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Selection of Human Ovarian Carcinoma Cells with High Dissemination Potential by Repeated Passage of the Cells *in vivo* into Nude Mice, and Involvement of Le^x-determinant in the Dissemination Potential

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Cells of the human tumor cell line RMG-1, derived from a clear-cell adenocarcinoma of the ovary, were injected intraperitoneally into nude mice, and the cells obtained from the tumor nodules in the mesenterium were found to form a larger number of, and larger-sized, tumor nodules than the original RMG-1 cells. The RMG-1-h cells, transferred into culture from the tumor nodules after a 4th *in vivo* passage, showed a dissemination potential as high as that of cells disseminating directly from the tissues, and exceedingly higher than that of RMG-1 cells. To assess the molecular bases of the different biological properties of RMG-1 and RMG-1-h cells, we compared the content and expression of various carbohydrate antigens in both cells. The chromosomal profile of RMG-1-h cells revealed their human origin and was identical to that of the original RMG-1 cells. In contrast to the broad histogram for the Le^x-bearing cells among RMG-1 cells in flow cytometry, the weakly and moderately positive cells toward anti-Le^x antibody were found to be eliminated from the histogram for the RMG-1-h cells, resulting in the enrichment of cells strongly expressing Le^x, which may account for the high dissemination potential. In addition, the adhesion of RMG-1 cells to mesothelial cells was found to be significantly inhibited by pretreatment of the cells with anti-Le^x antibody, indicating Le^x-mediated cell-to-cell interaction between ovarian cancer cells and mesothelial cells. By TLC-immunostaining, two Le^x-glycolipids, $III^3Fuc\alpha$-nLc_4Cer and $V^3Fuc\alpha$-nLc_6Cer were detected in both RMG-1 and RMG-1-h cells, and their total concentrations were not significantly different from each other. However, the hydrophobic moieties of Le^x-glycolipids in RMG-1-h cells were different from those in RMG-1 cells, suggesting that a difference in the structure of the hydrophobic moieties of Le^x is partly involved in the enhanced reactivity of RMG-1-h cells toward anti-Le^x antibody. Thus, the high dissemination potential of ovarian cancer cells was shown to be mediated by the Le^x-determinant and the Le^x-bearing cells are enriched by repeated *in vivo* passage of the cells into nude mice.

Key words: Glycolipid — TLC-immunostaining — Cell adhesion — Flow cytometry

Suppression of dissemination and metastasis, using chemotherapy and surgery, is an important issue in the treatment of gynecologic cancers, but the molecular mechanisms involved in dissemination and metastasis have not been extensively studied.[1, 2] In the case of ovarian tumors, a high peritoneal dissemination potential is frequently used as an indicator of malignancy. Once the tumor cells are dispersed in the abdominal cavity, they form solid tumors that are disseminated widely in the mesenterium and the peritoneum, making treatment more difficult. We have assumed that malignant cells with a high dissemination potential tend to interact with the mesenterium and the peritoneum by the expression of molecules related to cell-to-cell interaction. In fact, we recently compared GSL compositions on the surfaces of cells from benign and malignant ovarian tumors, and found that the difference in composition was closely related to the malignant potential of the cells and to their histological type.[3] GSLs play a role in cellular recognition and receptor function, and alterations of their synthesis and membrane organization occur in relation to cellular differentiation, proliferation, oncogenic transformation, and metastatic processes.[4] To analyze the molecular mechanism of dissemination, we have recently

Abbreviations used are: GSL, glycosphingolipid; FCS, fetal calf serum; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; BSA, bovine serum albumin; Hex, hexose; HexNAc, N-acetylhexosamine; IL-1, interleukin-1.

developed a procedure for evaluating the correlation between Le^x-expression and the degree of peritoneal dissemination *in vivo* after the intraperitoneal injection of cancer cells into nude mice.[5] Using the above procedure, we observed that repeated *in vivo* passage of ovarian cancer cells resulted in increases of their potential for peritoneal dissemination. A possible contribution of carbohydrate antigens to the adhesion of ovarian cancer cells to peritoneal mesothelial cells was also demonstrated in this study.

MATERIALS AND METHODS

Cell lines of ovarian cancer The ovarian cancer cell line RMG-1 was established with cells obtained from the tumor tissue of a patient with clear cell adenocarcinoma of the ovary, in our laboratory,[6] and another cell line RTSG, was established with cells obtained from the pleural fluid of a patient with poorly differentiated mucinous cystadenocarcinoma of the ovary.[7]

***In vivo* passage of tumor cells and transfer to culture** The RMG-1 cells cultured in a plastic dish were suspended with a cell scraper and injected intraperitoneally into 5 nude mice (1×10^7 cells/mouse). Eight weeks after the inoculation, the animals were killed under anesthesia. The numbers of dissemination sites in the mesenterium and peritoneum were counted, and the tumor size was measured using microcalipers. Tumor nodules excised from the animals were dissected in FCS-free Ham's F12 medium, and incubated in the same medium containing 0.25% collagenase at 37°C for 1 h, followed by incubation in a medium containing 0.25% trypsin at 37°C for 30 min. After filtration through a mesh (150 μm), the cells were collected by centrifugation at 700*g* for 2 min, and cultured on a plastic dish in Ham's F12 medium supplemented with 10% FCS.

Establishment of a cell line with a high dissemination potential Tumor nodules formed in the mesenterium and the peritoneum of individual nude mice were removed and, after dissection and filtration through a mesh (150 μm), they were implanted into the abdominal cavity of recipient nude mice. The tumor cells obtained from the nodules of the nude mice after the fourth *in vivo* passage were transferred for culture into Ham's F12 medium with 10% FCS, and the cells established from the cell line, the RMG-1-h cells, were found to maintain a dissemination potential as high as that of a cell suspension prepared directly from the tumor nodule.

Preparation and quantitation of GSLs GSLs from RMG-1 and RMG-1-h cells were prepared according to the method reported previously.[8] Briefly, the cells were suspended in water and then lyophilized. Lipids were extracted from the lyophilized powder with chloroform/methanol/water (20:10:1 and 10:20:1, v/v/v) and chloroform/methanol (1:1, v/v) at 45°C. The extracts were combined and the lipid-bound phosphorus and cholesterol contents in the total lipid extracts were determined using Bartlett's method[9] and gas-liquid chromatography with 5α-cholestane as the internal standard,[8] respectively. The total lipids were fractionated into neutral and acidic fractions using DEAE-Sephadex A-25 (acetate form) column chromatography. Neutral GSLs were prepared from the neutral lipid fraction by acetylation, Florisil column chromatography, and deacetylation,[10] and acidic GSLs were prepared from the acidic lipid fraction by mild alkaline hydrolysis for the cleavage of the ester-containing lipids, followed by dialysis. The acidic and neutral GSLs thus obtained were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) and chloroform/methanol/water (65:35:8, v/v/v), respectively, and visualized with orcinol-H_2SO_4 reagent. Lipid-bound sialic acid in the acidic GSL fraction was measured by the resorcinol-HCl method.[11] The concentration of each neutral GSL was determined densitometrically at an analytical wavelength of 420 nm and a control wavelength of 710 nm using a dual-wavelength TLC densitometer (CS-9000; Shimadzu Co., Kyoto) after visualization of the spots with orcinol-H_2SO_4 reagent. A standard curve for quantitation was prepared with 0.2 to 2 μg of GalCer from human brain, on the same TLC plate. The concentration of lipid-bound sialic acid was also determined densitometrically after visualization of the spots with resorcinol-HCl reagent and the density of the spots was determined at an analytical wavelength of 580 nm and a control wavelength of 710 nm.

Purification of GSLs The individual neutral GSLs were further purified using an Iatrobeads (6RS8060; Iatron Lab., Tokyo) column with a gradient of chloroform/isopropyl alcohol/water (85:15:0.2, v/v/v and 40:60:2, v/v/v) for mono- to trihexaosylceramides, followed by a gradient of chloroform/methanol/water (70:30:4, v/v/v and 10:90:4, v/v/v) for polar GSLs. The homogeneity of the isolated GSLs was examined by TLC with orcinol-H_2SO_4 reagent and by TLC-immunostaining with anti-blood group monoclonal antibodies.[12–14] Monoclonal anti-Le^a and anti-Le^b antibodies were purchased from Biotest Diagnostics (Frankfurt, Germany), and monoclonal anti-Le^x (NCC-LU-279) and anti-Le^y (NCC-ST433) antibodies were kindly donated by Dr. S. Hirohashi, National Cancer Center (Tokyo).[13]

TLC-immunostaining Neutral GSLs were chromatographed on plastic-coated TLC plates (Art Z12277-7; Sigma, St. Louis, MO) with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) and the plates were incubated with a blocking buffer (1% PVP, 1% ovalbumin, and 0.02% NaN_3 in PBS) at 37°C for 1 h and then with approximately 0.2 μg/ml of monoclonal anti-Le^a, anti-Le^b, anti-Le^x and anti-Le^y antibodies in 3% PVP in PBS at

37°C for 2 h. The plates were washed 5 times with 0.1% Tween 20 in PBS, and the bound antibodies were detected using peroxidase-conjugated anti-mouse IgM antiserum (Cappel Laboratories, Cochraville, PA), diluted 1:1000 (v/v) with 3% PVP in PBS and with the enzyme substrates H_2O_2 and 4-chloro-1-naphthol, as described previously.[12)]

Structural analysis of GSLs by FABMS The purified GSL was subjected to negative ion FABMS (JMS HX-110; JEOL Ltd., Tokyo) as follows. The GSL (5 μg) dissolved in 5 μl of chloroform/methanol (1:1, v/v) was mixed with 5 μl of triethanolamine on a sample holder for FABMS. Analysis was performed by bombardment with a neutral xenon beam having a kinetic energy of 4 keV, and the negative ions were detected by mass spectrometry.[15)] Mass number assignment was achieved by comparing the spectrum with that of perfluoroalkyl phosphazine (Ultra Mark; PCR Laboratories, Gainesville, FL).

Flow-cytometric analysis of cells stained with anti-Le^x antibody For comparison of the levels of Le^x expression on the cell surfaces of RMG-1 and RMG-1-h cells, the cells (5×10^6) were incubated with 5% (w/v) BSA in PBS for 30 min on ice, and with 20 μl of anti-Le^x antibody diluted serially in 5% BSA in PBS (50 ng, 25 ng and 12.5 ng). After having been washed 3 times with 500 μl of PBS, the cells were stained with 50 μl of fluorescein-conjugated goat anti-mouse IgM antiserum (usually diluted 1:10) at 4°C for 40 min and washed 3 times with 500 μl of PBS. After the removal of aggregated cells with a nylon filter (100 μm), fluorescence histograms of the stained cells were obtained with a flow cytometer (FACStar; Becton Dickinson, Mountain View, CA).

Assay of adhesion of human ovarian carcinoma-derived cells to mesothelial cells To identify the molecules involved in the adhesion of human ovarian carcinoma-derived cells to mesothelial cells, RMG-1 or RTSG (poorly differentiated adenocarcinoma of human ovary) cells were labeled with 5 (and 6)-carboxyfluorescein diacetate,[5)] and added to an ELISA plate bearing a mesothelial cell monolayer derived from omental tissue at a final concentration of 2.0×10^6 cells per ml (0.1 ml per well) in RPMI 1640 medium with 1% FCS. Prior to the reaction, the cells were pretreated with either monoclonal

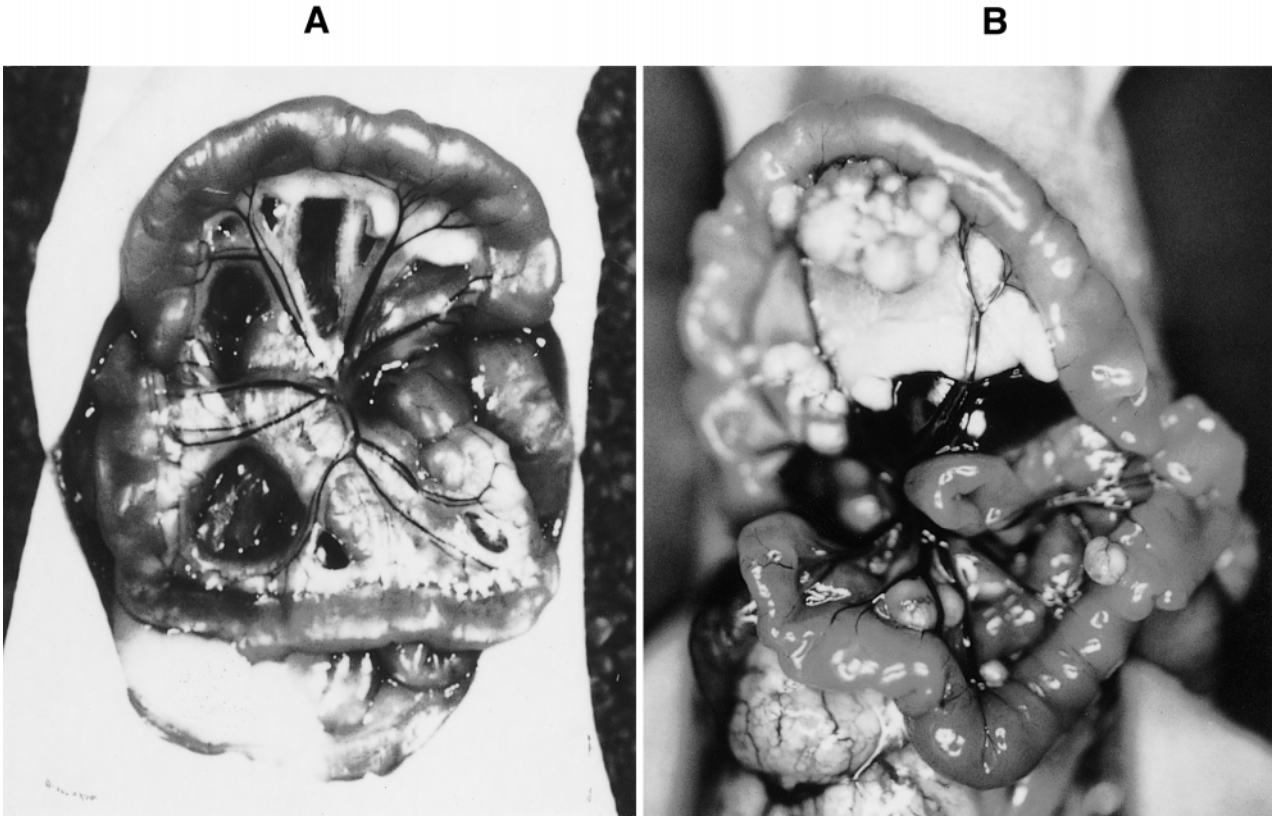

Fig. 1. Formation of tumor nodules after intraperitoneal injection of cells of human ovarian carcinoma-derived cell lines, RMG-1 (A), and RMG-1-h cells (B), obtained from a culture established from the cells of tumor nodules formed in the mesenterium after the 4th *in vivo* passage into nude mice. The formation of tumor nodules was examined 8 weeks after the injection of cells (5×10^6).

Table I. Formation of Tumor Nodules in the Abdominal Cavity of Nude Mice after Intraperitoneal Injection of Human Ovarian Tumor Cells, RMG-1 and RMG-1-h

	RMG-1	RMG-1-h
Rate of formation of tumor nodules	12/21	11/11
Number of nodules	2.2±2.8	6.0±3.6
Total size of nodules (mm^3)	330.8±546.3	15283.7±17870.4

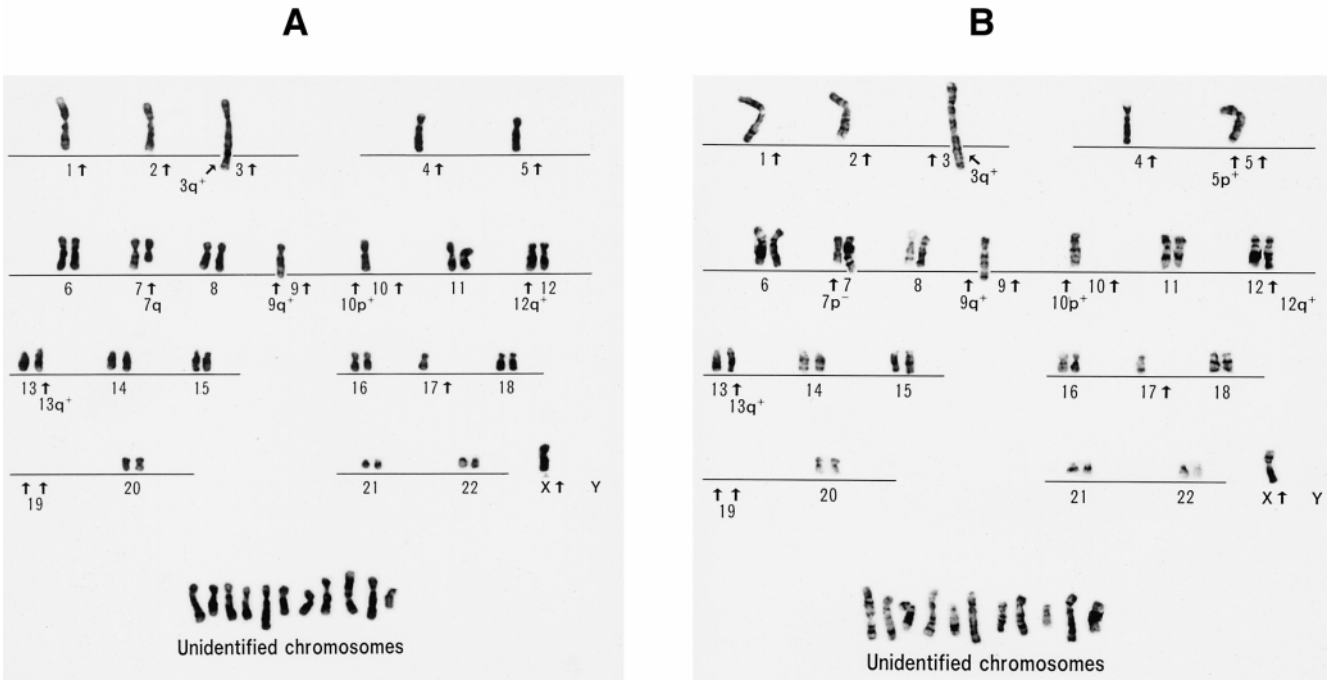

Fig. 2. Chromosomal analysis of RMG-1 (A) and RMG-1-h (B) cells.

anti-Le^x antibody or murine IgM (1 μg) at 4°C for 2 h. After incubation at 37°C for 20 min, the wells were filled with the same medium, sealed with tape, inverted, and centrifuged at 150*g* for 5 min. The fluid containing the nonadherent cancer cells was discarded, and the fluorescence intensity of each well was measured with an ELISA reader.

RESULTS

Establishment of a tumor cell line with high dissemination potential by repeated *in vivo* passaging After injection of RMG-1 cells, derived from a human ovarian carcinoma-derived cell line, into the abdominal cavity of nude mice, solid tumor nodules formed occasionally in the mesenterium. These nodules were dissected to make cell suspensions, which were injected into the abdominal cavities of other nude mice. The repeated *in vivo* passage of these cells into the abdominal cavities of the mice resulted in an increase in the rate of formation and the size of tumor nodules, and this property was maintained at the same level as that of a cell suspension made directly from a tumor nodule, even after the transfer of the cells into culture (Fig. 1). The cell line, RMG-1-h, established from cells derived from tumor nodules after the 4th *in vivo* passage, exhibited a potential for the formation of a larger number of, and larger-sized, nodules than the original RMG-1 cells in all the mice tested (Table I). In contrast to the formation of tumor nodules in 57% of mice after the injection of RMG-1 cells, tumor nodules appeared in all the mice injected with RMG-1-h cells, without exception. Thus, the dissemination potential of human ovarian tumor cells, when measured in terms of the rate of formation of tumor nodules after intraperito-

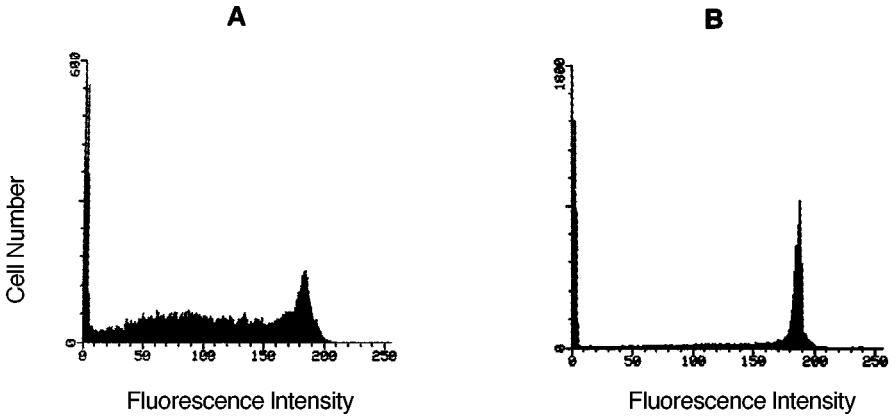

Fig. 3. Fluorescence histograms of RMG-1 (A) and RMG-1-h (B) cells stained with murine monoclonal anti-Le^x antibody. Cells (5×10^6) were stained with 12.5 ng of monoclonal anti-Le^x antibody in 20 μl of 5% BSA in PBS, followed by fluorescein-labeled anti-murine IgM antiserum (1:20) under the conditions described in the text.

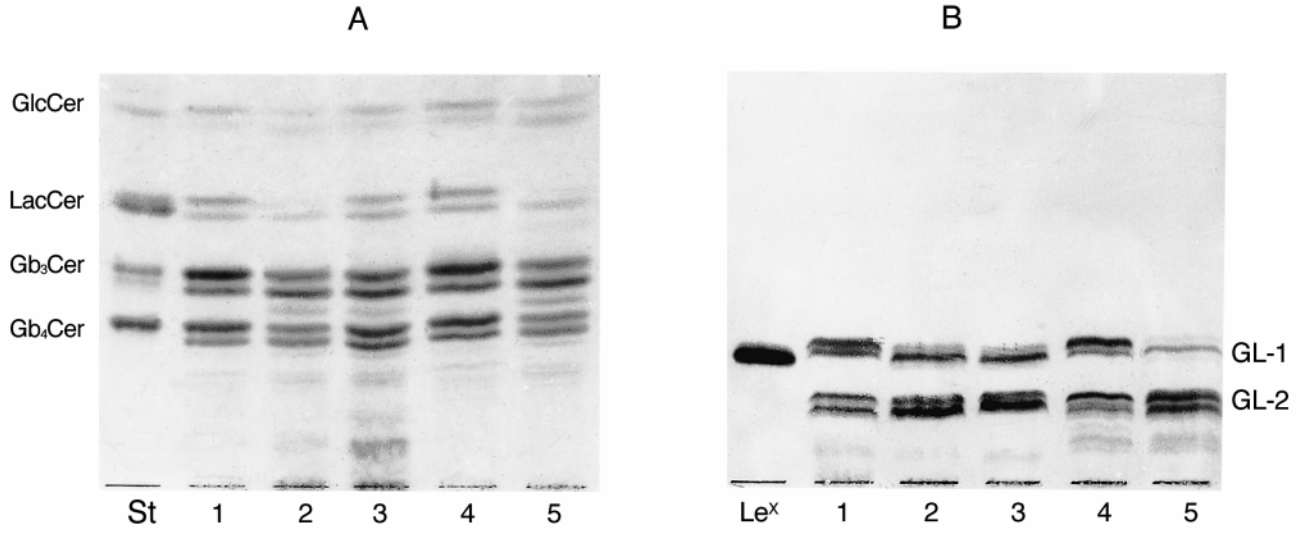

Fig. 4. TLC (A) and TLC-immunostaining (B) of neutral GSLs from RMG-1 (lanes 1 and 4) and RMG-1-h cells (lanes 2, 3 and 5). Neutral GSLs, corresponding to 0.5 mg of dry tissue weight, were chromatographed on a plastic-coated TLC plate with chloroform/methanol/water (65:35:8, v/v/v) and the spots were visualized with orcinol-H_2SO_4 reagent for (A) and with monoclonal anti-Le^x antibody (5 ng/ml) for (B). St, standard glycolipids mixture, GlcCer, LacCer, Gb_3Cer and Gb_4Cer from the top; Le^x, III^3Fucα-nLc_4Cer. Glycolipids, GL-1 and GL-2, were positive with monoclonal anti-Le^x antibody.

neal injection into nude mice, was significantly increased, probably due to the selection of tumor cells with a high dissemination potential during repeated *in vivo* passages.

Comparison of chromosomes between RMG-1 and RMG-1-h cells As shown in Fig. 2, the number of chromosomes in RMG-1-h cells was the same as that in RMG-1 cells, being 46, and their chromosomal profiles were essentially the same, confirming that the RMG-1-h cells established from cells obtained from tumor nodules in the mesenterium of nude mice after the 4th *in vivo* passage, are human-derived cells. The abnormalities of individual chromosomes, as distinct from those in normal human cells, were as follows: $3q^+$, $7q^-$, $9q^+$, $10p^+$, $12q^+$ and $13q^+$ for RMG-1 cells, and $3q^+$, $5p^+$, $7p^-$, $9q^+$, $10p^+$, $12q^+$ and $13q^+$ for RMG-1-h cells.

Expression of Le^x in RMG-1 and RMG-1-h cells Origi-

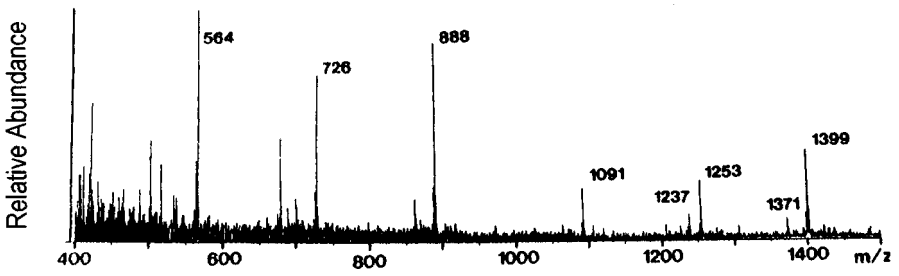

Fig. 5. Negative-ion FABMS of glycolipid (GL-1) stained with monoclonal anti-Le^x antibody, from RMG-1-h cells. About 5 μg of glycolipid was used for the analysis.

Table II. Concentrations of Various GSLs in RMG-1 and RMG-1-h Cells

Glycolipid	Concentration (μg/mg of dry weight)	
	RMG-1	RMG-1-h
CMH-1	0.31	0.11
CMH-2	0.12	0.20
LacCer-1	0.34	0.06
LacCer-2	0.24	0.15
Gb_3Cer-1	1.14	0.66
Gb_3Cer-2	0.56	0.72
Gb_4Cer-1	0.79	0.44
Gb_4Cer-2	0.44	0.45
III^{3}Fucα-nLc_4Cer-1	0.06	0.00
III^{3}Fucα-nLc_4Cer-2	0.03	0.04
V^{3}Fucα-nLc_6Cer-1	0.07	0.05
V^{3}Fucα-nLc_6Cer-2	0.03	0.09

Glycolipids 1 and 2 represent the upper and lower bands corresponding to the molecules with lignoceric and stearic acids, respectively.
Mean values for three samples are shown. Standard deviations were within 10%.

nal RMG-1 cells and the newly established RMG-1-h cells were stained using the indirect immunofluorescence technique with murine monoclonal anti-Le^x antibody. In contrast to the broad histogram for the Le^x-bearing cells in the RMG-1 cells, the weakly and moderately positive cells toward anti-Le^x antibody were found to have been eliminated from the histogram for the RMG-1-h cells, which consisted predominantly of strongly positive cells (Fig. 3), indicating that the high dissemination potential of RMG-1-h cells is closely related to the expression of Le^x, and the cells expressing Le^x strongly are selected by the repeated *in vivo* passaging. TLC-immunostaining with anti-Le^x antibody revealed two glycolipids having the Le^x-determinant (GL-1 and GL-2) in the neutral glycolipid fraction from both RMG-1 and RMG-1-h cells (Fig. 4), but the concentrations of GL-1 and GL-2 were less than 5% of the total concentration of neutral GSLs and were not significantly different between RMG-1 and RMG-1-h cells. The structures of GL-1 and GL-2 were confirmed by negative-ion FABMS, after purification using Iatrobeads column chromatography. The glycolipid GL-1 purified from RMG-1-h cells (lane 3 in Fig. 4) yielded the molecular ion at *m*/*z* 1399, corresponding to III^{3}Fucα-nLc_4Cer with stearoylsphingosine in the ceramide moiety, and fragment ions lacking one Hex and one fucose residue were detected at *m*/*z* 1237 and 1253, respectively, indicating that Hex and fucose occupy the nonreducing terminals of the carbohydrate chain (Fig. 5). Furthermore, fragment ions due to cleavage at the glycosidic linkages were obtained as follows: (ceramide-Hex-Hex-HexNAc)$^-$ at *m*/*z* 1091, (ceramide-Hex-Hex)$^-$ at *m*/*z* 888, (ceramide-Hex)$^-$ at *m*/*z* 726 and (ceramide)$^-$ at *m*/*z* 564. Hence, the structure of GL-1 was concluded to be Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1′ceramide, III^{3}Fucα-nLc_4Cer. Similarly, the glycolipid GL-2 (lane 3 in Fig. 4) was identified as Galβ1-4(Fucα-1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′-ceramide, V^{3}Fucα-nLc_6Cer. Both III^{3}Fucα-nLc_4Cer and V^{3}Fucα-nLc_6Cer exhibited two bands on TLC-immunostaining due to a difference in their fatty acid composition, the upper and lower bands being composed primarily of lignoceric and stearic acids, respectively. As clearly presented in Fig. 4, the relative concentrations of lignoceric acid-containing III^{3}Fucα-nLc_4Cer and V^{3}Fucα-nLc_6Cer were higher than those of their stearic acid-containing counterparts in the original RMG-1 cells, whereas the stearic acid-containing GSL molecules were the major molecular species in RMG-1-h cells. The concentration of stearic acid-containing Le^x-glycolipids in RMG-1-h cells was approximately twice that in RMG-1 cells, though the total concentration of Le^x-glycolipids in RMG-1-h cells was similar to that in RMG-1 cells (Table II). A similar difference in molecular species was observed with respect to neutral GSLs with two to four carbohydrate chains,

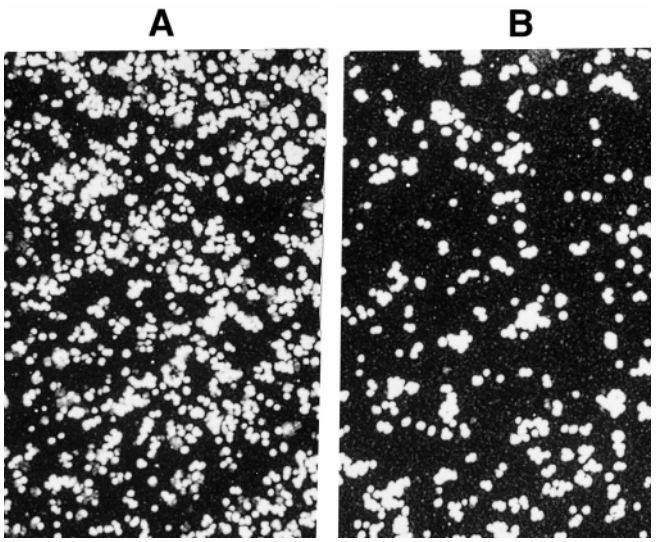

Fig. 6. Fluorescence micrographs of RMG-1 cells adhering to the mesothelial cell monolayer. RMG-1 cells stained with 5(6)-carboxyfluorescein diacetate were reacted with the mesothelial cell monolayer after treatment of RMG-1 cells with murine control IgM (A) and murine anti-Le^x antibody (B).

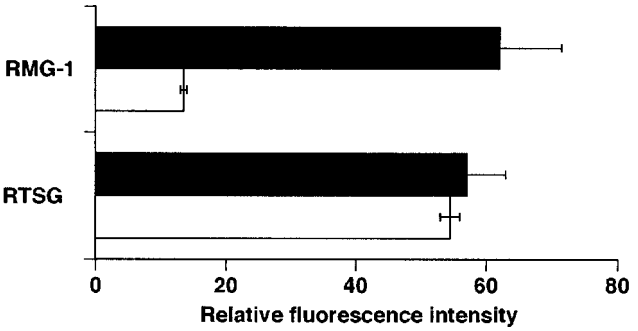

Fig. 7. Inhibition of cell adhesion of RMG-1 and RTSG cells to the mesothelial cell-monolayer with murine control IgM (closed column) and murine anti-Le^x antibody (open column). The relative fluorescence intensity of cells adhering to the mesothelial cell monolayer was measured with an ELISA reader.

between RMG-1 and RMG-1-h cells. The concentrations of lignoceric acid- and stearic acid-containing glycolipids were consistently higher in the RMG-1 and RMG-1-h cells, respectively, and hence, the higher concentration of stearic acid-containing Le^x-glycolipids in RMG-1-h cells seems to account for the enhanced expression of the Le^x-determinant, as determined by flow-cytometric analysis. On the other hand, Le^y, Le^a, Le^b, sialyl Le^a and sialyl Le^x were not detected by TLC-immunostaining using carbohydrate-specific antibodies, or even after spotting GSLs corresponding to 5 mg of dry tissue weight.

Assay of adhesion of RMG-1 cells to mesothelial cells

To explore the involvement of Le^x in the adhesion of RMG-1 cells to mesothelial cells, we carried out an inhibition assay of the adhesion with monoclonal anti-Le^x antibody. As shown in Figs. 6 and 7, the extent of adhesion of RMG-1 cells after treatment with anti-Le^x antibody was reduced to 23% compared to that after treatment with control immunoglobulin. However, no inhibition of adhesion of RTSG cells, which were negative toward anti-Le^x antibody, to mesothelial cells was observed after pretreatment with anti-Le^x antibody under the same conditions. A similar inhibitory effect of anti-Le^x antibody was observed in the adhesion of RMG-1-h cells to mesothelial cells. Thus, the adhesion of RMG-1 and RMG-1-h cells, but not of RTSG cells, to mesothelial cells was shown to be largely mediated by Le^x-determinant, accounting for the high dissemination potential of RMG-1-h cells with their higher expression of Le^x.

DISCUSSION

We demonstrated that cells from a human ovarian carcinoma cell line, established from cells recovered from tumor nodules in the mesenterium of nude mice, showed a higher potential for dissemination than the cells originally injected for the formation of these tumor nodules, and that repeated passage of the cells *in vivo* resulted in the increase in the dissemination potential. The RMG-1-h cells from a cell culture established from cells derived from the tumor nodules after the 4th *in vivo* passage formed tumor nodules in all the nude mice examined. The tumor nodules that formed in the abdominal cavity after injection of RMG-1-h cells were more numerous and larger in size than those formed after the intraperitoneal injection of RMG-1 cells, suggesting an enhanced dissemination potential of the RMG-1-h cells due to selection of cells with a higher propensity for adhesion or invasion. From the peritoneal dissemination model using mesothelial cells and ovarian cancer cells,[5] RMG-1 cells were found to be of the adhesion type, the number of dissemination sites being much higher than that of invasive-type cell lines. This finding is consistent with clinical observations, and indicates that dissemination is a characteristic of clear cell ovarian carcinoma.

In order to identify the molecules that are involved in the adhesion of cancer cells to the peritoneum, comparison of cell surface molecules on RMG-1 and RMG-1-h cells should be a useful approach, and should yield information on the biological properties of clear cell ovarian carcinoma. In particular, the expression of glycoconjugate molecules on the cell surface is frequently found to be associated with the metastatic phenotype,[16, 17] as well as with other cellular functions and characteristics, such as adhesiveness,[2, 18] immunogenicity, immune recognition

processes,[19–22] induction of platelet aggregation,[23, 24] and invasion.[25] Among the glycoconjugate molecules related to the above functions, sialyl Le^x [26] and sialyl Le^a [27, 28] antigens expressed on the surface of cancer cells have been found to be involved in the processes of vascular invasion and hematogenous metastasis. Sialyl Le^x antigen is known to be expressed frequently on lung and ovarian cancer cells, and to a lesser extent, on digestive organ cancer cells.[29] On the other hand, sialyl Le^a antigen is expressed on cancer cells of the pancreas and other digestive organs much more frequently than sialyl Le^x antigen,[27, 28] and is probably involved in the hematogenous metastasis of cancer cells originating in these organs.[29] However, we did not detect sialyl Le^x and sialyl Le^a antigens in the acidic GSLs of RMG-1 and RMG-1-h cells by TLC-immunostaining and flow cytometry. Instead, neutral GSLs with the Le^x determinant, $III^3Fuc\alpha$-nLc_4Cer and $V^3Fuc\alpha$-nLc_6Cer, were found in RMG-1-h cells, on which the level of Le^x expression was also shown to be significantly higher than that on RMG-1 cells by flow-cytometric analysis. In addition, the adhesion of RMG-1 and RMG-1-h cells to mesothelial cells was shown to be inhibited by monoclonal anti-Le^x antibody, while the antibody was unable to inhibit the adhesion of RTSG cells (without Le^x) to mesothelial cells. These observations clearly demonstrated that the Le^x-determinant is implicated in the adhesion of ovarian cancer cells.

When the histogram of RMG-1-h cells obtained by flow cytometry is compared with that of RMG-1 cells, the moderately positive cells toward anti-Le^x antibody, which comprised half of the RMG-1 cells, were effectively eliminated from the RMG-1-h cells by the repeated passage of the cells in the abdominal cavity of nude mice, resulting in the concentration of highly positive cells in the RMG-1-h cells. The Le^x determinant is known to be contained in glycolipids and glycoproteins, but no procedure for accurate quantitation of Le^x in the glycoproteins has been established yet, and accordingly we determined the concentration and the structure of Le^x-glycolipids. As shown in Table II, the total concentration of Le^x-glycolipids in RMG-1-h cells was not much different from that in RMG-1 cells, though the relative concentration of Le^x-glycolipids with stearic acid in RMG-1-h cells was significantly higher than that in RMG-1 cells, being comprised 100% and 33% of $III^3Fuc\alpha$-nLc_4Cer, and 64% and 30% of $V^3Fuc\alpha$-nLc_6Cer in RMG-1-h and RMG-1 cells, respectively. In addition, all GSLs from ceramide monohexoside to ceramide pentahexoside, as well as Le^x, in RMG-1-h cells were composed primarily of N-stearoylsphingosine, in contrast to the observation that the major ceramide of GSLs in RMG-1 cells was N-lignoceroylsphingosine. Although the relationship between the structure of the ceramide and the reactivity of the carbohydrate is not yet fully understood, and the involvement of glycoproteins bearing Le^x in its expression on the cell surface is unclear, our observations that RMG-1-h cells were stained more intensely than RMG-1 cells indicated that an increase in the concentration of shorter-chain fatty acid-containing Le^x contributed in part to the high exposure of the carbohydrate antigen in RMG-1-h cells. In addition, the more intense staining of RMG-1-h cells than RMG-1 cells with anti-Le^x antibody was attributed to the higher concentration of $V^3Fuc\alpha$-nLc_6Cer in RMG-1-h cells, suggesting that the longer carbohydrate chain is susceptible to binding with the antibody, as well as with receptor molecules in mesothelial cells. Since adhesion of RMG-1 cells to mesothelial cells was shown to be mediated by Le^x, it is possible that RMG-1 cells with a high expression of the Le^x-determinant readily diffused into the mesenterium and peritoneum to form solid tumor nodules, and *in vivo* passage resulted in the selection of these Le^x-bearing cells with a high dissemination potential.

The receptor for the Le^x-related antigens has been identified as ELAM-1,[30] a cell adhesion molecule expressed on the surface of cytokine-activated human endothelial cells. Cytokines such as IL-1 and tumor necrosis factor-α induce the expression of ELAM-1 on the surface of human endothelial cells,[31–33] and ELAM-1 is thought to be involved in the recruitment of leukocytes to stimulate the vascular endothelium during inflammation. On the other hand, the expression of Le^x-related antigens on the surface of cancer cells is suggested to occur during hematogenous metastasis of cancer cells.[26–28] However, since the above adhesion molecules so far identified have an affinity for sialylated or sulfated Le^x-structure, it seems likely that other molecules with an affinity for Le^x are also involved in the dissemination process. The selection of cancer cells with high expression of carbohydrate ligands might contribute to high risk of hematogenous metastasis and peritoneal dissemination in patients.

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