A Specific Cell Surface Glycoconjugate Controlling Cell Motility: Evidence by Functional Monoclonal Antibodies That Inhibit Cell Motility and Tumor Cell Metastasis†

Masayuki Miyake‡ and Sen-itiroh Hakomori*

The Biomembrane Institute, 201 Elliott Avenue West, Seattle, Washington 98119, and University of Washington, Seattle, Washington 98195

Received October 3, 1990; Revised Manuscript Received December 27, 1990

ABSTRACT: The biochemical basis of cell motility has been viewed as a complex process involving cell surface membrane proteins, integrin receptors, growth factors and their receptors, and cytoskeletal components [Rosen & Goldberg (1989) *In Vitro 25*, 1079]. The possible involvement of glycoconjugates at the cell surface in controlling cell motility has not been systematically investigated. We addressed this question using functional monoclonal antibodies (MAbs), which inhibit cell motility and the metastatic potential of tumor cells, as probes. Two such MAbs, derived from two independent processes of immunization and selection, were found to directed to a common specific carbohydrate structure, Fucα1→2Galβ1→R. MAb MIA-15-5 was established after immunization of mice with small cell lung carcinoma line PC7 and selected on the basis of inhibition of U937 and HEL cell migration. MAb MIA-22-20 was established after immunization with lung adenocarcinoma line MAC-10 and selected on the basis of inhibition of MAC-10 cell migration. These two MAbs were both IgM and were consistently reactive with the Fucα1→2Galβ1→R structure, regardless of the identity of the R group. Various other anti-H MAbs, specific to carrier isotype, did not affect cell motility. MAb MIA-15-5 reacted with 30–40% of high-metastatic variant BL6 of mouse melanoma B16 line but with only <5% of low-metastatic variant F1. Metastatic deposition to lung after injection of BL6 cells was inhibited if MAb MIA-15-5 was injected within 3 h but was not inhibited by injection of other anti-H antibodies under the same conditions. These results suggest that a specific surface carbohydrate structure may influence cell motility, since a MAb directed to this structure inhibits cell motility but does not affect cell proliferation. The MAb may mask the crucial structure or eliminate the subpopulation of tumor cells, thus reducing the metastatic potential.

Motility is an essential cellular function, closely related to the processes of development, organogenesis, and wound healing under physiological conditions and to various pathological processes involved in inflammation and tumor invasiveness/metastasis. Cell motility also constitutes a crucial determining factor in various regenerative/renewal processes such as angiogenesis, epitheliogenesis, hematopoiesis, and spermatogenesis. The molecular basis of cell motility is obviously highly complex and is imagined to be controlled by a number of molecular systems, e.g., cell adhesion molecules (e.g., FN[1] and laminin), their receptors (integrin superfamily), cytoskeletal components, a junctional unit connecting cytoskeletal components and membrane receptors, and various peptide growth factors [see Rosen and Goldberg (1989) for review]. Our recent studies indicate that FN-mediated cell motility is highly dependent on integrin receptor in only two out of 10 cell lines examined, although all cell lines showed integrin-dependent cell adhesion on FN (Straus et al., 1989). Thus, cell motility and cell adhesion appear to be phenotypically independent, although they are closely related.

Birchmeier et al. (1985) studied the correlation between cell adhesion and metastatic potential of tumor cells by use of MAbs that inhibit these phenotypes. They found that various MAbs affect phenotypic characteristics of melanoma cells such as adhesion, growth, migration, and morphology. The molecular basis of the effect of these MAbs was not clearly shown. In the present study, this approach was used to establish motility-inhibiting MAbs (MIA) following immunization of mice with human tumor cell lines characterized by high motility. Screening and selection of MAbs was performed on the basis of inhibition of tumor cell motility as measured by a modified Boyden chamber. Two independent MAbs showing strong inhibition of motility were established and found to be directed to a common carbohydrate structure. In this paper, we describe characterization of this structure, specificity of the motility-inhibiting MAbs, and their ability to reduce metastatic potential.

Materials and Methods

Determination of Cell Motility. Cell motility was determined by two assay systems, cell penetration and phagokinetic track. In the former system, cell migration was determined through a polycarbonate membrane assembly based on a Boyden chamber (Greenberg et al., 1981; McCarthy et al., 1983). The assembly (Transwell) was purchased from Costar Scientific (Cambridge, MA) with defined pore size (3-, 5-, and 8-μm diameter). Briefly, 100 μL of hybridoma supernatant

† This study was supported by National Cancer Institute Outstanding Investigator Grant CA42505 (to S.H.) and by funds from The Biomembrane Institute, in part under a research contract from Otsuka Pharmaceutical Co. This is the second report in a series, Studies on Mechanism of Cell Migration.

‡ Present address: Kitano Hospital, Medical Research Institute, 13-3 Kamiyama, Kita-ku, Osaka 530, Japan.

[1] Abbreviations: FN, fibronectin; HPLC, high-pressure liquid chromatography; HPTLC, high-performance thin-layer chromatography; MAb, monoclonal antibody; MIA, motility-inhibiting antibody; SDS, sodium dodecyl sulfate; SPG, sialylparagloboside; TLC, thin-layer chromatography.

0006-2960/91/0430-3328$02.50/0 © 1991 American Chemical Society

containing a known concentration of MAb was mixed with 500 μL of culture medium in the lower Transwell compartment, and 10^5 cells were placed in the upper compartment and cultured for 16 h. Cells in the lower chamber, found mostly (>95%) at the bottom of the lower compartment, were then counted. In the phagokinetic track assay, cell motility was determined on the basis of phagokinetic tracks on a gold sol particle-coated plate. Uniform carpets of gold particles were prepared on glass cover slips coated with BSA as previously described by Albrecht-Buehler (1977). The cover slips were rinsed extensively to remove nonadhering or loose gold particles before cell plating. Freshly trypsinized cells (1000–2000) were plated in a 3.5-cm plastic dish (Falcon, Oxnard, CA) and left in the incubator for 24 h. Phagokinetic tracks were visualized in dark-field microscopy at low power by using side illumination. Gold particles were detected as dark dots. Pictures were taken, areas cleared by a single cultured cell were traced on semitransparent paper of uniform thickness, and average areas were calculated. Since this method only measures minimum motility, it was used only for confirmation of penetration assay results. On the other hand, motility of some types of cells (e.g., variants of B16 melanoma) cannot be measured by penetration assay, since these cells hardly migrate through polycarbonate membrane. However, B16 motility can be measured easily by phagokinetic track assay. A wound healing assay, as previously described (Straus et al., 1989), was also used to estimate cell migration of adherent cells.

Selection of MAbs Displaying Inhibition of Cell Motility. Initially, cell motility of more than 120 human tumor cell lines was examined by the penetration assay described above. Only a few lines were found to be highly motile: PC7 (small cell lung carcinoma), U937 (monocytic leukemia), HEL (erythroleukemia), and MAC-10 (lung adenocarcinoma) (these lines were able to penetrate Transwell membranes with pore diameters of 8, 3, 3, and 5 μm, respectively). All other tumor cell lines tested were less motile, and nontransformed cells were immotile. Subsequently, mice were immunized with one of the highly motile cell lines as above, and their splenocytes were subsequently fused with HAT-sensitive mouse myeloma cell line SP2. Antibody-secreting hybridomas were selected on the basis of their ability to inhibit cell motility (see above) in the motile cell lines. Reduction of cell motility was expressed as the number of cells able to penetrate the polycarbonate membrane in the presence of mouse IgM without MAb (for details see Figure 1 legend).

Determination of MAb Specificity. The antibodies selected were tested against panels of glycolipids as well as proteins coated on plastic plates. Glycolipids in ethanol solution (10–20 ng/well) or proteins (or glycoproteins) in cellular extracts with 1% Triton X-100 (protein concentration 5–10 μg/mL) were incubated in a vinyl well (Costar Scientific) and tested for antibody binding as described previously (Kannagi & Hakomori, 1986). Some of the MAbs selected on the basis of inhibition of cell motility were found to bind glycolipids or carbohydrates, while others bound glycoproteins or proteins; only the former are described in this paper. MAbs in the former category were further purified, and specificity of antigen binding was examined by using panels of glycosphingolipids prepared as described previously (Hakomori, 1983). Purified glycolipid antigens were prepared from human blood cells and various tissues purified by HPLC on Iatrobeads 6RS-1080 column in 2-propanol–hexane–water system as described previously (Watanabe & Arao, 1981) and further purified on HPTLC. Solid-phase antibody binding on each glycolipid antigen was performed on plastic wells as previously described and by TLC immunostaining (Kannagi & Hakomori, 1986; Magnani et at., 1980).

The following MAbs were used as controls: 1B2 (anti-*N*-acetyllactosamine) (Young et al., 1981), BE2 (anti-type 2 chain H) (Young at al., 1981), AH6 (anti-Le^y) (Abe et al., 1983), 17-206 (anti-type 1 chain H) (Welt et al., 1988), and MBr1 (anti-globo-H) (Bremer at al., 1984).

Glycoprotein or protein antigens were also characterized after cells were extracted with Laemmli's sample buffer (Laemmli, 1970), followed by slab gel electrophoresis, Western blotting, and immunostaining (Towbin et al., 1979). Alternatively, cells were washed, harvested with EDTA, and solubilized by sonication in 50 mM Tris-HCl (pH 7.5) buffer containing 0.5% NP40. Insoluble materials were removed by centrifugation and the soluble fraction was subjected to slab gel electrophoresis (Laemmli, 1970) for Western blotting and autoradiography with rabbit anti-mouse IgM (ICN, Lisle, IL) and ^{125}I-protein A as described previously (Towbin et al., 1979).

Antibody Binding to Various Cell Lines. A variety of motile and immotile human cell lines, as well as high-metastatic (BL6) and low-metastatic (F1) variants of the mouse melanoma B16 cell line originally established by Fidler (1975) and Hart (1979), were donated by Dr. J. Starkey (Department of Microbiology, Montana State University). Their reactivities with MAb were tested by immunofluoresence with FITC-conjugated goat anti-mouse MAbs (TAGO, Burlingame, CA) and by cytofluorometry.

Determination of Lung Metastatic Deposition of BL6 Cells and Its Inhibition by MAbs. In order to obtain background values of metastatic potential, varying numbers of BL6 cells (10^3, 5×10^3, 10^4, 5×10^4) were injected intravenously in C57/BL mice; after 14 days of incubation the animals were killed and lung deposits of B16 tumor were measured. With this background value, a defined number of BL6 cells (10^4) were injected with or without combined administration of various doses of antibody at various times as indicated in the legend for Figure 6. The effect of injection of control MAbs was also studied under conditions as described in this legend.

Results

Selection of MAbs MIA-15-5 and MIA-22-20, Displaying Inhibition of Cell Migration but Not Affecting Cell Proliferation. Using criteria as described under Materials and Methods, two antibody-secreting hybridomas were selected from 50 obtained after 12 fusions of splenocytes from mice immunized with two solid tumor lines of four human tumor cell lines showing high motility. MAb MIA-15-5 (isotype IgM), derived from mice immunized with human small cell lung carcinoma line PC7, showed significant inhibition of cell migration of U937, HEL, and MAC-10 lines. MAb MIA-22-20 (isotype IgM) was derived from mice immunized with human lung adenocarcinoma line MAC-10 and showed motility inhibition of this same line. A few other MAbs were established that showed even stronger inhibition of tumor cell motility than MIA-15-5 and MIA-22-20. These MAbs were directed to a protein structure and will be described elsewhere.

The effect of MAb MIA-15-5 was observed consistently, regardless of the number of cells added to the Boyden chamber assembly, although the number of MAC-10 cells penetrating to the lower compartment reached a plateau after 3.3×10^5 cells were added to the upper compartment (Figure 1). In contrast, the effect of MAb concentration was dose-dependent and led to constant inhibition at 8–16 μg/mL for MIA-15-5 (Figure 2, top) and at 15–32 μg/mL for MIA-22-20 (Figure 2, bottom). Control IgM showed no inhibitory effect on cell

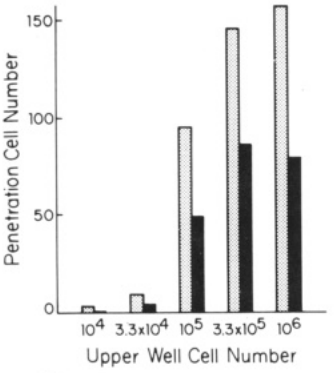

FIGURE 1: Effect of MAb MIA-15-5 on MAC-10 cell motility. MAC-10 cells were seeded in the upper compartment of 6.5-mm Transwell plates with 5-μm pore diameter (see Materials and Methods), with the indicated number of cells. In the lower compartment, mouse IgM (10 μg/mL; shaded columns) or MAb MIA-15-5 (10 μg/mL; solid columns) was added. Numbers of cells migrated into the lower compartment were counted after a 16-h incubation.

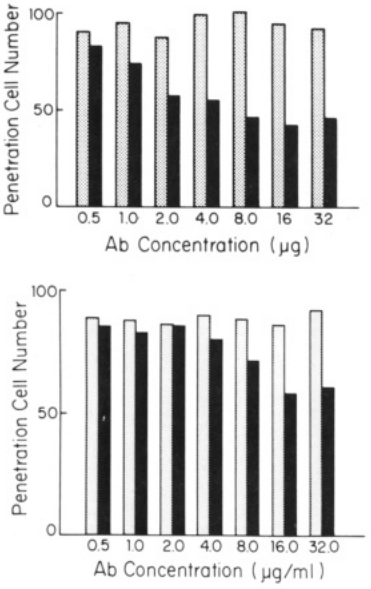

FIGURE 2: Dose-dependent effect of MAbs MIA-15-5 and MIA-22-20 on cell migration. Cell migration was determined with MAC-10 cells in Transwell plates as described for Figure 1. Increasing concentration of MAb was placed in the lower compartment as indicated on the abscissa. Top panel, dose-dependent effect of MAb MIA-15-5 (solid column) and comparison with control mouse IgM (shaded column). Note that a constant inhibitory effect was produced by MIA-15-5 concentrations from 8 to 15 μg/mL. Bottom panel, effect of MAb MIA-22-20 (solid column) and comparison with control mouse IgM (shaded column). A constant inhibitory effect was produced by MIA-22-20 concentrations from 16 to 32 μg/mL.

motility regardless of concentration (Figure 2, shaded columns). These results clearly indicate that cell motility inhibition was stronger with MIA-15-5 than with MIA-22-20. Neither MAb had any inhibitory effect on growth of MAC-10 cells at the same concentration that effectively inhibited cell motility. Cell numbers counted at day 1, 2, 3, 4, and 5 after addition of 10^5 MAC-10 cells at day 0 were (all numbers $\times10^5$) 1.8 ± 0.3, 2.1 ± 0.4, 3.3 ± 0.5, 5.5 ± 0.6, and 8.4 ± 0.7, respectively, regardless of the presence or absence of control mouse IgM MAb or MAb MIA-15-5 at 10 or 50 μg/mL. MIA-15-5 also did not inhibit growth of cell lines PC7 or U937 (data not shown).

Motility-Inhibitory Effect of MIA-15-5 As Compared with Various Other MAbs Directed to H-Structures. Since the epitopes defined by both MIA-15-5 and MIA-22-20 were identified as H-structures (see Characterization of Epitope),

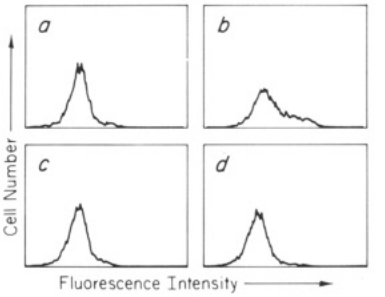

FIGURE 3: Antigen expression in mouse melanoma cell lines BL6 and F1 as defined by MAb MIA-15-5. Antigen expression was determined by cytofluorometric analysis as described under Materials and Methods. (a) BL6 cells with mouse IgM mixture. (b) BL6 cells stained with MIA-15-5. (c) F1 cells with mouse IgM mixture. (d) F1 cells stained with MIA-15-5. Note that BL6, but not F1, showed significant staining with MIA-15-5.

inhibitory effects by various MAbs on MAC-10 cell motility were compared. A total of 10^5 cells were added in the upper compartment of Transwell plates (see Figure 1 legend for experimental conditions), and the numbers of cells migrating to the lower chamber were counted in the presence of 20 μg/mL of various MAbs: MIA-15-5, mouse IgM (nonimmune), 1B2 (anti-*N*-acetyllactosamine), BE2 (anti-type 2 chain H), AH6 (anti-Le^y), 17-206 (anti-type 1 chain H), and MBr1 (anti-globo-H). Results were 25 ± 5, 95 ± 8, 98 ± 15, 90 ± 5, 91 ± 15, 102 ± 10, and 97 ± 20 cells, respectively. Clearly, only MIA-15-5 had a significant inhibitory effect on MAC-10 cell migration. The other MAbs, which had no inhibitory effect, included ones directed to type 1 chain H, type 2 chain H, and globo-H (see Discussion).

Reactivity of MAb MIA-15-5 with Mouse Metastatic Melanoma Line BL6 and Inhibition by MIA-15-5 of Metastatic Deposition in Syngenic Mice. Significant reactivity of MAb MIA-15-5 was observed with a subpopulation of high-metastatic variant BL6 of mouse melanoma line B16 (Figure 3b), in contrast to the lack of such reactivity in low-metastatic variant F1 (Figure 3d). Control IgM showed no reactivity with BL6 cells (Figure 3a). When MAb MIA-15-5 was injected 3 h after intravenous injection of 10^4 BL6 cells in syngenic C57/BL mice, clear inhibition of melanoma colony formation in lung was observed (Figure 4, top). Identical doses of other MAbs did not have this effect. This inhibitory effect by MAb MIA-15-5 was pronounced only when MAb injection occurred 3 h or less after injection of BL6 cells; it was negligible when the delay was 1 day and nonexistent when the delay was 3 days (Figure 4, bottom).

Inhibition of Phagokinetic Motility of B16/BL6 Melanoma Cells by MAb MIA-15-5. Phagokinetic motility of various kinds of cancer cells was tested by using a gold particle coating method as described above. However, we were unable to detect significant motility in this way except for mouse melanoma cells. Areas of the particle-clear zone for high-metastatic melanoma B16/BL6 cells were measured after a 24-h incubation, and the average area was calculated. In the presence of control mouse IgM (10 μg/mL), the clearance area was 6960 ± 2220 μm^2 (average of 50 unselected cells). In contrast, the same cells in the presence of 5 and 10 μg/mL MIA-15-5 showed clearance areas of 4980 ± 1960 and 4200 ± 1740 μm^2, respectively. A typical example of inhibition of BL6 motility in the presence of MIA-15-5 is shown in Figure 5.

Characterization of Epitope. Both MAbs MIA-15-5 and MIA-22-20 showed similar reactivities to various glycolipids bearing H structure. However, MIA-15-5 showed moderate reactivity with Le^b as well as Le^y (Figure 6, top), whereas MIA-22-20 showed no reactivity with Le^b (Figure 5, bottom).

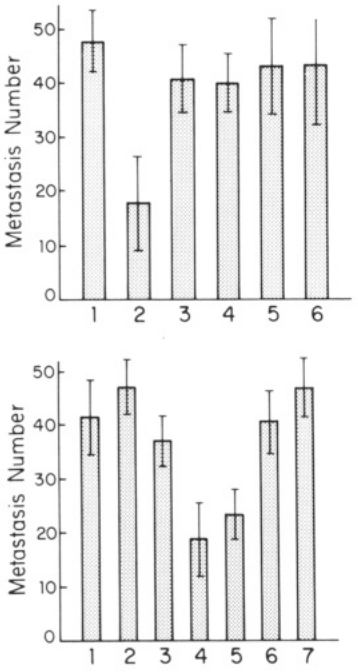

FIGURE 4: Effect of various MAbs on metastatic potential of mouse melonoma BL6 cells. High-metastatic BL6 cells were injected intravenously into syngenic C57/BL mice. Top panel: After 3 h, various MAbs were injected, as follows: Lane 1, mouse IgM mixture, 20 μg; lane 2, MAb MIA-15-5, 20 μg; lane 3, MIA-15-5, 5 μg; lane 4, MAb AH6 (anti-Le^y), 20 μg; lane 5, MAb 1B2 (anti-*N*-acetyllactosamine), 20 μg; lane 6, MAb BE2 (anti-type 2 chain H), 20 μg. Metastatic deposits in lung were examined after 15 days (see Materials and Methods). Results shown represent mean values from five mice. Bottom panel: Time course effect of injection of various MAbs on BL6 metastatic deposits. In all experiments, 20 μg of antibody was injected. Experimental groups consisted of seven C57/BL mice; results shown represent mean values with standard error bars. Lane 1, MAb BE2 was injected immediately after BL6 inoculation; lane 2, MAb AH6 was injected immediately after BL6 inoculation; lane 3, MAb MIA-15-5 was injected 24 h before BL6 inoculation; lane 4, MIA-15-5 was injected immediately after BL6 inoculation; lane 5, MIA-15-5 was injected 3 h after BL6 inoculation; lane 6, MIA-15-5 was injected 24 h after BL6 inoculation; lane 7, MIA-15-5 was injected 3 days after BL6 inoculation.

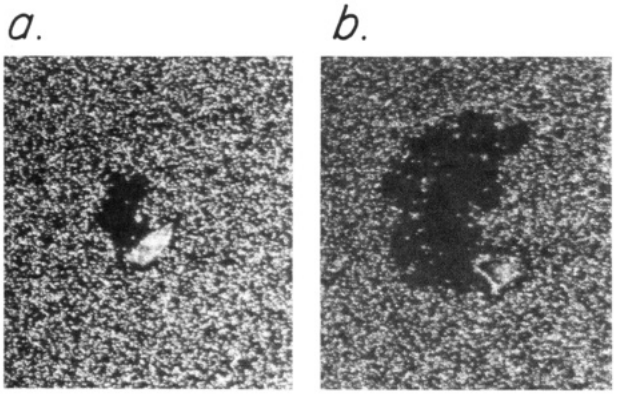

FIGURE 5: Effect of MAb MIA-15-5 on motility of metastatic mouse melanoma BL6 cells as determined by pharmacokinesis on gold colloid method. Since BL6 cells showed little transmembrane motility as determined by the Transwell plate method, the effect of MIA-15-5 on BL6 motility was also studied by pharmacokinesis on a gold sol coated surface, as described under Materials and Methods. (a) BL6 motility exhibited in medium containing MIA-15-5 (10 μg/mL). (b) BL6 motility exhibited in medium containing mouse IgM (10 μg/mL).

Immunoblotting of various glycolipids separated on TLC showed strong reactivity with all lacto series glycolipids having H or Le^y structure (Figure 7); i.e., both MAbs MIA-15-5 and MIA-22-20 bind to the Fucα1→2Gal epitope with lacto series

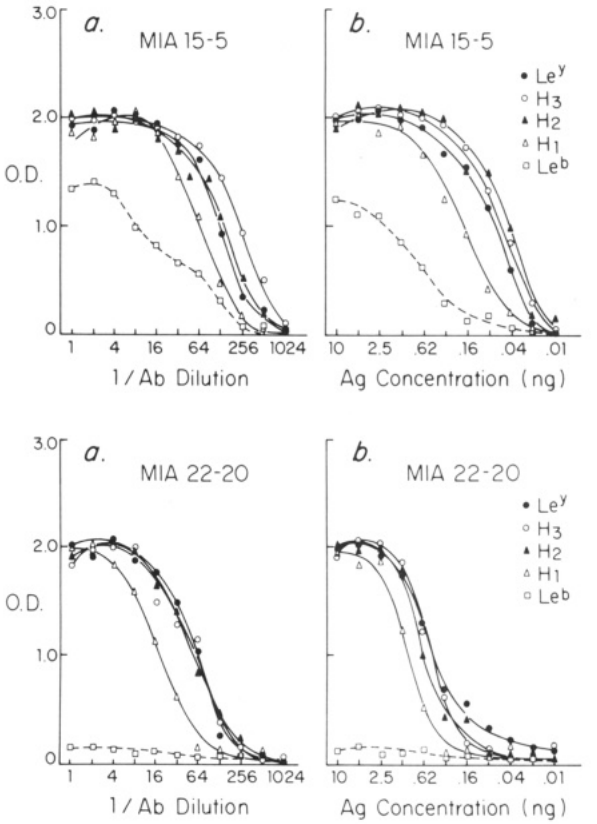

FIGURE 6: Specificity of MAbs MIA-15-5 and MIA-22-20. Antigen-binding specificity of these two MAbs was determined with various glycosphingolipid antigens coated on plastic wells. Top panel, left side (a): Reactivities of MIA-15-5 with a constant quantity (10 ng/well) of various glycolipids coated on plastic well with various concentrations of antibody. Right side (b): Reactivities of a constant concentration of MIA-15-5 (10 μg/mL) with various concentrations of antigen (10–0.01 ng/well). Bottom panel: Reactivities of MIA-22-20 with glycolipids under various conditions, as described for the top panel.

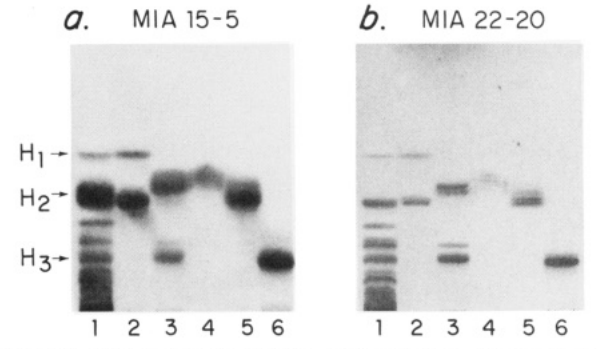

FIGURE 7: Binding of MAbs MIA-15-5 and MIA-22-20 on TLC-separated glycolipids. (a) Binding of MIA-15-5 was determined by immunostaining on TLC plates as described under Materials and Methods. Lane 1, mixture of neutral glycolipids extracted from MAC-10 cells; lane 2, H1 and H2 glycolipids from human O erythrocytes; lane 3, Le^y glycolipid from lung cancer tissue (upper band) and H3 glycolipid from human O erythrocytes; lane 4, Le^b glycolipid from lung cancer tissue; lane 5, H2 glycolipid from lung cancer tissue; lane 6, trifucosyl Le^y from lung cancer tissue. All quantities: 1 μg. (b) Binding of MIA-22-20 under the same conditions as described for (a).

structures (see Table I). MIA-15-5 reacted with globo–H on TLC immunostaining and on ELISA assay, while MIA-22-20 did not. Neither of these MAbs reacted with ganglio-H. On Western blotting of glycoprotein of MAC-10 cells, four bands (M_r 97K, 77K, 45K, and 32K) were clearly demonstrated (Figure 8). The pattern was consistent in the presence vs absence of 2-mercaptoethanol.

Table I: Structures of Glycolipid Antigens Used in This Study and Their Reactivities with MAbs MIA-15-5 and MIA-22-20

name	structure	MIA-15-5 ELISA	MIA-15-5 TLC	MIA-22-20 ELISA	MIA-22-20 TLC
II^{3}NeuAcLacCer (GM3)	NeuAcα2→3Galβ1→4Glcβ1→1Cer	−	−	−	−
IV^{3}NeuAcnLc$_4$ (2→3SPG)	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	−	−	−	−
IV^{6}NeuAcnLc$_4$ (2→6SPG)	NeuAcα2→6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	−	−	−	−
III^{3}FucIV^{3}NeuAcnLc$_4$ (sialyl Le^x penta)	NeuAcα2→3Galβ1→4GlcNAcβ1(3↑Fucα1)→3Galβ1→4Glcβ1→1Cer	−	−	−	−
Gb3 (CTH)	Galα1→4Galβ1→4Glcβ1→1Cer	−	−	−	−
Gb4 (globoside)	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer	−	−	−	−
nLc$_4$ (paragloboside)	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	−	−	−	−
IV^{2}FucnLc$_4$ (H$_1$)	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	+	+	+	+
III^{3}FucnLc$_4$ (Le^x penta)	Galβ1→4GlcNAcβ1(3↑Fucα1)→3Galβ1→4Glcβ1→1Cer	−	−	−	−
III^{4}FucLc$_4$ (Le^a)	Galβ1→3GlcNAcβ1(4↑Fucα1)→3Galβ1→4Glcβ1→1Cer	−	−	−	−
III^{3}IV^{2}Fuc$_2$nLc$_4$ (Le^y)	Galβ1→4GlcNAcβ1(2↑Fucα1,3↑Fucα1)→3Galβ1→4Glcβ1→1Cer	+	+	+	+
nLc$_6$ (i active antigen)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	−	−	−	−
VI^{2}FucnLc$_6$ (H$_2$)	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	+	+	+	+
II^{3}NeuAcIV^{2}FucGg4 (ganglio-H)	Fucα1→2Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer	−	−	−	−
III^{4}IV^{2}Fuc$_2$Lc$_4$ (Le^b)	Galβ1→3GlcNAcβ1(2↑Fucα1,4↑Fucα1)→3Galβ1→4Glcβ1→1Cer	+	+	−	−
V^{2}FucGb5 (globo-H)	Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer	+	+	−	−
III^{3}V^{3}Fuc$_2$nLc$_6$ (di-Y$_2$; dimeric Le^x)	Galβ1→4GlcNAcβ1(3↑Fucα1)→3Galβ1→4GlcNAcβ1(3↑Fucα1)→3Galβ1→4Glcβ1→1Cer	−	−	−	−
III^{3}V^{3}VI^{2}Fuc$_3$nLc$_6$ (tri-Le^y)	Galβ1→4GlcNAcβ1(2↑Fucα1,3↑Fucα1)→3Galβ1→4GlcNAcβ1(3↑Fucα1)→3Galβ1→4Glcβ1→1Cer	+	+	+	+
H$_3$	Fucα1→2Galβ1→4GlcNAcβ1→6 / Fucα1→2Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	+	+	+	+
extended I antigen	Galβ1→4GlcNAcβ1→6 / Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	−	−	−	−

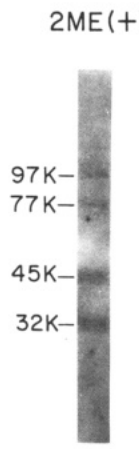

FIGURE 8: Western blot immunostaining of MAC-10 cells with MAb MIA-15-5. MAC-10 cells lysates were electrophoresed through 12% acrylamide slab gels under reducing conditions. Antigens were electroblotted to the nitrocellulose membrane and immunostained with MAb MIA-15-5.

DISCUSSION

Cell motility is a highly complex process, dependent on pericellular adhesion molecules (e.g., FN and laminin), their integrin receptors, cytoskeletal components, and a junctional unit connecting cytoskeletal components and membrane receptors. Involvement of the cell-binding and Hep-2 domains of FN and integrin receptor in determining cell motility for certain (but not all) types of cell has become increasingly clear (Straus et al., 1989; McCarthy et al., 1983; Makabe et al., 1990). In addition, a large variety of growth factors and autocrine factors have been shown to stimulate cell motility (Rosen & Goldberg, 1989). Essentially all the factors so far described that modulate cell motility are proteins, and there has been little attention paid to carbohydrates performing similar roles, despite the fact that cell surface proteins and lipids are heavily glycosylated. In order to further identify cell surface molecules involved in control of cell motility, the present study utilized an approach based on (i) selection of a few lines showing high motility out of a large number of human tumor cell lines, (ii) immunization of mice with the high-motility cell lines and selection of MAbs showing inhibition of cell motility in a polycarbonate membrane penetration assay, and (iii) characterization of the epitope defined by the motility-inhibiting MAbs.

By use of this approach, several motility-inhibiting MAbs were selected, including MIA-15-5 and MIA-22-20. Interestingly, these two MAbs, established at different times with different cell lines as immunogens, were found to be directed to the same carbohydrate epitope (H/Le^y). Other previously established anti-H and anti-Le^y MAbs showed no inhibition of tumor cell motility under the same conditions. Both MIA-15-5 and MIA-22-20 showed a strong reactivity with both type 1 and type 2 chain H as well as Le^y (difucosyl type 2 chain). MIA-15-5, which displayed high antimotility activity, showed cross-reactivity even with Le^b and globo-H, whereas MIA-22-20, which displayed low antimotility activity, did not show such cross-reactivity. Other MAbs directed to the H epitope with restricted carrier structure, e.g., BE2 (vs type 2 chain H; Fucα1$\rightarrow$2Galβ1$\rightarrow$4GlcNAc), 17-206 (vs type 1 chain H; Fucα1$\rightarrow$2Galβ1$\rightarrow$3GlcNAc), and MBr1 (vs globo-H; Fucα1$\rightarrow$2Galβ1$\rightarrow$3GalNAcβ1$\rightarrow$3Galα1$\rightarrow$4Gal), had no effect on cell motility. Thus, MAbs affecting cell motility were directed to the H structure (Fucα1$\rightarrow$2Galβ1$\rightarrow$R) and were reactive regardless of the identity of the carrier R structure (structures and reactivities are summarized in Table I). The motility-inhibitory effect of MIA-15-5 may be partially due to its wide specificity, not affected by carrier structure, and its nonrestricted high affinity. A mixture of various anti-H and anti-Le^y MAbs did not have a similar motility-inhibitory effect as MIA-15-5 (data not shown). We conclude that the inhibitory effect of MIA-15-5 is closely dependent on its inherent properties (i.e., both specificity and affinity, nonrestricted by carrier structure), which allow it to bind at the cell surface in random directions, thereby restricting membrane perturbation, an essential step in the process of cell motility. However, the exact mechanisms controlling cell motility through specific carbohydrate structures such as H (as described in this paper) require extensive further chemical and physical study. Recently, a technique for measurement of molecular motion of carbohydrate residues in bilayers has been developed, based on a new mode of ^{2}H NMR spectroscopy (Renou et al., 1989; Auger et al., 1990). Application of this technique in studies of the motility-inhibitory mechanism of MIA-15-5 is a promising approach to be considered.

Another finding of interest is that the epitope defined by MIA-15-5 is expressed in high-metastatic B16 melanoma variant BL6 but not in low-metastatic variant F1. Administration of MIA-15-5 produced a clear, dose-dependent reduction of BL6 metastatic potential in syngenic mouse lung. The reduction may be due to antibody-dependent elimination of high-metastatic populations expressing H/Le^y or to masking by the antibody of such epitopes, which are essential for cell motility.

Cell surface carbohydrates of a specific type play a major role in defining tumor cell motility and, consequently, metastatic potential, although the precise mechanism remains unknown. Two motility-inhibiting MAbs (MIA-15-5 and MIA-22-20) were isolated from independent immunization and selection processes. The fact that these two MAbs display similar specificity (to H/Le^y antigen) may not be incidental. H and Le^y structures have been found to be highly expressed in a variety of human tumors. Expression of these epitopes may be instrumental in defining cell social behavior of tumor cells, leading incidentally to their recognition as tumor-associated antigens [Abe et al., 1983; Welt et al., 1988, Brown et al., 1983; Lloyd et al., 1983; Bremer et al., 1984; Tsuji et al., 1987; for a review see Hakomori (1989)]. In B16 melanoma, expression of sialyl type 2 chain and Le^x has been correlated with metastatic and less metastatic properties, respectively (Tao & Burger, 1977; Finne et al., 1980). Expression of H and Le^y structures in B16 melanoma cells was not examined in these earlier studies; however, H/Le^y expression may be correlated with high metastatic potential, as suggested by the present study. A study with phagokinetic tracks of BL6 cells indicated strong inhibition of motility by MIA-15-5, which could be correlated with reduction of metastatic potential. This effect by the antibody may be due partially to antibody-dependent elimination of high-metastatic populations expressing the H/Le^y epitope.

Since neither MIA-15-5 nor MIA-22-20 produced clear inhibition of cell growth or cell adhesion (data not shown), motility inhibition through the H/Le^y structure may be independent of the cell adhesion process. Our previous study suggests that cell adhesion and motility processes are independent but partially overlapping (Straus et al., 1989). Although H/Le^y is now shown to be a mediator of cell motility, the mechanism for this process remains completely unknown. Each cell type may express a different pattern of H/Le^y in glycolipids and proteins. In MAC-10 cells, >50% of H/Le^y was expressed in glycolipids, and a relatively small proportion

was expressed in four proteins (M_r 97K, 77K, 45K, and 32K). The possibility therefore exists that antibody-dependent inhibition of cell motility operates through these glycoproteins. Since the integrin receptor is involved in cell motility (Straus et al., 1989; Makabe et al., 1990), we considered the interesting possibility that MAb-reactive H structure is present in this receptor. However, none of the four proteins (see above) had relative molecular weights corresponding to that of integrin receptor. Since the majority of H/Le^y in MAC-10 cells was found in glycolipid, there is a greater possibility that the effect of MAb is via direct binding on the lipid bilayer through glycolipid, which could restrict membrane motility through an unexplored mechanism. Possible interaction through H/Le^y with other carbohydrates, based on carbohydrate–carbohydrate interactions (Kojima & Hakomori, 1989), is under consideration, and studies along this line are now in progress.

ACKNOWLEDGMENTS

We thank Dr. Kenneth Lloyd (Sloan-Kettering Institute, New York) for donating MAb 17-206, Edward Nudelman for various glycolipids, and Dr. Stephen Anderson for editing and preparation of the manuscript.

Registry No. Fucα1→2Galβ, 16741-18-7.

REFERENCES

Abe, K., McKibbin, J. M. & Hakomori, S. (1983) *J. Biol. Chem. 258*, 11793–11797.

Albrecht-Buehler, G. (1977) *Cell 11*, 395–404.

Auger, M., Van Calsteren, M.-R., Smith, I. C. P., & Jarrell, H. C. (1990) *Biochemistry 29*, 5815–5821.

Birchmeier, W., Imhof, B. A., Goodman, S. L., & Vollmers, H. P. (1985) in *Gene expression during normal and malignant differentiation* (Andersson, L. C., Gahmberg, C. G., & Ekblom, P., Eds) pp 33–55, Academic Press, London.

Bremer, E. G., Levery, S. B., Sonnino, S., Ghidoni, R., Canevari, S., Kannagi, R., & Hakomori, S. (1984) *J. Biol. Chem. 259*, 14773–14777.

Brown, A., Feizi, T., Gooi, H. C., Embleton, M. J., Picard, J. K., & Baldwin, R. W. (1983) *Biosci. Rep. 3*, 163–170.

Fidler, I. J. (1975) *Cancer Res. 35*, 218–224.

Finne, J., Tao, T.-W., & Burger, M. M. (1980) *Cancer Res. 40*, 2580–2587.

Greenberg, J. H., Seppa, S. T., Seppa, H. E. J., & Hewitt, A. T. (1981) *Dev. Biol. 87*, 259–266.

Hakomori, S. (1983) *Sphingolipid biochemistry* (Kanfer, J. N., & Hakomori, S., Eds.) pp 1–165, Plenum Press, New York.

Hakomori, S. (1989) *Adv. Cancer Res. 52*, 257–331.

Hart, I. R. (1979) *Am. J. Pathol. 97*, 587–592.

Kannagi, R., & Hakomori, S. (1986) *Handbook of experimental immunology, Vol. 4 (Applications of immunological methods in biomedical sciences)* (Weir, Z., & Herzenberg, Z., Eds.) pp 117.1–117.20, Blackwell Scientific, Boston, MA.

Kojima, N., & Hakomori, S. (1989) *J. Biol. Chem. 264*, 20159–20162.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lloyd, K. O., Larson, G., Stromberg, N., Thurin, J., & Karlsson, K.-A. (1983) *Immunogenetics 17*, 537–541.

Magnani, J. L., Smith, D. F., & Ginsburg, V. (1980) *Anal. Biochem. 109*, 399–402.

Makabe, T., Saiki, I., Murata, J., Ohdate, Y., Kawase, Y., Taguchi, Y., Shimojo, T., Kimizuka, F., Kato, I., & Azuma, I. (1990) *J. Biol. Chem. 265*, 14270–14276.

McCarthy, J. B., Palm, S. L., & Furcht, L. T. (1983) *J. Cell Biol. 97*, 772–777.

Renou, J.-P., Giziewicz, J. B., Smith, I. C. P. & Jarrell, H. C. (1989) *Biochemistry 28*, 1804–1814.

Rosen, E. M., & Goldberg, I. D. (1989) *In Vitro 25*, 1079–1087.

Straus, A. H., Carter, W. G., Wayner, E. A., & Hakomori, S. (1989) *Exp. Cell Res. 183*, 126–139.

Tao, T.-W., & Burger, M. M. (1977) *Nature 270*, 437–438.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Tsuji, Y., Yoshioka, M., Ogasawara, T., Takemura, T., & Isojima, S. (1987) *Cancer Res. 47*, 3543–3550.

Watanabe, K., & Arao, Y. (1981) *J. Lipid Res. 22*, 1020–1024.

Welt, S. W., Yin, B. W. T., Furukawa, K., Karlsson, K.-A., Old, L. J. & Lloyd, K. O. (1988) *Carbohydr. Res. 178*, 333–336.

Young, W. W., Jr., Portoukalian, J., & Hakomori, S. (1981) *J. Biol. Chem. 256*, 10967–10972.