GM3 Ganglioside Inhibits CD9-Facilitated Haptotactic Cell Motility: Coexpression of GM3 and CD9 Is Essential in the Downregulation of Tumor Cell Motility and Malignancy†

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ABSTRACT: A cooperative inhibitory effect of GM3, together with CD9, on haptotactic cell motility was demonstrated by a few lines of study as described below. (i) Haptotactic motility of colorectal carcinoma cell lines SW480, SW620, and HRT18, which express CD9 at a high level, is inhibited by exogenous GM3, but not by GM1. (ii) Motility of gastric cancer cell line MKN74, which expresses CD9 at a low level, was not affected by exogenous GM3. Its motility became susceptible to and inhibited by exogenous GM3, but not GM1, when the CD9 level of MKN74 cells was converted to a high level by transfection with CD9 cDNA. Findings i and ii suggest that haptotactic tumor cell motility is cooperatively inhibited by coexpression of CD9 and GM3. (iii) This possibility was further demonstrated using cell line ldlD 14, and its derivative expressing CD9 through transfection of its gene (termed ldlD/CD9). Both of these cell lines are defective in UDP-Gal 4-epimerase and cannot synthesize GM3 unless cultured in the presence of galactose (Gal^+), whereas GM3 synthesis does not occur when cells are cultured in the absence of Gal (Gal^-). Haptotactic motility of parental ldlD cells is low, and shows no difference in the presence and absence of Gal. In contrast, the motility of ldlD/CD9 cells is very high in Gal^- whereby endogenous GM3 synthesis does not occur, and is very reduced in Gal^+ whereby endogenous GM3 synthesis occurs. (iv) Photoactivatable ^3H-labeled GM3 added to HRT18 cells, followed by UV irradiation, causes cross-linking of GM3 to CD9, as evidenced by ^3H labeling of CD9, which is immunoprecipitated with anti-CD9 antibody. These findings suggest that CD9 is a target molecule interacting with GM3, and that CD9 and GM3 cooperatively downregulate tumor cell motility.

Cell motility is a basic cell physiological process, involving many factors and mechanisms (for a review, see ref *1* and the Discussion). Factors which modulate cell motility play a major role in defining pathobiological processes such as tumor metastasis, inflammatory response, and wound healing. Only a few studies have appeared regarding the effect of gangliosides on cell motility (*2*, *3*).

During our search for mAbs[1] directed to cell surface molecules and capable of modulating cell motility rather than adhesion, a few mAbs were cloned and cell surface molecules involved in cell motility were identified (*4*). Along this line of study, motility-regulatory protein (MRP) was identified by its mAb M31−15, selected on the basis of its inhibitory effect on tumor cell motility. The amino acid sequence of MRP was found to be identical to that of CD9 (*5*). In subsequent studies, CD9 (MRP) expression and its down-regulation were correlated with tumor metastasis, malignancy, and patient survival in human breast cancer (*6*), colon carcinoma (*7*), and melanoma (*8*). CD9 is one of the best characterized tetraspan membrane proteins (TM4SF, or tetraspanin) (*9*), and interacts with other transmembrane proteins such as integrins and other tetraspanins (*10*−*13*) to form complexes which facilitate cell adhesion (*14*−*16*), cell motility (*17*−*19*), and signaling (*20*). Expression of CD9 at the egg cell membrane plays an essential role in the gamete fusion process for fertilization, as demonstrated in CD9-deficient mice (*21*, *22*).

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[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Gal^+, medium with Gal; Gal^-, medium without Gal (i.e., ITS medium); GSL, glycosphingolipid; IP, immunoprecipitation; ITS, insulin−transferrin−selenium; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TM4SF, transmembrane-4 superfamily (same as tetraspanin); GM3, NeuAcα2→3Galβ1→4Glcβ1→1Cer. Other gangliosides such as GM1, GM2, etc., are abbreviated according to Svennerholm's list (*62*).

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None of these previous studies paid attention to ganglioside cofactors which modulate CD9 function. We report here a functional cooperative effect of ganglioside GM3 and CD9 on haptotactic cell motility, based on three lines of study: (i) the effect of exogenously added GM3 on human tumor cell lines with high versus low levels of CD9 expression, (ii) the effect of endogenous GM3 expression in mutant ldlD 14 cells expressing CD9 (when these cells are cultured in galactose-containing medium, only GM3 synthesis occurs) and (iii) the direct interaction of GM3 with CD9, using photoactivatable GM3.

MATERIALS AND METHODS

Cell Culture, Antibodies, Reagents, and Flow Cytometry. Human colon cancer cell lines SW480, SW620, and HRT18 (all from ATCC) and differentiated human gastric cancer cell line MKN74 (*23*) (donated by M. Adachi, Japan Immunoresearch Laboratories, Takasaki, Japan) were cultured in DMEM with 10% FBS. Cell line ldlD 14, a CHO cell mutant deficient in UDP-Glc 4-epimerase, originally established by Krieger and colleagues (*24*), was kindly donated by M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). The cell line was maintained, and the glycosylation pattern was altered by addition of Gal and/or GalNAc in serum-free insulin−transferrin−selenium (ITS) medium (Collaborative Biomedical Products, Bedford, MA) as described previously (*25*). Briefly, cells were maintained in Ham's F-12 medium supplemented with 5% FBS. For observation of an effect of Gal and/or GalNAc, the medium was changed on day 1 to Ham's F-12 plus ITS without FBS and with or without Gal (20 μM) and/or GalNAc (200 μM). The effect of glycosylation on cellular function was determined on day 3.

Anti-human CD9 mouse mAb M-L13 was from Pharmingen (San Diego, CA). Matrigel was from Collaborative Biomedical Products. Antibodies used for IP of integrin receptors in human colon carcinoma HRT18 cells were as follows: anti-β1 mAb ZH1 (*26*), anti-α3 mAb P1B5, and anti-α6 mAb G0H3 (from Pharmingen). Anti-GM3 mouse mAb DH2 (*27*) was established in this laboratory. GM3 having *N*-acetylneuraminic acid was prepared from dog erythrocytes (*28*), purified by high-performance liquid chromatography on Iatrobeads 6RS-8010, and shown to be free of contaminants. GM1 was prepared from bovine brain and free of contaminants. The levels of expression of CD9 and GM3 in human cancer, ldlD, and its transfectants were determined by flow cytometry on EPICS XL, using specific mAbs and reagents with control cells treated with mouse IgG or IgM, as described previously (*29*). The intensity of antigen expression was indicated by "expression index", i.e., the mean fluorescence intensity of specific antigen divided by the mean fluorescence intensity of control Ig.

Preparation of CD9 cDNA and Its Transfection into MKN74 and ldlD Cells. cDNAs for CD9 were generated by RT-PCR using human placental RNA (Clontech). The PCR product was cloned into the pCDNA3 plasmid (Invitrogen). CD9/pCDNA3 was transfected into MKN74 and ldlD 14 cells as described previously (*30*).

Haptotactic Transwell Cell Motility Assay. The assay was performed using a 6.5 mm Transwell assembly (8 μm pore size; Costar, Cambridge, MA) as described previously (*31*, *32*), with some modification. Briefly, the lower surface of the filter was coated by including a 10 μL solution (containing 1.5 μg Matrigel in distilled water), and the filter was dried at room temperature. Cells were harvested in 0.5 mM EDTA and suspended (5×10^5 cells/mL) in Ham's F-12 (no ITS medium, to avoid proliferation) for ldlD cells, and DMEM containing 0.25% BSA for human tumor cells. One hundred microliters of each cell suspension was placed in the upper compartment of the Transwell chamber, and 0.6 mL Ham's F-12 for ldlD cells and DMEM for human cancer cells with 0.25% BSA were added in the lower compartment. Cells were incubated for 24 h and fixed by methanol. The cells on the upper filter surface were removed by swab, and the number of cells that migrated to the lower surface was counted after hematoxylin staining. The Transwell membrane field was divided into nine areas, and the cell numbers at the center of five areas (center, upper right, upper left, lower right, and lower left) were counted at 200× magnification.

Determination of the Level of Expression of GM3 and Other GSLs. Packed cells (10^6), harvested by rubber scraper in Ca^{2+}/Mg^{2+}-free PBS, were homogenized by sonication in 2 mL of a 2-propanol/hexane/water mixture (55:25:20, v/v/v) for 5 min. The sonicate was centrifuged, and the pellet was extracted twice with the same solvent. Combined extracts were subjected to the acetylation procedure, and the GSL fraction was separated by Florisil (Sigma) chromatography from phospholipid, cholesterol, and glycerides. Acetylated GSLs were deacetylated by sodium methoxide in CM 2:1 (*33*), neutralized with Dowex 50, and evaporated to dryness under an N_2 stream, and the residue was dissolved in 50 μL of a 2-propanol/hexane/water mixture (55:25:20). Ten microliter aliquots were analyzed on an HPTLC plate and developed with a chloroform/methanol/water mixture (55:30:5), and GSLs were revealed with 0.2% orcinol in 2 M sulfuric acid.

Identification of CD9 Using SDS−PAGE and Western Blotting. Cells were harvested with a rubber scraper and solubilized in lysis buffer with protease inhibitors [140 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA (pH 7.2), 1% Triton X-100, 2 mM phenylmethanesulfonyl fluoride, and 0.05−0.1 trypsin inhibition unit/mL of aprotinin], and the protein content of the lysates was determined with a BCA kit (Pierce, Rockford, IL), using BSA as a standard. Lysates were subjected to SDS−PAGE under nonreducing conditions, electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA), and subjected to Western blotting with a chemiluminescence system (Pierce) as described previously (*32*).

Effect of Exogenous GM3 on ldlD/CD9 Cell Motility. GM3 was dissolved in DMEM or Ham's F-12 medium by sonication, and incubated at room temperature for 24 h. ldlD/CD9 cells were grown in Ham's F-12 with ITS for 48 h, and then incubated with various concentrations of a GM3 solution (0, 10, 50, and 100 μM). The medium for human cancer cells was changed to 2% FBS containing DMEM when adding various concentrations of GM3 or GM1, and incubated for 24 h. The Transwell cell motility assay was performed as described above.

^{3}H-Labeled Photoactivatable GM3 and Cross-Linking Experiments with CD9. ^{3}H-labeled photoactivatable GM3 having a 1-amino-2-nitro-4-azidophenyl group at the ω

position of the fatty acid, linked to the amino group of sphingosine, i.e., [11-^{3}H(Neu5Ac)]GM3-N3), α-Neu5[3]Ac-(2−3)-*β*-Gal-(1,4)-*β*-Glc-(1-)-{(2*S*,3*R*,4*E*)-2-[12-(2-nitro-4-azidophenyl)aminododecanoyl]amino-3-hydroxyoctadeca-4-ene}, was synthesized as described previously (*34*, *35*).

[11-^{3}H(Neu5Ac)]GM3-N3 (photoactivatable GM3), and nonradioactive natural GM3 from dog erythrocytes, dissolved in methanol at a molar ratio of 1:40, were evaporated under a nitrogen stream. Since the concentration of the original solution of photoactivatable GM3 was 1.67 *μ*g/mL methanol, 7.5 mL of this solution, containing 12.5 *μ*g photoactivatable GM3, was mixed with 500 *μ*g GM3 in methanol in 0.25 mL of a normal GM3 stock solution containing 2 mg/mL. This methanol mixture (consisting of photoactivatable and normal GM3 in a 1:40 ratio) was evaporated to dryness under a nitrogen stream; the residue was dissolved in DMEM containing 2% FBS, and the final GM3 concentration was adjusted to 50 *μ*M. HRT18 cells were grown in DMEM supplemented with 1% sodium pyruvate and 10% FBS to confluency in a 15 cm culture dish. To this was added a 10 mL solution of photoactivatable GM3 with normal GM3 in a 2% FBS/DMEM mixture, and then the mixture was cultured for 5 h at 37 °C. The cells were washed four times with a 2% FBS/DMEM mixture (for each washing, the medium was kept on the cell layer for 30 min at room temperature, and then aspirated). Next, cells were washed twice with PBS at 4 °C. Mixing of photoactivatable and normal GM3, application to cell culture, and washing were all performed in the dark. While dishes with the cell monolayer were kept on ice, UV irradiation was carried out at 366 nm, with the lamp (Spectroline, model ENF-240C, Spectronics Corp., Westbury, NY) 5 cm above the monolayer, and continued for 30 min. Next, the cell layer was washed three times with PBS precooled at 4 °C; cells were scraped off and centrifuged at 1000 rpm for 10 min, and the pellet was lysed in 1 mL lysis buffer [140 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA (pH 7.2), and 1% Triton X-100], with occasional agitation on a Vortex mixer and vigorous pipetting in a 4 °C cold room over the course of 1 h. The lysate was centrifuged at 12 000 rpm for 20 min, and the protein content was determined using BSA as a reference and subjected to IP. For IP, quantities of antibody added per 100 *μ*g protein in the lysate supernatant above were as follows: 5 *μ*g anti-CD9, 8 *μ*g anti-α3, 5 *μ*g anti-α4, 5 *μ*g anti-α6, 8−10 *μ*g anti-*β*1, and 5 *μ*g mouse IgG as a control. Each mixture, in a cold room, was combined with 10 *μ*L protein-Sepharose beads for separation of the immunoprecipitate. An 8% polyacrylamide gel was used for SDS−PAGE followed by Western blotting as described above. Digital autoradiography of the PVDF membranes was performed with a Beta-Imager 2000 instrument (Biospace, Paris, France). The detection time was 48 h. The amount of radioactivity associated with individual proteins was determined with the specific Beta-Vision software provided by Biospace.

RESULTS

Haptotactic Motility of Colonic Carcinoma Cell Lines Expressing a High Level of CD9, and Its Susceptibility to Exogenously Added GM3. Three colonic carcinoma cell lines (SW480, SW620, and HRT18) express high levels of CD9 as indicated by a flow cytometry expression index of 25−36 (Figure 1A). However, the level of GM3 expression at the surface of these cells was minimal, since anti-GM3 mAb DH2 did not react unless the cells were preincubated with a high concentration (50 *μ*M) of GM3. Preincubation of cells with a low concentration (10 *μ*M) of GM3 was not sufficient to show clear reactivity with DH2 (Figure 1B). Haptotactic motility as determined by migration of these cells through Matrigel-coated membranes varied significantly (Figure 1C, white bar), but was equally inhibited when the cells were preincubated with 10 or 50 *μ*M GM3 (Figure 1C, shaded and solid bars in groups a−c), compared to control cells (white bars). This inhibitory effect was statistically significant ($p < 0.05$ for SW480 and HRT18; $p < 0.01$ for SW620). However, the motility was not inhibited by preincubation with 10 or 50 *μ*M GM1, as observed typically for HRT18 cells (Figure 1C, shaded and solid bars in group d). The discrepancy between GM3 expression as probed by reactivity with mAb DH2 and the GM3 effect on haptotactic motility is due to the existence of a "threshold value" of the GM3 concentration for detectability by DH2 (see the Discussion).

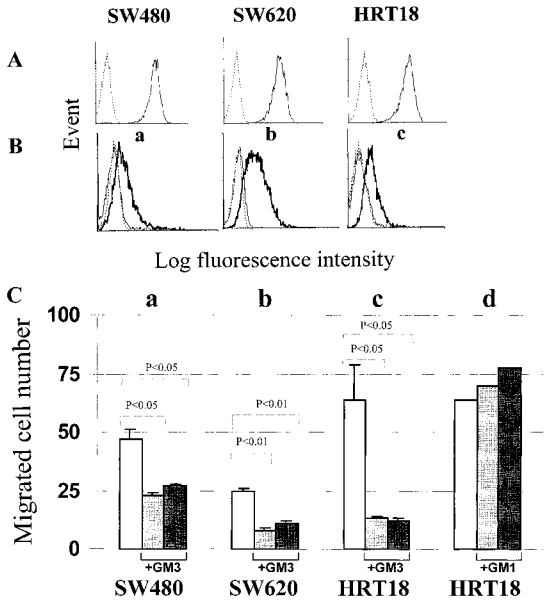

FIGURE 1: Expression patterns of CD9 and GM3 in three colonic carcinoma cell lines, and their motility susceptibility to exogenous GM3. (A) CD9 expression patterns of SW480, SW620, and HRT18, as determined by flow cytometry (expression indexes of 36.2, 25.0, and 28.9, respectively): (dotted line) control mouse IgG and (solid line) anti-CD9 mAb. (B) GM3 expression induced by preincubation with various concentrations of exogenous GM3, as determined by flow cytometry with anti-GM3 mAb DH2: (dotted line) 0, (thin line) 10, and (thick line) 50 *μ*M. Control cells treated with mouse IgG gave the same results as the cells whose data are depicted with a dotted line. (C) Effect of preincubation with various concentrations of exogenous GM3 on the Transwell cell motility assay of SW480 (a), SW620 (b), and HRT18 (c), and effect of exogenous GM1 on HRT18 (d). The ordinate is the number of migrated cells: (white) 0, (light gray) 10, and (black) 50 *μ*M GM3. The statistical significance of differences in motility reduction among groups a−c is indicated with a *p* value.

Haptotactic Motility of Gastric Carcinoma Cell Line MKN74 Expressing a Low Level of CD9, and That Expressing a High Level of CD9 through Its Gene Transfection: Difference in Susceptibility to Exogenous GM3 Addition. The level of CD9 expression in gastric carcinoma cell line MKN74 was extremely low as compared to that in the colonic carcinoma cell lines described in the preceding

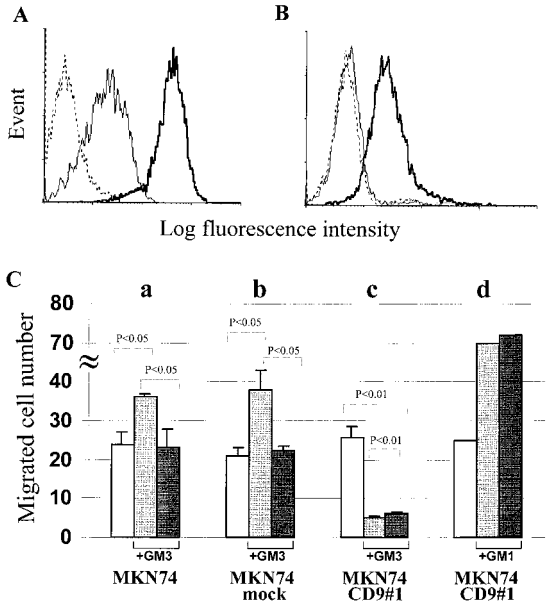

FIGURE 2: Expression patterns of CD9 and GM3 in gastric carcinoma cell line MKN74 and its CD9 gene transfectant, relationship with haptotactic cell motility, and susceptibility to exogenous GM3 and GM1. (A) Flow cytometric patterns of CD9 expression in MKN74 and its CD9 gene transfectant (MKN74/CD9 clone 1): (dotted line) control with mouse IgG, (thin line) with anti-CD9 mAb of MKN74, and (thick line) with anti-CD9 mAb of MKN74/CD9 clone 1. The CD9 expression index for MKN74 is 1.29, and that for MKN74/CD9 is 34.8. (B) GM3 expression induced by preincubation with GM3, as determined by flow cytometry with anti-GM3 mAb DH2 of MKN74: (dotted line) 0, (thin line) 10, and (thick line) 50 μM. MKN74vec1 (transfected with vector) and MKN74/CD9 clone 1 showed the same degree of GM3 expression, which was the same as the control (dotted line). Control cells treated with mouse IgG gave the same results as the cells whose data are depicted with a dotted line. (C) Effect of exogenous GM3 addition on the Transwell cell motility assay of MKN74, MKN74vec1, and MKN74/CD9 clone 1: (white) 0, (light gray) 10, and (black) 50 μM. Note that only MKN74/CD9 showed strong motility inhibition by exogenous GM3, whereas motility enhancement was observed for 0.5 μM GM3 in MKN74 and GM1 in MKN74/CD9.

section (expression index 1.29, compared to a value of 25−36). The level of CD9 expression was increased, becoming comparable to those of the colonic cell lines (expression index 34.8) after transfection of the CD9 gene ("MKN74/CD9 cells", clone 1) (Figure 2A). The level of GM3 expression in MKN74 was as low as in the colonic cell lines, but increased when cells were incubated with 50 μM, but not 10 μM, GM3 (Figure 2B). Haptotactic motility of native or mock-transfected MKN74 cells through Matrigel-coated membrane was not inhibited by 10 or 50 μM GM3. Rather, the motility was enhanced by preincubation with 10 μM GM3 ($p < 0.05$; Figure 2C, groups a and b). In contrast, motility of MKN74/CD9 cells was strongly inhibited by 10 or 50 μM GM3 ($p < 0.01$ in both cases; group c). Preincubation of the same cells with GM1 showed enhancement, rather than inhibition, of motility (group d). MKN74/CD9 cells showed a discrepancy between GM3 expression as detected by mAb DH2 and the GM3 effect on motility due to the existence of a threshold value of the GM3 concentration for detectability by DH2, similar to the case in colonic carcinoma cell lines (see the Discussion).

Endogenous GM3 Synthesis and CD9 Status in ldlD 14 and ldlD/CD9 Clones 24 and 28. The results described in

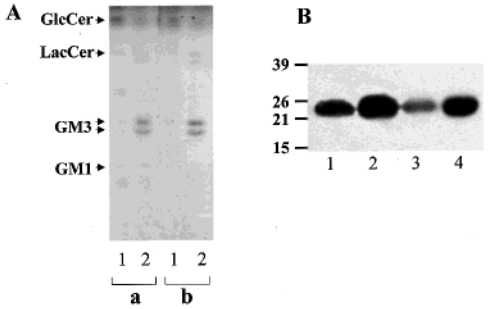

FIGURE 3: Endogenous GM3 synthesis and CD9 glycosylation status in ldlD 14 and ldlD/CD9 clones 24 and 28. (A) GSL and ganglioside profiles of ldlD 14 (a) and ldlD/CD9 (b) grown in the absence of Gal (lane 1) and in the presence of Gal (lane 2). Positions of GlcCer, LacCer, GM3, and GM1 are shown at the left margin. Note that GM3 is present only in cells grown in Gal^+, while higher gangliosides are absent under this condition. (B) SDS−PAGE profile of CD9 in ldlD/CD9 cells revealed by Western blotting with anti-CD9 mAbs. Cells grown in ITS (lane 1), ITS with Gal (lane 2), ITS with GalNAc (lane 3), and ITS with Gal and GalNAc (lane 4). Numbers at the left indicate positions of molecular weight markers ($M_r \times 10^{-3}$). Note that there is no molecular weight change of CD9 upon addition of Gal and/or GalNAc in culture medium.

the two preceding sections indicate that haptotactic motility is inhibited in the copresence of GM3 and CD9. If CD9 is absent or at a low level, exogenous GM3 does not inhibit (rather, enhances) motility (see the preceding section). To confirm the effect of copresence of GM3 and CD9 in defining haptotactic motility, we employed a mutant cell system of ldlD 14 cells expressing CD9 through its gene transfection (ldlD/CD9 clones 24, 28, and 47), in which GM3 is synthesized when cells are grown in ITS medium containing Gal (Gal^+), but GM3 is absent when cells are grown in ITS medium without Gal (Gal^-). This is because ldlD cells are deficient in UDP-Gal 4-epimerase, which converts UDP-Glc to UDP-Gal (*24*).

The band corresponding to GM3 was absent in ldlD 14 or ldlD/CD9 cells grown in the absence of Gal (Figure 3A, lane 1, group a for ldlD 14 and group b for ldlD/CD9). GM3 bands (appearing as a doublet) were seen in cells grown in the presence of Gal (lane 2, both groups a and b as described above). A band corresponding to GlcCer was unchanged in both ldlD 14 and ldlD/CD9 cells regardless of the presence or absence of Gal. A faint band corresponding to LacCer appeared in cells grown in the presence of Gal (lane 2, groups a and b). GM1, GM2, GD1a, and GD1b are absent in cells grown in medium containing both Gal and GalNAc, since the cells do not express β1→4GalNAc transferase for synthesis of GM2, GM1, and other higher gangliosides. The electrophoretic mobility of the CD9 band was unchanged in Gal^- versus Gal^+ or $GalNAc^+$ conditions (Figure 3B, lanes 1 vs 2 and 3 vs 4). In ldlD cells expressing CD82, the electrophoretic mobility of CD82 changed greatly when cells were grown in the presence of Gal (data not shown). Thus, GM3 is the sole ganglioside synthesized in ldlD 14 or ldlD/CD9 cells grown in the presence of Gal, and is the sole glycosylation factor that affects cellular phenotype in CD9-expressing ldlD cells.

Effect of Endogenous and Exogenous GM3, and the Presence of CD9, on Haptotactic Motility: Demonstration in ldlD 14 and ldlD/CD9 Cells Grown in ITS Medium with or without Gal. Haptotactic motility of two clones of ldlD/CD9 (clones 24 and 28) (Figure 4Aa, white and shaded

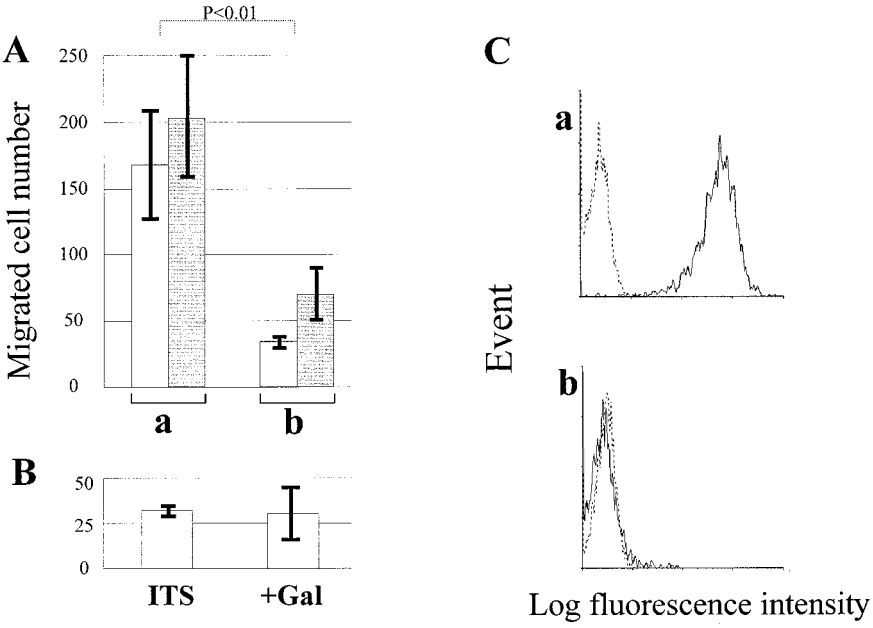

FIGURE 4: Haptotactic motility of ldlD and its CD9 gene transfectant (ldlD/CD9), as determined by the Transwell assay (panels A and B). (A) Cell motility of two ldlD/CD9 clones, 24 (white) and 28 (gray), in ITS medium (a), or ITS with Gal (b). The statistical significance of differences between two groups is indicated with a *p* value. (B) Cell motility of parental ldlD in ITS with (right) or without (left) addition of Gal. (C) Flow cytometric patterns showing expression of CD9: (a) ldlD/CD9 (clone 24) and (b) parental ldlD. Similar patterns were obtained with other clones.

columns, respectively) was much higher than that of parental ldlD 14 without CD9 (Figure 4B, left bar). CD9 is highly expressed in ldlD/CD9 (Figure 4Ca), but is not expressed in parental ldlD 14 (Figure 4Cb). Motility of ldlD/CD9 clones 24 and 28 was greatly inhibited when cells were grown in the presence of Gal, whereby endogenous GM3 synthesis occurred (Figure 4Ab). In contrast, motility of parental ldlD 14 without CD9 was not affected by addition of Gal, even though GM3 synthesis occurred (Figure 4B, right bar). These results indicate that the copresence of GM3 and CD9 is essential for downregulation of cell motility.

To confirm this finding, we examined the effect of exogenous GM3 on haptotactic motility of mock-transfected ldlD 14 and that of ldlD/CD9 cells. Motility was very high in ldlD/CD9 cells in Gal$^-$ ITS medium without exogenous GM3 (Figure 5, solid bar for 0 μM GM3), but was greatly reduced when various concentrations (10, 50, and 100 μM) of GM3 were added (Figure 5, solid bars). Motility of mock-transfected ldlD 14, which do not express CD9, was unaffected by the presence or absence of GM3 (Figure 5, bars). The level of GM3 expressed in ldlD/CD9 cells increased steadily in correlation with the amount of GM3 being incubated (Figure 5, inset).

Cross-Linking of Photoactivatable GM3 with CD9 in HRT18 Cells. The results of the above studies all indicate that coexpression of CD9 and GM3 is essential to down-regulation of tumor cell motility, and that CD9 and GM3 are organized in close association with each other. To test association of GM3 with CD9 at the cell surface, we applied the approach with photoactivatable GM3 developed by Sonnino and colleagues (*34*, *35*), using colonic carcinoma HRT18 cells, which express a high CD9 level and whose haptotactic motility is highly susceptible to exogenous GM3. The cells were preincubated with photoactivatable GM3 (Figure 6, left panel), irradiated, lysed, and immunoprecipitated with mAbs to integrins β1, α6, α4, α3, and CD9.

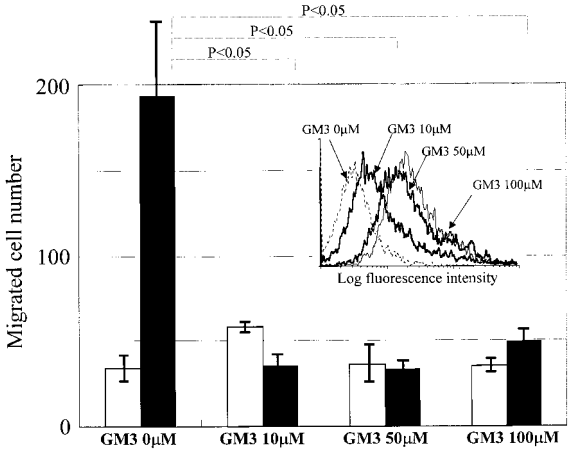

FIGURE 5: Effect of exogenous GM3 addition on Transwell cell motility of ldlD (vec-transfected, white) and ldlD/CD9 clone 28 cells (black). Cells were grown in ITS (Gal$^-$) medium with addition of GM3 as described in Materials and Methods. The ordinate is the migrated cell number in the presence of various concentrations of GM3 (0, 10, 50, and 100 μM; abscissa). The inset shows the level of GM3 expression induced by preincubation of cells with various concentrations of exogenous GM3, as determined by flow cytometry with anti-GM3 mAb DH2.

Mouse IgG was used as a nonspecific control. No radioactive band was co-immunoprecipitated with mAbs to integrin β1, α6, α4, or α3. A major radioactive band corresponding to CD9, in addition to minor radioactive bands, was co-immunoprecipitated with anti-CD9 mAb (Figure 6, right panel). The same results were reproduced in three separate experiments. These results indicate that exogenously added GM3 interacted and cross-linked with CD9 but not with α3, α4, α6, or β1 integrin under these conditions.

DISCUSSION

Cell adhesion and cell motility are closely correlated processes. Motility is controlled by protruding and ruffled

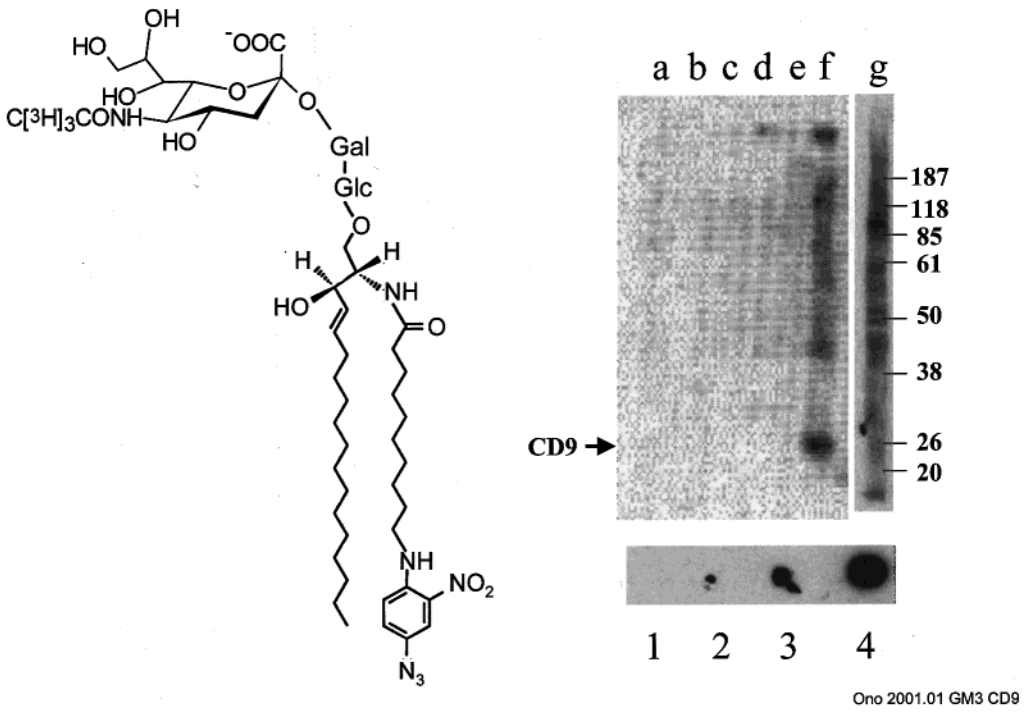

FIGURE 6: Interaction of CD9 and other membrane proteins with photoactivatable GM3. HRT18 cells were used because they express high levels of CD9 and are susceptible to GM3 (see panels Ac and Cc of Figure 1). Cells were treated with photoactivatable ^{3}H-labeled GM3 (structure shown in the left panel) along with regular GM3, washed, and irradiated (see Materials and Methods). Cells were then lysed and subjected to IP with normal mouse IgG as a control (lane a), anti-integrin β1 (lane b), anti-α6 (lane c), anti-α4 (lane d), anti-α3 (lane e), or anti-CD9 (lane f). Lane g contained the total lysate of HRT18 cells (containing 60 μg of protein). 1−4 in the lower right panel show spots of ^{3}H-labeled photoactivatable GM3 having 10, 10^2, 10^3, and 10^4 cpm, respectively, as a standard. The arrow denotes the location of CD9.

lamellipodia and filopodia, where small, transient adhesion sites termed "focal complexes" are found. Adhesion receptors at which actin stress fibers terminate constitute larger adhesion sites underlying the cell body, termed "focal adhesion". The organization of adhesion receptors, cytoplasmic signaling molecules, and cytoskeletal fibers at focal complexes versus focal adhesion may determine the degree of cell motility versus adhesion. The adhesion strength should be moderate to maximize cell motility. The major signal transducers that are involved are FAK, Rho, Rac, Cdc42, etc., although the overall signaling processes defining cell motility versus adhesion are highly complex; e.g., for recent reviews, see refs *36*−*39*.

Recently, two classes of membrane components which modulate cell motility have received increased attention. One class is the transmembrane-4 superfamily (TM4SF; same as tetraspanin), in particular, CD9 and CD82, the members of which form complexes with integrin receptors and modulate or facilitate integrin-dependent cell adhesion and motility. Membrane components that modulate motility were found during a search for monoclonal antibodies that inhibit cell motility (*4*, *5*), and "motility-regulatory protein (MRP)" having an amino acid sequence identical to that of CD9 was identified (*5*); its level of expression was high in primary colonic cancer and downregulated in metastatic deposits (*7*). Another tetraspanin, CD82, was originally cloned as anti-metastatic gene product KAI-1 (*40*), the level of expression of which is inversely correlated with the degree of metastasis in prostate cancer (*41*) and in non-small cell lung cancer (*42*). However, expression of CD82 with full N-glycosylation, not that with impaired N-glycosylation, is required to suppress cell motility (*30*).

The second class of factors which modulate motility is gangliosides, as reported previously (*2*, *3*) and in the study presented here, although effects of gangliosides on integrin-dependent cell adhesion were described in a few previous studies (*43*−*45*). A remarkable feature of gangliosides is their multiple functioning, i.e., capability to interact with many functional proteins such as growth factor receptors, adhesive receptors, and signal transducers. They modulate signal transducers through inhibition or activation of tyrosine kinases associated with growth factor and hormone receptors (*46*−*51*). They themselves are adhesion molecules, when clustered and organized with signal transducers such as Src family kinases and small G-proteins at GSL microdomains (*52*). In association with cell adhesion through carbohydrate−carbohydrate interaction (*53*, *54*), signal transducer molecules are activated, leading to phenotypic changes (*55*−*58*). GM3 has a clear inhibitory effect on angiogenesis and growth of mouse brain tumor EPEN, and this effect is released by conversion of GM3 to GM2 through GM2 synthase gene transfection (*59*). Suppression of GD3 in rat F11 tumor cells reduced the extent of angiogenesis and tumor growth through reduction of vascular growth factor (*3*). Interestingly, motility of F11 cells was reduced when GD3 synthesis was inhibited by the antisense approach (*3*), perhaps due to enhanced synthesis of GM3. Strong inhibition of motility and metastasis of Balb/c mouse osteosarcoma cell line FBJ-LL by GD1a was reported (*2*). The target molecule was imagined to be integrin, but not clearly identified. These processes of ganglioside-dependent inhibition of cell motility may be facilitated by CD9.

Three lines of observation in the study presented here provide a new notion that GM3 interacts with and affects

function of tetraspanin CD9, leading to downregulation of haptotactic cell motility: (i) Colonic carcinoma cell lines having an inherently high CD9 level are susceptible to exogenous GM3, which inhibits haptotactic motility. Exogenous GM1 did not inhibit motility. (ii) Tumor cell lines with a low CD9 level (e.g., MKN74) require a high level of expression of CD9 through its gene transfection before they become susceptible to GM3-dependent inhibition of haptotactic motility. In contrast, exogenous GM1 enhanced motility in MKN74/CD9 cells. (iii) Endogenous GM3 per se does not affect cell motility unless CD9 coexists, as demonstrated using the ldlD mutant expressing CD9 through its gene transfection (ldlD/CD9). The presence of CD9 enhanced motility of ldlD 14 cells in the absence of GM3 (i.e., in Gal^-), whereas motility of the same cells was inhibited in the presence of endogenous GM3 synthesis in Gal^+. The inhibitory effect of exogenous GM3 on ldlD/CD9 was demonstrated in ITS medium with GM3 preincubation. Since no N- or O-glycosylation was observed for CD9 in ldlD/CD9 cells in Gal^+, the only factor involved is GM3.

These findings, taken together, give rise to the idea of a cooperative effect of GM3 and CD9 in inhibiting haptotactic motility. It should be noted that preincubation of cells with 10 μM GM3 barely increased the level of GM3 expression as indicated by flow cytometry (Figures 1B and 2B, and the inset of Figure 5), yet motility was greatly reduced to the same level as in cells incubated with 50 μM GM3, whereby a clear increase in the level of GM3 expression was observed (Figures 1B, 1C, 2B, 2Cc, and 5). A low concentration (5 μM) of GM3 may be sufficient for incorporation onto the cell surface and association with CD9, but may not be sufficient for detection by mAb DH2, because there is a threshold value for anti-GM3 mAb reactivity, either DH2 (*27*) or, more clearly, M2590 (*60*). Failure to demonstrate GM3 dose-dependent inhibition of motility of CD9-expressing tumor cells may also be due to the existence of a threshold value of the GM3 concentration causing motility inhibition. Preincubation with a low concentration (5 μM) of GM3 in cells cultured in 2% FBS caused GM3 expression to a minimal degree. However, when incubation was performed under serum-free conditions (ITS medium), 5 μM GM3 was sufficient to cause GM3 expression that could be detected by DH2 on flow cytometry (Figure 5 inset). The effect of GM3 is not directed to the integrin receptor, since cells without a high level of CD9 expression did not show change of motility or adhesion. Thus, the target of GM3 must be CD9. This point was confirmed by experiments with photoactivatable GM3; i.e., incubation with photoactivatable GM3 revealed preferential binding of [^{3}H]GM3 to CD9 but not to other membrane proteins, including integrin receptors β1, α6, α4, and α3 (α5 was not examined). Besides the major CD9−[^{3}H]GM3 cross-linked band immunoprecipitated with anti-CD9, several minor bands with higher molecular masses as detected could be the CD9−membrane protein complex, since CD9 is known to form complexes (*12*, *14*, *15*). The exact mechanism by which motility of the examined cells is so sensitive to GM3 in the presence of CD9 remains to be elucidated.

GM3 and CD9 together with integrin receptors α5 and α3 coexist in the same membrane domain, separable as detergent-insoluble low-density membrane components under certain conditions when cells are lysed in lysis buffer containing 1% polyoxyethylene oleoyl ether (Brij). However, such a complex is dissociated and solubilized in 1% Triton X-100, and is translocated into a high-density membrane fraction (unpublished observation). This situation is similar to that of the integrin, CD82, and GM3 complex as observed recently in ldlD/CD82 cells (*61*).

Further studies on the organizational status of integrins, CD9, GM3, and signal transducers such as Src kinases and G-proteins are in progress.

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REFERENCES

1. Goldberg, I. D. (1991) *Cell motility factors*, Birkhauser Verlag, Basel, Switzerland.
2. Hyuga, S., Yamagata, S., Tai, T., and Yamagata, T. (1997) *Biochem. Biophys. Res. Commun. 231*, 340−343.
3. Zeng, G., Gao, L., Birkle, S., and Yu, R. K. (2000) *Cancer Res. 60*, 6670−6676.
4. Miyake, M., and Hakomori, S. (1991) *Biochemistry 30*, 3328−3334.
5. Miyake, M., Koyama, M., Seno, M., and Ikeyama, S. (1991) *J. Exp. Med. 174*, 1347−1354.
6. Miyake, M., Nakano, K., Ieki, Y., Adachi, M., Huang, C.-L., Itoi, S., Koh, T., and Taki, T. (1995) *Cancer Res. 55*, 4127−4131.
7. Cajot, J.-F., Sordat, I., Silvestre, T., and Sordat, B. (1997) *Cancer Res. 57*, 2593−2597.
8. Si, Z., and Hersey, P. (1993) *Int. J. Cancer 54*, 37−43.
9. Maecker, H. T., Todd, S. C., and Levy, S. (1997) *FASEB J. 11*, 428−442.
10. Berditchevski, F., Bazzoni, G., and Hemler, M. E. (1995) *J. Biol. Chem. 270*, 17784−17790.
11. Mannion, B. A., Berditchevski, F., Kraeft, S.-K., Chen, L. B., and Hemler, M. E. (1996) *J. Immunol. 157*, 2039−2047.
12. Horvath, G., Serru, V., Clay, D., Billard, M., Boucheix, C., and Rubinstein, E. (1998) *J. Biol. Chem. 273*, 30537−30543.
13. Sugiura, T., and Berditchevski, F. (1999) *J. Cell Biol. 146*, 1375−1389.
14. Masellis-Smith, A., and Shaw, A. R. (1994) *J. Immunol. 152*, 2768−2777.
15. Hadjiargyrou, M., and Patterson, P. H. (1995) *J. Neurosci. 15*, 574−583.
16. Anton, E. S., Hadjiargyrou, M., Patterson, P. H., and Matthew, W. D. (1995) *J. Neurosci. 15*, 584−595.
17. Ikeyama, S., Koyama, M., Yamaoka, M., Sasada, R., and Miyake, M. (1993) *J. Exp. Med. 177*, 1231−1237.
18. Shaw, A. R., Domanska, A., Mak, A., Gilchrist, A., Dobler, K., Visser, L., Poppema, S., Fliegel, L., Letarte, M., and Willett, B. J. (1995) *J. Biol. Chem. 270*, 24092−24099.
19. Schmidt, C., Künemund, V., Wintergerst, E. S., Schmitz, B., and Schachner, M. (1996) *J. Neurosci. Res. 43*, 12−31.
20. Tai, X. G., Yashiro, Y., Abe, R., Toyooka, K., Wood, C. R., Morris, J., Long, A., Ono, S., Kobayashi, M., Hamaoka, T., Neben, S., and Fujiwara, H. (1996) *J. Exp. Med. 184*, 753−758.
21. Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M., and Boucheix, C. (2000) *Science 287*, 319−321.
22. Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., and Mekada, E. (2000) *Science 287*, 321−324.
23. Nishihira, T., Kasai, M., Kitamura, M., Hirayama, K., Akaishi, T., and Sekine, Y. (1985) in *In vitro models for cancer research, Vol. I: Carcinomas of the esophagus and colon* (Webber, M. M., Ed.) pp 65−79, CRC Press, Boca Raton, FL.

24. Kingsley, D. M., Kozarsky, K. F., Hobbie, L., and Krieger, M. (1986) *Cell 44*, 749−759.
25. Krieger, M., Reddy, P., Kozarsky, K., Kingsley, D., Hobbie, L., and Penman, M. (1989) *Methods Cell Biol. 32*, 57−84.
26. Zheng, M., Fang, H., and Hakomori, S. (1994) *J. Biol. Chem. 269*, 12325−12331.
27. Dohi, T., Nores, G., and Hakomori, S. (1988) *Cancer Res. 48*, 5680−5685.
28. Klenk, E., and Heuer, K. (1960) *Zeitschrift für Verdauungs und Stoffwechselkrankheiten 20*, 180−183.
29. Handa, K., White, T., Ito, K., Fang, H., Wang, S., and Hakomori, S. (1995) *Int. J. Oncol. 6*, 773−781.
30. Ono, M., Handa, K., Withers, D. A., and Hakomori, S. (1999) *Cancer Res. 59*, 2335−2339.
31. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. (1987) *Cancer Res. 47*, 3239−3245.
32. Ichikawa, D., Handa, K., Withers, D. A., and Hakomori, S. (1997) *Cancer Res. 57*, 3092−3096.
33. Saito, T., and Hakomori, S. (1971) *J. Lipid Res. 12*, 257−259.
34. Sonnino, S., Nicolini, M., and Chigorno, V. (1996) *Glycobiology 6*, 479−487.
35. Chigorno, V., Palestini, P., Sciannamblo, M., Dolo, V., Pavan, A., Tettamanti, G., and Sonnino, S. (2000) *Eur. J. Biochem. 267*, 4187−4197.
36. Small, J. V., Kaverina, I., Krylyshkina, O., and Rottner, K. (1999) *FEBS Lett. 452*, 96−99.
37. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. (2000) *Oncogene 19*, 5606−5613.
38. Stupack, D. G., Cho, S. Y., and Klemke, R. L. (2000) *Immunol. Res. 21*, 83−88.
39. Somlyo, A. P., and Somlyo, A. V. (2000) *J. Physiol. 522*, 177−185.
40. Dong, J.-T., Lamb, P. W., Rinker-Schaeffer, C. W., Vukanovic, J., Ichikawa, T., Isaacs, J. T., and Barrett, J. C. (1995) *Science 268*, 884−886.
41. Dong, J. T., Suzuki, H., Pin, S. S., Bova, S., Schalken, J. A., Isaacs, W. B., Barrett, J. C., and Isaacs, J. T. (1996) *Cancer Res. 56*, 4387−4390.
42. Adachi, M., Taki, T., Ieki, Y., Huang, C., Higashiyama, M., and Miyake, M. (1996) *Cancer Res. 56*, 1751−1755.
43. Cheresh, D. A., Pytela, R., Pierschbacher, M. D., Klier, F. G., Ruoslahti, E., and Reisfeld, R. A. (1987) *J. Cell Biol. 105*, 1163−1173.
44. Zheng, M., Fang, H., Tsuruoka, T., Tsuji, T., Sasaki, T., and Hakomori, S. (1993) *J. Biol. Chem. 268*, 2217−2222.
45. Fang, L. H., Lucero, M., Kazarian, T., Wei, Q., Luo, F.-Y., and Valentino, L. A. (1997) *Clin. Exp. Metastasis 15*, 33−40.
46. Bremer, E. G., Hakomori, S., Bowen-Pope, D. F., Raines, E. W., and Ross, R. (1984) *J. Biol. Chem. 259*, 6818−6825.
47. Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986) *J. Biol. Chem. 261*, 2434−2440.
48. Yates, A. J., VanBrocklyn, J., Saqr, H. E., Guan, Z., Stokes, B. T., and O'Dorisio, M. S. (1993) *Exp. Cell Res. 204*, 38−45.
49. Mutoh, T., Tokuda, A., Miyada, T., Hamaguchi, M., and Fujiki, N. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5087−5091.
50. Rebbaa, A., Hurh, J., Yamamoto, K., Kersey, D., and Bremer, E. G. (1996) *Glycobiology 6*, 399−406.
51. Li, R., Manela, J., Kong, Y., and Ladisch, S. (2000) *J. Biol. Chem. 275*, 34213−34223.
52. Hakomori, S., Handa, K., Iwabuchi, K., Yamamura, S., and Prinetti, A. (1998) *Glycobiology 8*, xi−xviii.
53. Kojima, N., and Hakomori, S. (1989) *J. Biol. Chem. 264*, 20159−20162.
54. Kojima, N., and Hakomori, S. (1991) *Glycobiology 1*, 623−630.
55. Yamamura, S., Handa, K., and Hakomori, S. (1997) *Biochem. Biophys. Res. Commun. 236*, 218−222.
56. Iwabuchi, K., Yamamura, S., Prinetti, A., Handa, K., and Hakomori, S. (1998) *J. Biol. Chem. 273*, 9130−9138.
57. Iwabuchi, K., Handa, K., and Hakomori, S. (1998) *J. Biol. Chem. 273*, 33766−33773.
58. Prinetti, A., Iwabuchi, K., and Hakomori, S. (1999) *J. Biol. Chem. 274*, 20916−20924.
59. Manfredi, M. G., Lim, S., Claffey, K. P., and Seyfried, T. N. (1999) *Cancer Res. 59*, 5392−5397.
60. Nores, G. A., Dohi, T., Taniguchi, M., and Hakomori, S. (1987) *J. Immunol. 139*, 3171−3176.
61. Ono, M., Handa, K., Withers, D. A., and Hakomori, S. (2000) *Biochem. Biophys. Res. Commun. 279*, 744−750.
62. Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P., and Strannegard, O. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1947−1950.

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