation was represented by the aggregation index,²⁶⁾ N30/N0 , where N0 is the total number of particles (single cells plus cell clusters) per well at 0 min of incubation and N30 is that at 30 min of incubation. The experiment was done in triplicate.

Inhibition of hepatic metastasis Cells (2×10^6) with high metastatic potential (LS-LM4 cells) were treated with 0.1% trypsin and 0.02% EDTA under a Ca^{2+} -free condition for 10–15 min at 37°C. The cells were washed twice with CMF-HBSS, then incubated with anti-mouse IgG antibody (control), G125 antibody or E117 antibody (each 300 $\mu\text{g/ml}$) at 4°C for 1 h. Cells (2×10^6) treated with 50 μl of antibody were injected into the spleen of SCID mice. The mice were killed 18 days after the injection. We measured the number and size of macroscopic metastatic

tumors in the liver, as well as the weight of the liver and the spleen.

Statistics The significance of differences between the mean values within groups was tested by using an unpaired two group *t* test (Student's *t* test or Welch's *t* test) with $P < 0.05$ as the criterion of significance. Differences between mean values of different groups were tested by one-way analysis of variance (ANOVA), followed by either Fisher's protected least significant difference or Dunnett's *t* test. All calculations were performed on a Macintosh microcomputer using "Microsoft Excel" and "Stat View" software.

RESULTS

Establishment of cells with high metastatic potential to the liver All SCID mice ($n=5$) that received intrasplenic injection of LS174T cells developed hepatic metastasis. Cells from the hepatic tumors were further injected into the spleen of SCID mice, and after 4 cycles, cells with high metastatic potential (LS-LM4 cells) were established. The number of metastatic tumors generated by LS-LM4 cells was increased about 2-fold as compared with LS174T cells, and the size of the metastatic tumors generated by LS-LM4 cells was increased 1.7-fold (Table I). The tumor volume and tumor volume index were both markedly increased in the case of LS-LM4 cells. Doubling times *in vitro*, however, were not different.

Changes in expression levels of cell surface antigens Expression levels of 2 (CEA and sLe^a) of 6 cell surface antigens were increased significantly in LS-LM4 cells. The percentage of CEA-positive LS-LM4 cells (56%) was much higher than that of LS174T cells (30%). sLe^a expression was positive in 30% of LS-LM4 cells and 11% of LS174T cells. Expression of sLe^x, E-cadherin or VLA β -1 was observed in more than 90% of cells, but there was no significant difference between the two cell lines. The results for E-cadherin expression analyzed with SHE78-7 and HECD-1 antibody were similar. NCAM expression was minor, and no significant difference was observed between the two cell lines (Fig. 1).

Table I. Hepatic Metastasis and Doubling Time in LS174T and LS-LM4 Cells

Cell line	Doubling time <i>in vitro</i> (h)	Hepatic metastasis				
		Incidence ^{a)}	Mean number of nodules	Mean diameter of nodules (mm)	Mean tumor volume ^{b)} (mm ³)	Mean tumor volume index ^{c)}
LS174T	29.5	5/5	36	2.6	545	17.2
LS-LM4	29.6	5/5	73	4.4	3060	120.0

a) Number of mice with hepatic metastasis/number of mice which received splenic injection.

b) Tumor volume: $(\text{diameter of nodule}/2)^3 \times 4\pi/3$, summed for all nodules.

c) Tumor volume index: tumor volume/interval between splenic injection and autopsy (days).

Inhibition of homotypic cell adhesion Homotypic cell adherence of LS-LM4 cells was significantly higher than that of LS174T cells ($P<0.01$, Fig. 2). G125 antibody significantly inhibited homotypic adhesion of LS-LM4 cells in a dose-dependent manner (Fig. 2). The radioactivity was 62% less than the control when cells were treated with 100 $\mu\text{g/ml}$ of G125 antibody under a Ca^{2+} -free condition ($P<0.01$). E117 antibody did not inhibit homotypic cell binding (only data for 100 $\mu\text{g/ml}$ of E117 antibody are shown). The G125 and E117 antibodies showed no cytotoxicity as evaluated by the WST-1 method (data not shown).

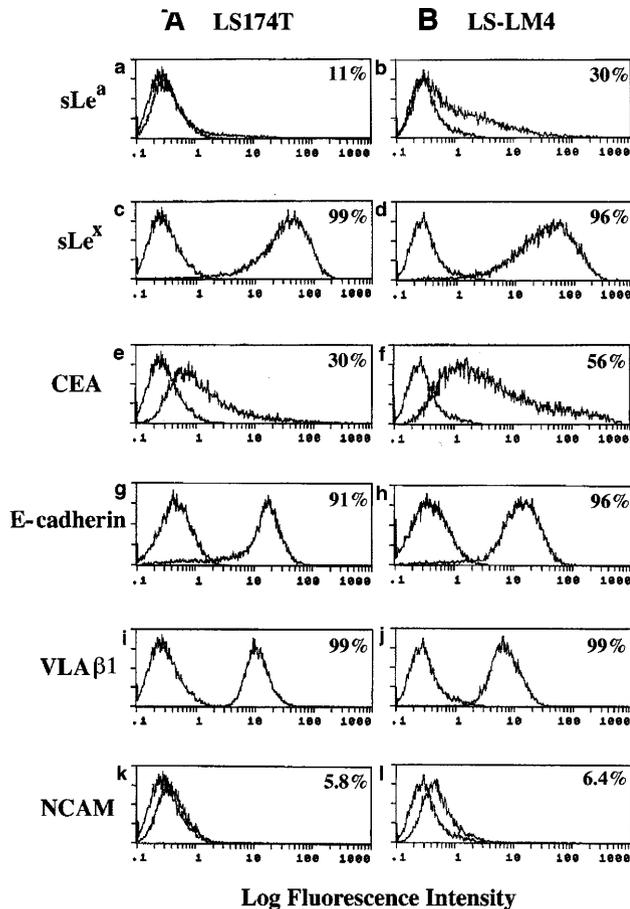


Fig. 1. Histogram and percentage of positive cells for 6 adhesion molecules in LS174T cells and LS-LM4 cells. The expression levels of the 6 cell surface antigens were analyzed using flow cytometry. In every histogram, the plot on the left is the control. The cut-off level is 2% less than the maximum level of the control. A (LS174T), a, c, e, g, i and k. B (LS-LM4), b, d, f, h, j and l. sLe^a, a and b; sLe^x, c and d; CEA, e and f; E-cadherin, g and h; VLA β 1, i and j; NCAM, k and l. Vertical axis, number of cells; horizontal axis, log fluorescence intensity.

Inhibition of cell aggregation Under a Ca^{2+} -free condition, G125 antibody inhibited cell aggregation. The values of aggregation index after treatment with 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$ of G125 antibody were 0.12 and 0.17 greater than the control, respectively ($P<0.05$, Fig. 3A). E117 antibody did not inhibit cell aggregation (only data for 200 $\mu\text{g/ml}$ of E117 antibody are shown). A phase contrast microscope revealed many aggregates composed of more than ten cells in the control and E117 antibody treatment groups. The contact area was small. The aggregates in the control were decreased in number and size by G125 antibody treatment (Fig. 3B-a, b and c). Addition of Ca^{2+} caused cells to adhere tightly and form huge aggregates whose contact areas were larger than that of the control. The values of the aggregation index after treatment with 0.4 mM Ca^{2+} and 1 mM Ca^{2+} were 0.32 and 0.47 less than that of the control under a Ca^{2+} -free condition, respectively. Anti-E-cadherin antibody (SHE78-7) inhibited the cell aggregation. Values of the aggregation index after treatment with 30 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$ of SHE78-7 antibody were 0.36 and 0.47 greater than that in the control at 1 mM Ca^{2+} , respectively ($P<0.01$). Large aggregates were not observed, but some aggregates of more than ten cells remained, though the contact areas were small. Anti-E-cadherin antibody (60 $\mu\text{g/ml}$) plus G125 antibody (100 $\mu\text{g/ml}$) inhibited the number of cell aggregates more markedly than anti-E-cadherin antibody alone at 1 mM Ca^{2+} . The aggregation index after treatment with both anti-E-cadherin antibody and G125 antibody was 0.12 greater than that after treatment with anti-E-cadherin antibody

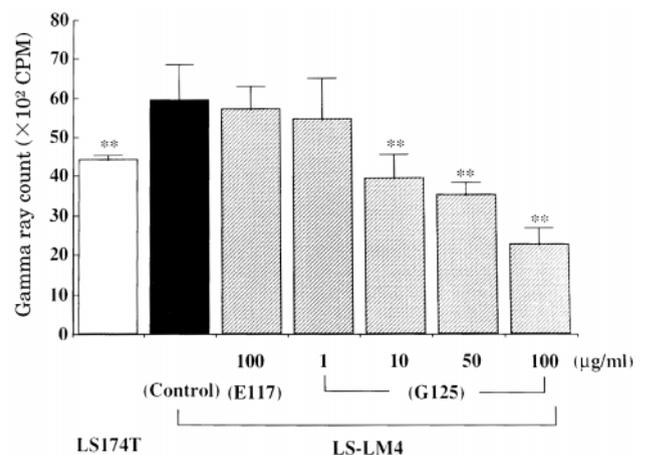


Fig. 2. Cell adhesion assay with anti-CEA antibody. Cells were labeled with $\text{Na}_2^{51}\text{CrO}_4$. LS-LM4 cells were incubated with anti-CEA antibody (G125 or E117) at 1, 10, 50 or 100 $\mu\text{g/ml}$, or anti-mouse IgG antibody (control). Adherent cells were harvested and their radioactivity was measured in an Autowell gamma counter. Bars; standard deviation. ** $P<0.01$ vs. control and E117 antibody treatment.

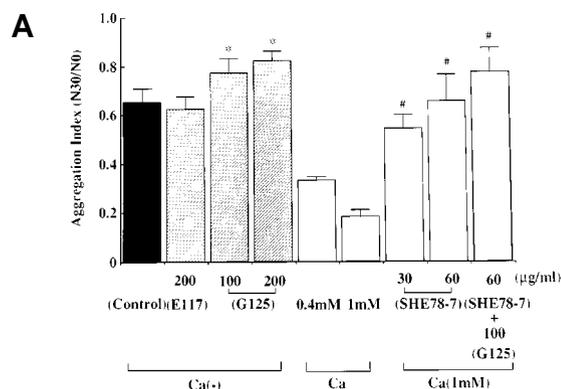


Fig. 3. Cell aggregation assay using LS-LM4 cells with anti-CEA antibody (G125 or E117) and anti-E-cadherin antibody (SHE78-7) at the indicated concentration or anti-mouse IgG antibody (control) in the presence or absence of Ca²⁺. A, Aggregation represented by aggregation index. Bars, standard deviation, * $P < 0.05$ vs. control (Ca²⁺-free condition), # $P < 0.01$ vs. control (1 mM Ca²⁺). B, Photographs of cells under each condition using a phase contrast microscope ($\times 100$). a, b and c, Ca²⁺-free condition; d, e and f, 1 mM Ca²⁺. a, Control; b, E117 (200 µg/ml); c, G125 (200 µg/ml); d, control; e, SHE78-7 (60 µg/ml); f, SHE78-7 (60 µg/ml) and G125 (100 µg/ml) antibody treatment.

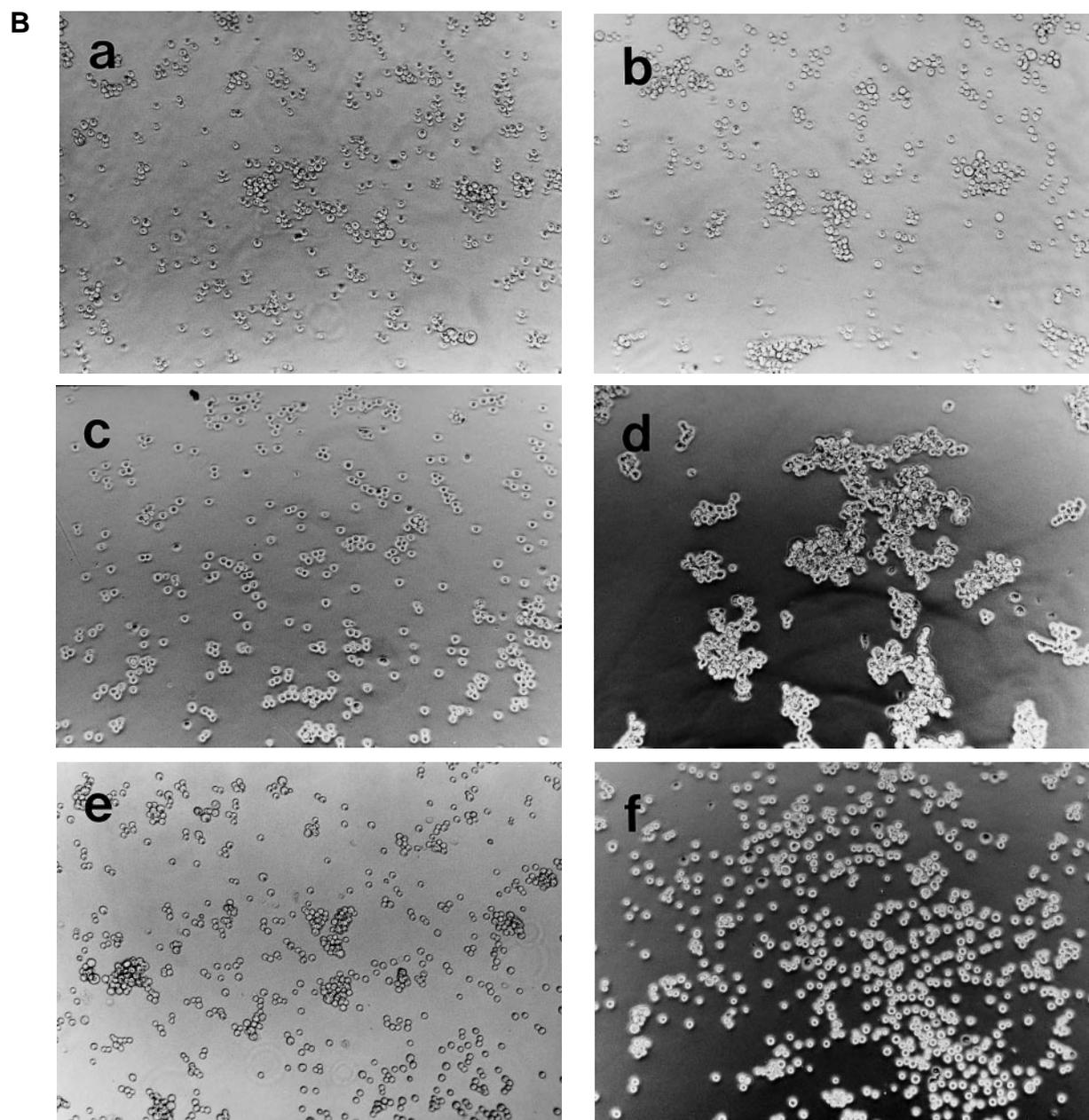


Table II. Inhibition of Hepatic Metastasis of LS-LM4 Cells by Anti-CEA Antibody

Antibody	Hepatic metastasis					Weight of ^{b)} spleen (g)
	Incidence ^{a)}	Weight of ^{b)} liver (g)	Number of nodules	Diameter of nodules (mm)	Tumor volume ^{c)} (mm ³)	
Control ^{d)}	5/5	1.63±0.36	115±52.2	1.29±0.17	191±85.0	1.25±0.44
E117 ^{d)}	6/6	1.67±0.22	149±20.5	1.19±0.10	204±67.6	1.03±0.50
G125 ^{d)}	6/6	1.32±0.13	29±23.0	0.77±0.17	17.7±20.8	1.13±0.26

a) Number of mice with hepatic metastasis/number of mice which received splenic injection.

b) Weight of normal liver, 1.30±0.16 g; weight of normal spleen, 0.045±0.01 g.

c) Tumor volume: (diameter of nodule/2)³ × 4 π/3, summed for all nodules.

d) Antibody concentration was 300 μg/ml or 50 μl/mouse body. Mice were killed 18 days after the intrasplenic injection.

* P<0.05, ** P<0.01. Values are means±SD.

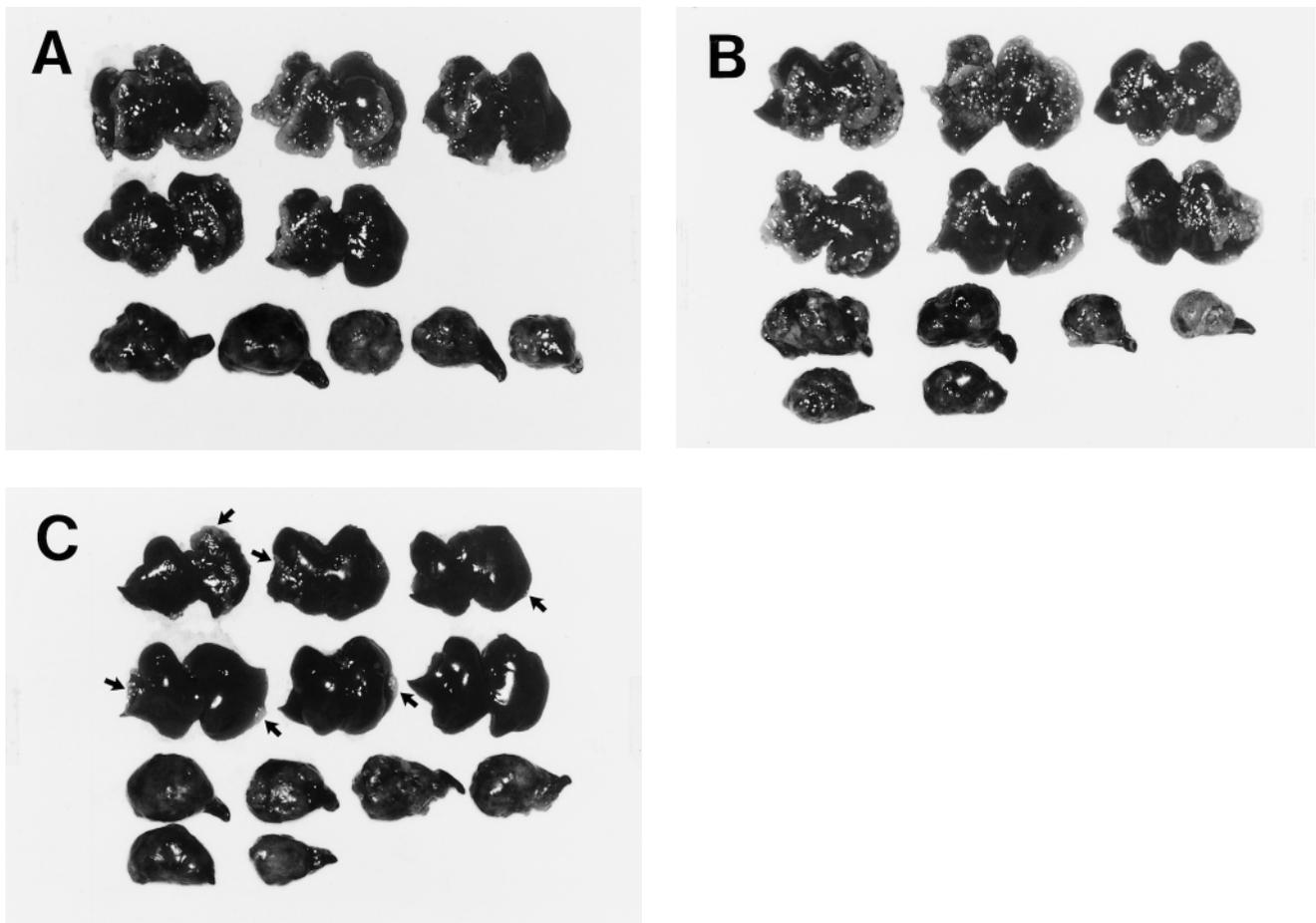


Fig. 4. Hepatic metastasis and spleen after splenic injection of LS-LM4 cells treated with anti-CEA antibody (G125 or E117) or anti-mouse IgG antibody (control). Each antibody was used at 300 μg/ml and 50 μl/mouse. A, control; B, E117; C, G125 antibody treatment. Arrows indicate nodules of hepatic metastasis in C.

alone and 0.59 greater than that in the control ($P < 0.01$). The aggregates after treatment with anti-E-cadherin antibody were decreased in number and size by anti-E-cadherin antibody plus G125 antibody treatment (Fig. 3A, 3B-d, e and f).

Inhibition of hepatic metastasis There was no significant difference among the groups treated with control, E117 or G125 antibody in incidence of hepatic metastasis or in liver weight. However, the number and diameter of nodules and total tumor volume of hepatic metastasis were significantly different between the group treated with G125 antibody and the control. There was a 75% decrease in the number of hepatic metastatic nodules in the G125 antibody-treated group as compared with the control group ($P < 0.01$). Similarly, a 40% decrease in the diameter of metastatic nodules and a 90% decrease in total tumor volume of hepatic metastasis were seen in the G125 antibody-treated group as compared with the control ($P < 0.01$). There were significant differences in the number and diameter of nodules and total tumor volume of hepatic metastasis between the group treated with G125 antibody and that with E117 antibody. There was no significant difference in spleen weight among the three groups (Table II, Fig. 4).

DISCUSSION

We established cells with high metastatic potential to the liver (LS-LM4) by successive repetitions of splenic injection of liver-metastatic cells in SCID mice. The tumor volume and tumor volume index were increased with LS-LM4 cells, even though the *in vitro* doubling times of LS-LM4 cells and the parental LS174T cells were similar. This suggests that LS-LM4 cells colonized the liver in greater numbers than LS174T cells. To investigate the factors which promote colonization to the liver, we assessed the changes in expression of cell surface antigens and in homotypic cell binding between cells with enhanced metastatic potential (LS-LM4) and their parent cells (LS174T).

Among cell surface antigens whose expression was changed between the two cell lines, we were interested in CEA, because its expression was increased remarkably in LS-LM4 cells. Our finding is in good agreement with the fact that high CEA-producing cancer cell lines generally have higher potential of hepatic metastasis as compared with low CEA-producing cancer cell lines.¹¹ CEA has been shown to act as an intercellular adhesion molecule which promotes cell aggregation via homotypic interactions.^{16, 17} We therefore examined whether anti-CEA antibody would inhibit the homotypic binding and hepatic metastasis. So far, little is known about the relationship between metastatic potential and homotypic binding mediated by CEA in cell lines obtained after serial passage via hepatic metastasis.

E-Cadherin loses its function easily upon enzyme treatment in a Ca^{2+} -free condition, but not in the presence of Ca^{2+} .²⁷ By treatment with 0.1% trypsin and 0.02% EDTA at 37°C for 10–15 min under a Ca^{2+} -free condition, the domain of E-cadherin which mediates homotypic cell adhesion was degraded,²⁸ and we confirmed that cells treated under this condition could not aggregate and could not bind to anti-E-cadherin antibody in flow cytometry in the presence of 2 mM CaCl_2 (data not shown). The cell adhesion assay in this study and the experiments on hepatic metastasis were done under this condition, so it is presumed that E-cadherin did not influence the results.

As judged by cell aggregation assay, LS-LM4 cells were not completely separated into single cells by treatment with 0.01% trypsin and 0.01% EDTA in the presence of 0.5 mM CaCl_2 . Phase contrast microscopy revealed some adhesion through dead cells, so we used a modified aggregation index. The results obtained after various antibody treatments again suggested that the aggregation through CEA was independent of that through E-cadherin. The extent of aggregation through CEA presumably depends on the number of cells which express CEA and the number of molecules of CEA per cell. Though the aggregation mediated by CEA was less than that mediated by E-cadherin, the aggregation through CEA clearly played a key role in the hepatic metastasis under the conditions of this study. Nevertheless, some other adhesion system(s) might be involved since the aggregation of this cell line was not completely prevented by anti-CEA antibody and anti-E-cadherin antibody in combination.

Our finding that G125 antibody, but not E117 antibody, inhibited the homotypic binding of LS-LM4 cells, is consistent with the study by Hashino *et al.*, who used CHO cells.¹⁹ The data indicate that the N-domain of CEA is important for homotypic binding. Updike and Nicolson²⁹ have shown that increased homotypic intercellular binding favors the metastatic process, probably because cell aggregates breaking away from the primary tumor have a greater chance than single cells of evading the immune surveillance systems and surviving in the circulation to lodge eventually in other organs, resulting in frequent and large metastasis.

Kupffer cells, liver fixed macrophages, are responsible for detection and removal of tumor cells and modulating the host immune response to the early stage of hepatic metastasis. However, this important role of Kupffer cells seems to be limited in extent and capacity and only applicable to a restricted number of tumor cells arriving at the liver.³⁰ The cells do carry a specific binding protein for CEA and clear it from the circulation.³¹ We found that anti-CEA antibody did not influence the cell growth *in vitro*. It also had no effect *in vivo* because there was no significant difference in the spleen weight among the three

groups, which means that complement-mediated or antibody-dependent cell-mediated cytotoxicity was not involved in the inhibition of hepatic metastasis. We speculate that clusters of less than about ten cells were removed by Kupffer cells, but that larger clusters survived in the liver.

We examined other adhesion molecules which have been reported to be possibly involved in the process of cancer metastasis. However, there was no significant difference in the expression of sLe^x, VLAβ-1 or NCAM antigen between LS-LM4 cells and LS174T cells.

Both sLe^a and sLe^x antigens, which serve as ligands for adhesion of cancer cells to the endothelial leukocyte adhesion molecule-1 (ELAM-1) on vascular endothelial cells,^{32, 33)} are reportedly involved in liver metastasis.^{7, 8)} In this study, expression of sLe^x was not changed between the two cell lines, though more than 90% of the cells expressed sLe^x. Expression of sLe^a was higher in LS-LM4 cells than in LS174T cells. However, the percentage of positive cells was low even in LS-LM4 cells. These data suggest that the increase of sLe^a expression may not have been a major factor in the development of hepatic metastasis in this study.

In this study, we did not investigate the possible role of the cadherin-catenin complex system.¹³⁾ There is a tendency for cells with epithelial morphology (differentiated tumors) to maintain high levels of E-cadherin.³⁴⁾ In our case, more than 90% of LS174T and LS-LM4 cells ex-

pressed E-cadherin, in accordance with the fact that LS174T cells are a differentiated colon cancer cell line. Though high expression of E-cadherin is related to hepatic metastasis,¹⁴⁾ it may not account for the acquired metastatic potential of LS-LM4 cells in this study, because there was no significant difference of E-cadherin expression between LS-LM4 cells and LS174T cells.

In conclusion, we showed that increased homotypic binding of LS-LM4 cells is mediated by CEA, and anti-CEA antibody inhibited the hepatic metastasis of these cells with high metastatic potential. The results suggest that the increased metastatic potential to the liver is related to the increased homotypic binding through CEA. Thus, cancers with high levels of CEA expression may have a high probability of developing hepatic metastasis.

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