Cell Adhesion in a Dynamic Flow System as Compared to Static System

GLYCOSPHINGOLIPID-GLYCOSPHINGOLIPID INTERACTION IN THE DYNAMIC SYSTEM PREDOMINATES OVER LECTIN- OR INTEGRIN-BASED MECHANISMS IN ADHESION OF B16 MELANOMA CELLS TO NON-ACTIVATED ENDOTHELIAL CELLS*

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The molecular basis of specific cell recognition is of central importance in current cell biology. Studies to date suggest that four combinations of molecular “families” provide the basis for most recognition events: (i) various adhesive proteins are recognized by members of the integrin family (1-3); (ii) members of the immunoglobulin family interact with each other or with integrins (3, 4); (iii) carbohydrates (CHOs)1 are recognized by members of the lectin family (5-7), particularly selectins (8, 9); (iv) CHOss interact with other CHOss (10). These studies were all based on static adhesion systems; i.e., adhesion molecules are coated on solid-phase and incubated under static conditions with cells expressing specific receptors or CHO epitopes. Using such a static system, we previously reported the specific adhesion, spreading, and enhanced motility of GM3-expressing cells on Gb3- or LacCer-coated solid-phase, based on GM3/Gb3 or GM3/LacCer interaction (11, 12).

Adhesion of neutrophils and monocytes to endothelial cells has been observed under dynamic flow conditions, designed to mimic the microvascular environment (13, 14). Selectin-dependent adhesion of neutrophils under dynamic flow conditions appears to occur preferentially over integrin-dependent adhesion (15). Tumor cell adhesion to activated endothelial cells, as mediated by integrin receptors expressed on tumor cells, and by ICAMs or selectins expressed on activated endothelial cells, has received considerable attention recently (16, 17). However, there is still no firm evidence for involvement of these adhesion molecules in the very initial interaction between circulating tumor cells and non-activated endothelial cells, particularly under dynamic flow conditions. We now present evidence that initial adhesion of B16 melanoma cells to non-activated mouse and human endothelial cells is mediated by interaction between GM3 and LacCer, which are highly expressed on B16 cells and endothelial cells, respectively. This GM3/LacCer interaction apparently occurs prior to activation of endothelial cells, and prior to involvement of ICAMs or selectins, and predominates over integrin- or lectin-mediated cell adhesion in a dynamic flow system.

The abbreviations used are: CHO, carbohydrate; ConA, concanavalin A; Gb3, GalNAcα2→3Galβ1→4Glcβ1→Cer; GM3, NeuAcα2→3Galβ1→4Glcβ1→Cer; GSL, glycosphingolipid; ICAM, intercellular adhesion molecule; IL-1, interleukin-1; LacCer, lactosylceramide (Galβ1→4Glcβ1→Cer); mAb, monoclonal antibody; PBS, phosphate-buffered saline containing 0.1% bovine serum albumin; PC, phosphatidylcholine; PG, paragloboside (nLc,); SPG, sialosylparagloboside; TLC, thin layer chromatography. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature ([1977] Lipids 12, 455-463); however, the suffix -0seCer is omitted.

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**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—B16 melanoma variant cell lines F10, F1, and BL6 were kindly donated by Dr. Isaiah J. Fidler, M. D. Anderson Cancer Center, Houston, TX. F10 and F1 plated high and low lung colonization potential, respectively (18); BL6 produces lung metastases from subcutaneously grown tumors (19). Wheat germ agglutinin-resistant B16 clone WA4, which shows no lung colonization potential (20), was donated by Dr. Max Burger, BioCenter, University of Basel, Switzerland. All these cell lines were characterized by surface expression of Gal-(1,3)GalNAc (with mAb DH2 (27), anti-LacCer T5A7 (28), anti-Gg3 2D4 (29), anti-SLe" SNH3 (30), and anti-H/Le"/LeY MIA-15-5 (31)). Origins, properties, and isoforms of these mAbs are described in the references cited.

**Cell Surface Labeling and Characterization of GSLs in Endothelial Cells** (Including Mouse Lung Microvascular Endothelial Cells)—Human and mouse endothelial cells were cultured in 25-cm² Corning flasks until confluence, and cell surface CHO's were labeled as previously described (32). Cell layers were washed with PBS (pH 7.0), treated with 0.1% trypsin in PBS (pH 7.0) for 1 h at 37 °C, washed with 20 mM Tris-HCl (pH 8.4) containing 150 mM NaCl, and reduced with 0.5 mCi of NaB3H4 in 1 ml of HCl (pH 8.4) for 30 min at room temperature. Next, 1 mM NaBH4 in 20 mM Tris-HCl (pH 8.4) was added and incubated for 30 min at room temperature (32). Cells were collected by rubber scraper and washed with PBS. GSLs were extracted two times from labeled cells with a mixture of 10 pg/ml mouse IgG and IgM. In separate experiments, BL6 cells were treated with 10 μg/ml of mAbs directed against various GSLs, lectins, or adhesion molecules for 1 h at 4 °C, washed with PBS at a concentration of 1 × 10⁶ cells/ml. Methyl-β-D-lactoside and lactose were dissolved in PBS at a concentration of 200 μM. Liposomes (1 μl) were made from 500 nmol of cholesterol, 500 nmol of dipalmitoyl PC, and 200 nmol of GSL in PBS as described previously (11). In this case, GSL concentration was 200 μM. All reactions were done in the presence of 100 μM of galactosynase and 500 μM of NaB3H4. Cells were diluted 2-fold with 100 μl of PBS. 100 μl of BL6 cell suspension (2 × 10⁶ cells) was added to 100 μl of oligosaccharide solution or liposome suspension and incubated for 30 min at 37 °C. After incubation, mixtures of cells with oligosaccharide or liposome were plated on plates coated with LacCer (1 μg/well), human endothelial cells, or mouse endothelial cells, as described above. After incubation at 37 °C for 30 min, wells were washed and adherent cells collected and counted as described above.

**Cell Adhesion Through Glycosphingolipids in Dynamic Flow System**—In order to study surface expression of GSLs on animal lung microvascular endothelial cells, lungs were perfused with PBS (pH 7.0) followed by 0.1% of Ga oxidase washed in PBS (pH 7.0) via a pulmonary artery using a syringe. After 30 min, the lung was perfused with 5 μCi of NaB3H4 in PBS (pH 7.4) and then perfused with cold NaBH4. This was followed by extraction of lung, preparation of GSL fraction, TLC separation, and autoradiography as described above.

**Binding of B16 Cell Variants to GSLs, Lectins, Glycoproteins, and Laminin, and to Endothelial Cells, in a Static Adhesion System**—For preparation of GSL-coated plastic wells, 100 μl of GSL (1 μg) in absolute ethanol was placed on each well of 96-well flat-bottom assay plates (Falcon Probind) and dried at 37 °C. 100 μl of the appropriate concentration of brefinnure or laminin was placed on the adhesion potential, untreated at 4 °C. Wells were coated for longer periods or in duplicate, and non-specific binding sites of each well were coated with 1% bovine serum albumin in PBS at 37 °C for at least 1 h. Wells were then washed with PBS and used as fibronection- or laminin-coated surfaces.

B16 melanoma variant BL6, F10, F1, and WA4 were cultured in 10 ml of RPMI 1640 containing 10% fetal calf serum in 25-cm² Celltak, or dishes containing 0.1% BSA and文化izing for 16 h. For measurement of cell adhesion, labeled cells were detached by treatment in 0.02% EDTA at 37 °C. Detached cells were collected and washed twice with PBS. Cells were suspended in PBS at a density of 2 × 10⁶ cells/ml, and 100-μl aliquots of suspension were added to each well of 96-well plates co-coated with GSL and fibronectin. After addition of cells, plates were centrifuged at 100 × g for 1 min and incubated for 30 min at 37 °C. Plates were washed with PBS, and remaining adherent cells were collected by cell harvester and counted by scintillation counter. Suspensions of human and mouse endothelial cells (8 × 10⁶/ml) were placed in 96- or 48-well flat-bottom plates (purchased respectively from Falcon, Lincoln Park, NJ, and Costar, Cambridge, MA) pretreated with 0.5% gelatin at 37 °C for 1 h. Endothelial cells were cultured until confluence. Medium was removed, endothelial cells were washed with PBS, and labeled B16 cells (1 × 10⁶ cells/well (volume 100 μl) for 96-well plates, 3 × 10⁶ cells/well (volume 300 μl) for 48-well plates) in PBS were placed in each well and incubated at 37 °C for 30 min. In IL-1 stimulation experiments, endothelial cells were treated with 5 units/ml of human recombinant IL-1β (Boehringer Mannheim, 1000 units/ml) for at least 4 h at 37 °C before addition of B16 cells. After incubation, cells were washed twice with PBS, and remaining cells were collected by trypsinization, collected, and counted by scintillation counter.

**Assay for Inhibition of Cell Adhesion by Oligosaccharides, GSL-liposomes, mAbs, and Enzymatic Pretreatment of Cells—**BL6 cells were harvested with 0.02% EDTA, washed with PBS, and suspended in PBS at a concentration of 1 × 10⁶ cells/ml. Methyl-β-D-lactoside and lactose were dissolved in PBS at a concentration of 200 μM. Liposomes (1 μl) were made from 500 nmol of cholesterol, 500 nmol of dipalmitoyl PC, and 200 nmol of GSL in PBS as described previously (11). In this case, GSL concentration was 200 μM. 100 μl of 200 μM of GSL containing 2F5 was added to 100 μl of PBS. After incubation, mixtures of cells with oligosaccharide or liposome were plated on plates coated with LacCer (1 μg/well), human endothelial cells, or mouse endothelial cells, as described above. After incubation at 37 °C for 30 min, wells were washed and adherent cells collected and counted as described above.

BL6 cells (1 × 10⁶/ml) were treated with 10 μg/ml of mAbs directed against various GSLs, lectins, or adhesion molecules for 1 h at 4 °C. After incubation at 37 °C for 30 min, cells were washed twice with PBS, and untreated BL6 cells were added to treated endothelial cells. As controls, BL6 cells or endothelial cells were treated with a mixture of 10 μg/ml mouse IgG and IgM.

**Adhesion Assay in a Dynamic Flow System**—A parallel plate laminar flow chamber connected to a peristaltic infusion pump (model CR55, Harvard Apparatus, Cambridge, MA) was used to simulate the flow shear stresses present in physiological microvascular environments. The flow chamber consists of a glass plate on which a parallel, transparent plastic surface is attached with a Silastic rubber gasket; there is a 114-mm gap between the glass plate and the pump outlet. This gap is connected to an inlet and outlet. A laminar flow with defined shear stress is achieved by manipulation of the infusion pump, which is connected to the flow chamber. Endothelial cells are grown as a monolayer, or adhesion molecules are coated on the glass plate, and a laminar flow of cell suspension is passed through the chamber. Cell movements are observed under an inverted phase-contrast microscope (Diaphot-TMD Nikon) and recorded by time-lapse videocassette recorder. Adhesion is observed as rolling followed by stopping

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of cells. This assembly is essentially the same as that described by Lawrence et al. (13, 15). Number of cells bound during 3 min at different shear stresses from 0.4 to 4.8 dynes/cm² were counted from several fields recorded on videotape. Wall shear stress (T) was calculated by the equation of Lawrence et al. (13, 14):

\[ T = 3 \mu Q/2ba^2 \]

where \( \mu \) = coefficient of viscosity (1.0 cP), \( Q \) = volumetric flow rate (cm³/s), \( a \) = half channel height (in this case, \( 5.7 \times 10^{-3} \) cm), and \( b \) = channel width (1.3 cm).

Coating of Adhesion Molecules or Endothelial Cells on Glass Plates in the Dynamic Flow System—For lectins, fibronectin, laminin, and GSLs used in this study, 10 μl of solution having a concentration of 20-200 μg/ml was placed on a marked area (0.5-cm diameter) on a glass plate (38 x 75 mm, Corning Glassworks, Corning, VA) and dried in a refrigerator at 4 °C. Dried plates were immersed in PBS at 37 °C for 1 h and washed extensively with several changes of PBS. For GSL coating, GSL-liposomes were prepared from 200 μg of GSL, 200 μg of cholesterol, and 400 μg of PC in 1 ml of PBS as described previously (11). 10 μl of GSL-liposome solution was placed on glass plate and dried at 4 °C, and plates were washed with PBS as described above. Quantity of adsorbed molecules was determined using 125I labeling for lectins, fibronectin, or laminin, or [3H]cholesterol labeling for GSL-liposomes. Under these conditions, almost the entire quantity of protein, regardless of whether fibronectin, laminin, or lectin, was adsorbed on the glass plate. For example, when 100 μg/ml fibronectin was applied, 12.5 ± 1.8 ng/mm² was adsorbed. Likewise, almost all GSL-liposome dried on the glass plate was adsorbed; e.g. when 200 μg/ml GSL-liposome was applied, 31.3 ± 5.2 ng GSL/mm² was adsorbed.

Endothelial cells were coated by placing 100 μl of a suspension containing 2 × 10⁵ mouse or human endothelial cells on glass plates and culturing in a CO₂ incubator at 37 °C until confluence.

Plates coated with adhesion molecules or endothelial cells were affixed in a flow chamber, and a suspension of B16 melanoma cells was passed through the chamber as described in the preceding section. B16 cells were harvested from culture by 0.02% EDTA in PBS, and SUSPENDED in PBS at a concentration of 1 × 10⁷/ml.

RESULTS

Patterns of GSL Surface Expression on Mouse Melanoma B16 Variants, Human and Mouse Endothelial Cells, and Mouse Lung Microvascular Endothelial Cells—GSL compositions of BL6, F10, and F1 cells were essentially the same, consisting of GlcCer, GM3, LacCer, and a minor quantity of SPG. WA4 was characterized by a much lower chemical quantity of GM3, but higher quantities of SPG, PG, and IV²FucαLcα. These B16 variants differed markedly in surface reactivity with anti-GM3 mAb DH2 (BL6 ≈ F10 > F1 >> WA4) (Fig. 1A). Mouse endothelial cells contained GlcCer, LacCer, and Gb4 or PG as neutral GSLs, a major ganglioside with the same mobility as GD1α, and relatively small quantities of GM3 (Fig. 1B). GD3 was absent from both mouse and human endothelial cells, as indicated by lack of immunostaining with anti-GD3 mAb (data not shown). Based on labeling experiments, LacCer was the major GSL exposed at the cell surface in both mouse and human endothelial cells. In the Gal oxidase/NaB₃H₄ experiment with mouse lung microvascular endothelial cells, three GSL bands were observed: LacCer, Gb3 (or Gg3), and Gb4 (Fig. 1C). Human endothelial cells contained GlcCer, LacCer, Gb4, and PG as neutral GSLs, and GM3 and SPG as major gangliosides (data not shown), in agreement with a previous report (34).

Adhesion of Mouse Melanoma B16 Variants to Non-activated Human and Mouse Endothelial Cells Is Based on GM3/LacCer Interaction, and Occurs Prior to Integrin-mediated Adhesion—B16 melanoma variants BL6, F10, F1, and WA4, which show declining metastatic potential and reactivity with anti-GM3 mAb DH2 in that order (see “Experimental Procedures”), also showed relative adhesion to LacCer- or Gg3-coated plates in the same order (i.e. BL6 > F10 > F1 >> WA4) at longer incubation times (Fig. 2, C and D). BL6, F10, and F1 cells showed similar integrin-dependent adhesion to fibronectin-coated plates (Fig. 2A), whereas adhesion of WA4 cells was much lower (data not shown; see “Discussion”). It
should be noted that BL6 adhesion to LacCer- or Gg3-coated plates occurred earlier than integrin-dependent adhesion to fibronectin or laminin (10–20 min versus 30–50 min under comparable experimental conditions) (Fig. 2B). This finding suggests that cell adhesion based on GSL/GSL interaction is a faster process than integrin-dependent adhesion, which may explain the differences observed for static adhesion systems versus dynamic flow systems (see following section).

Since high quantities of cell surface LacCer are expressed in human and mouse endothelial cells, including mouse lung microvascular endothelial cells, we compared adhesion (and inhabitation of adhesion) of BL6 cells to human and mouse endothelial cells, and to LacCer-coated plates. All three types of adhesion were inhibited by LacCer or Gg3 at 50–100 μM concentration, and by methyl- or ethyl-β-lactoside at 50–100 mM concentration, but not by free lactose or methyl-β-N-acetylglactosaminide at the latter concentration (Fig. 3). The difference in adhesion of the four B16 variants to human and mouse endothelial cells was particularly obvious when endothelial cells were in the non-activated state (Fig. 4, A and B), and adhesion was ≈10 times higher to mouse endothelial cells than to human endothelial cells (maximum binding 1–1.5 × 10^6 versus 1–2.5 × 10^4 cells/well, respectively). For mouse endothelial cells, differences in adhesion of the B16 variants were obvious even after activation (Fig. 4D). Since the degree of LacCer expression on mouse endothelial cells is similar to that on human endothelial cells, the much greater adhesion of melanoma cells to mouse endothelial cells is assumed to be due to expression of unidentified adhesion molecules on non-activated mouse endothelial cells, and to a synergistic effect of GM3/LacCer and integrin-dependent adhesion, as previously reported (35). In human endothelial cells, differences in adhesion of B16 variants were less clearly observed after IL-1β stimulation (Fig. 4C), suggesting that adhesion following activation is based mainly on integrin receptors, ICAMs, or selectins.

BL6 adhesion to non-activated human or mouse endothelial cells was blocked by pretreatment of BL6 cells with mAb DH2 or α-sialidase (Fig. 5, A and B), but no such effect was observed for activated human endothelial cells (Fig. 5C). Conversely, BL6 adhesion to non-activated endothelial cells was blocked by pretreatment of endothelial cells with anti-LacCer mAb TS5A7 (Fig. 5, D and E), but this effect was not observed for activated human endothelial cells (Fig. 5F). These findings suggest that B16 adhesion to non-activated human and mouse endothelial cells is based on GM3/LacCer interaction.

**Adhesion of BL6 Cells to GSL-coated Glass Plates Is Predominant Over That to Lectin- or Fibronectin-coated Surfaces in a Dynamic Flow System**—A dynamic flow system as described by Lawrence et al. (13–15), in which a cell suspension flows over a GSL-, lectin-, or fibronectin-coated glass plate and is recorded on videotape (see "Experimental Procedures"), is designed to mimic conditions in the microvascular environment in which tumor cell metastatic deposition takes place.

In such a system, adhesion of BL6 cells to glass plates coated with Gg3 or LacCer was high, but no adhesion to surfaces coated with PG or GM3 was observed (Fig. 6A). Adhesion to surfaces coated with ConA or an E. coralloidendron lectin that recognizes N-acetyllactosamine residue (24) was not pronounced unless very high concentrations (≥200 μg/ml) of these lectins were applied (Fig. 6B), despite the fact that much lower concentrations (10–20 μg/ml) of these lectins were sufficient to cause strong adhesion and spreading of BL6 cells in a static system (Fig. 6C). Fibronectin or laminin, even applied at very high concentrations (≥100 μg/ml), had no significant effect on BL6 adhesion in a dynamic system (Fig. 6B), whereas fibronectin or laminin concentrations as low as 20 μg/ml produced strong adhesion and spreading in a static system (Fig. 6C). Fibronectin-dependent adhesion, even with 200 μg/ml concentration, required much longer incubation (20–30 min) as compared to ConA-dependent adhesion. Adhesion based on GM3/Gg3 interaction was much weaker than ConA- or fibronectin-dependent adhesion in a static system (Fig. 6C).

BL6 adhesion to LacCer-coated glass plates in a dynamic system was obvious even at high shear stress (1.5–3.0 dynes/cm²) and remarkable at low shear stress (<1.0 dynes/cm²).
on lectins (5-7), selectins takes place under dynamic flow conditions, i.e. in a moving system to lectin- and fibronectin-dependent adhesion under conditions. In this study, we have (i) demonstrated that adhesion has been clearly documented, there have been no comparative studies of adhesion under static versus dynamic flow conditions. (ii) Order of adhesion of the B16 variants on mouse endothelial cells is inhibited by pretreatment of BL6 cells with anti-GM3 mAb DH2 (Fig. 8B).

**Discussion**

Cell adhesion mediated by various mechanisms (see Introduction) has been repeatedly studied under static conditions. Physiologically, however, adhesion of blood cells (or tumor cells) among themselves (aggregation) or to endothelial cells takes place under dynamic flow conditions, i.e. in a moving bloodstream. While the role of CHO in cell adhesion based on lectins (5-7), selectins (8, 9), or GSL/GSL interaction (10) has been clearly documented, there have been no comparative studies of adhesion under static versus dynamic flow conditions.

In this study, we have (i) demonstrated that adhesion of melanoma cells to non-activated endothelial cells is based on GM3/LacCer interaction, and (ii) compared this adhesion system to lectin- and fibronectin-dependent adhesion under dynamic flow conditions.

There are several lines of evidence that adhesion of B16 melanoma cells to non-activated endothelial cells depends on GM3/LacCer interaction: (i) GM3 is the major GSL expressed on B16 cells, and the order of adhesion of the four B16 variants on endothelial cells (both human and mouse) is the same as their order of GM3 expression (BL6 > F10 > F1 > WA4). (ii) LacCer is the major cellular surface-exposed GSL strongly labeled by Gal oxidase/NaB3H4 in human endothelial cells (34), mouse endothelial cells, and mouse lung microvascular endothelial cells. (iii) Order of adhesion of the B16 variants on LacCer- or Gg3-coated plates is the same as their order of GM3 expression. (iv) Adhesion of B16/BL6 (the highest GM3 expression) to human and mouse endothelial cells is inhibited by pretreatment of BL6 cells with anti-GM3 mAb DH2 or with sialidase (which eliminates cell surface GM3). (v) Adhesion of BL6 to human or mouse endothelial cells is inhibited by pretreatment of the endothelial cell monolayer with anti-LacCer mAb TSAT, but not with other mAbs including anti-ELAM-1. 3B7. On the other hand, pretreatment of BL6 cells with anti-Gal-binding lectin mAb 5D7 did inhibit adhesion (on mouse endothelial cells only), supporting the previous suggestion that Gal-binding lectin associated with B16 melanoma plays some role in B16/endothelial cell adhesion (25). Binding of mAb 5D7 to B16 variants, measured by...
coated surfaces was negligible at low concentration

concentration
cell binding

various GSLs or adhesive proteins in dynamic flow and static

binding lectin in adhesion of melanoma cells to endothelial
cells adhered strongly to plates coated with 10 μg/ml fibronectin or laminin; however, an incubation period of >30 min was required for this adhesion process, in contrast to the rapid adhesion based on GSL/GSL interaction. In the dynamic system, B16 cell adhesion to fibronectin- or laminin-coated glass surfaces was negligible even at a fibronectin or laminin concentration of 100 μg/ml, and even at low shear stress (<1.0 dynes/cm²). B16 cells did not adhere to glass plates coated with GM3-liposomes, PG-liposomes, and control PC-cholesterol liposomes (lacking GSL). In these experiments, liposomes were applied on glass and air-dried in a refrigerator (see “Experimental Procedures”). The lack of adhesion to GM3 and other liposomes indicates that B16 cell adhesion to LacCer-liposome-coated plates is highly specific.

Blood-borne tumor metastasis is generally believed to be initiated by adhesion of tumor cells to microvascular endothelial cells (36, 37) and to activate platelets, leading to tumor

FIG. 6. Adhesion of BL6 cells to glass plates coated with various GSLs or adhesive proteins in dynamic flow and static systems. Panel A, freshly harvested BL6 cells were suspended in PBS (1 × 10^6/ml) and injected into a dynamic flow system (see text) under various shear stresses as shown on the abscissa. GSL-liposomes (200 μg of GSL/ml) were coated on a marked area (0.5-cm diameter) of the glass plate. Quantity of GSL adsorbed on the marked area was calculated as 31.3 ± 5.2 ng/mm². After 3 min of flow, the number of adherent cells on the marked area was counted. □, GSL; △, LacCer; ○, GM3; ▼, PG; ▽, control liposome without GSL. Panel B, procedure as in panel A, except that glass plates were coated with various adhesive proteins instead of GSL-liposomes. When a 100 μg/ml solution of fibronectin was applied, a quantity of fibronectin adsorbed on a marked area of 0.5-cm diameter was found to be 12.5 ng/mm². After 3 min of flow, the number of adherent cells on the marked area was counted. □, GSL; △, LacCer; ○, GM3; ▼, PG; ▽, control liposome without GSL. Panel C, glass plates were coated with ConA, fibronectin, and Gaβ3 at the same concentrations as in panels A and B and tested under static conditions. Labeled BL6 cells (1 × 10^6/ml; 180,000 cpm) were added to each plate and incubated at 37 °C for various durations as shown on the abscissa. ▼, ConA (200 μg/ml); ○, Gaβ3 (200 μg/ml); △, fibronectin (100 μg/ml); ▽, Gaβ3 (200 μg/ml).

cytolysis, was minimal. Therefore, the role of Gal

binding lectin in adhesion of melanoma cells to endothelial
cells appears to be minor compared to the role of GM3/ LacCer interaction.

While BL6 adhesion to LacCer- or Gaβ3-coated plates was evident at an earlier time than adhesion to fibronectin- or laminin-coated plates under static conditions, maximal strength of fibronectin-, laminin-, or ConA-mediated adhesion was greater than that of adhesion based on GSL/GSL interaction. Adhesion under dynamic flow conditions differed in many important respects. In the dynamic system, BL6 adhesion to LacCer- or Gaβ3-coated surfaces was stronger than integrin-dependent adhesion to fibronectin- or laminin-coated surfaces, and adhesion to ConA or Erythrina lectin-coated surfaces was negligible at low concentration (20 μg/ml) even at low shear stress, but became evident at high concentration (~200 μg/ml). In the static system, as little as 10-20 μg/ml of these lectins was sufficient to induce strong cell binding or spreading. Similarly, in the static system, BL6

FIG. 7. Inhibition by various reagents of BL6 cell adhesion to LacCer-coated plates in a dynamic system. The glass plate in the dynamic system was coated with 200 μg/ml LacCer-liposomes. BL6 cells were harvested, washed, and suspended in PBS (1 × 10^6 cells/ml). The cell suspension was mixed with 1 ml of mAb DH2 (10 μg/ml) or 1 ml of sialidase (0.1 unit/ml) at 4 °C for 30 min, washed, resuspended in PBS at 1 × 10^6 cells/ml, and injected into the flow chamber. In another experiment, 1 × 10^6 BL6 cells were suspended in 9 ml of PBS plus 1 ml of 0.5 M ethyl-β-lactoaside, and the cell suspension (1 × 10^6 cells/ml) was incubated at 37 °C for 30 min and injected into the flow chamber. Cell adhesion during 3 min at various shear stresses was quantified. □, untreated BL6 cells (control); △, cells treated with 0.1 M ethyl-β-lactoaside; ▲, sialidase; ●, anti-GM3 mAb DH2.

FIG. 8. Inhibition by various reagents of B16 cell adhesion to mouse endothelial cells in a dynamic system. Mouse endothelial cells were cultured on glass plates which were then placed in the flow chamber (see text). B16 cells (1 × 10^6/ml) in PBS were passed through the chamber, and the number of adherent cells was counted. Panel A, adhesion of untreated B16 variants. □, BL6; △, F10; ●, F1; ▽, WA4. Panel B, adhesion of BL6 cells treated with: △, mAb DH2; ▲, lactose (0.1 M); ●, ethyl-β-lactoaside (50 mM); ○, control (no treatment).
cell-platelet or tumor cell-neutrophil aggregation, a major cause of microembolism and metastatic deposition. Expression of selectin GMP-140, which recognizes the tumor-associated antigens sialosyl-Le^a and sialosyl-Le^b, may play an important role in tumor cell adhesion and aggregation (38, 39). Results of the present study make clear the pathobiological significance of GM3/LacCer interaction as the basis for specific adhesion of melanoma cells to non-activated endothelial cells under dynamic flow conditions. The possibility that this represents the initial event in tumor cell metastasis is suggested by the apparent correlation of strength of adhesion (under high shear stress) with relative metastatic potential of the melanoma cells. Recent interest has been focused on the possible mediation of tumor cell adhesion by ICAMs or selectins which are expressed on activated endothelial cells (16, 17). For this process, a sufficient local concentration of factors secreted from tumor cells (e.g. TGFβ or TNFα) is required to activate endothelial cells in situ. In dynamic microvascular flow, adhesion of circulating tumor cells to non-activated endothelial cells is a prerequisite for the process of endothelial cell adhesion by tumor cells, and therefore an essential step in initiation of metastasis. Tumor-associated lectins which recognize the Gal residue of N-acetyllactosamine have been assumed to play a role in such adhesion (25, 40), and Gal-binding lectin on endothelial cells has been claimed to mediate adhesion of tumor cells to endothelial cells (41).

It is highly plausible that initial adhesion of tumor cells, under physiological dynamic flow conditions, to non-activated endothelial cells is based on GSL/GSL interaction, which induces activation of endothelial cells, and is subsequently reinforced by induction of selectin or ICAM expression on activated endothelial cells. This trend is particularly pronounced in a dynamic flow system, in which blood flow mediates GSL/GSL interaction at the endothelial cell surface, and integrin-dependent adhesion is relatively minor. It should be noted that BL6 adhesion to LacCer strongly stimulated cell migration, in agreement with our previous report (35), thereby promoting transendothelial migration. Adhesion of tumor cells to endothelial cells could induce activation of endothelial cells through TGFβ or TNFα. Thus, a series of "cascade" reactions could be triggered by GSL/GSL interaction between tumor cells and non-activated endothelial cells. In vivo metastasis based on GSL/GSL interaction can be blocked by oligosaccharides, GSL derivatives, or GSL-liposomes. Studies based on this approach are in progress.

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REFERENCES

1. Hynes, R. O. (1987) Cell 48, 549–556.
2. Ruoslabadi, E. J. (1991) J. Clin. Invest. 87, 1–5.
3. Hembler, M. E. (1990) Annu. Rev. Immunol. 8, 305–400.
4. Springer, T. A. (1990) Annu. Rev. Cell Biol. 6, 359–402.
5. Barondes, S. H. (1981) Annu. Rev. Biochem. 50, 207–231.
6. Sharon, N. (1984) Biol. Cell 51, 235–246.
7. Zuckerman, K. (1988) J. Biol. Chem. 263, 9557–9560.
8. Springer, T. A., and Laasko, A. L. (1991) Nature 349, 196–197.
9. Brandley, B. K., Swidler, S. J., and Robbina, P. W. (1990) Cell 63, 861–863.
10. Hakomori, S. (1991) Pure & Appl. Chem. 63, 473–482.
11. Kojima, N., and Hakomori, S. (1988) J. Biol. Chem. 263, 20159–20162.
12. Kojima, N., and Hakomori, S. (1991) J. Biol. Chem. 266, 17652–17658.
13. Lawrence, M. B., Smith, C. W., Eskin, S. G., and McLaiister, L. V. (1990) Blood 75, 227–237.
14. Lawrence, M. B., McIntyre, L. V., and Eskin, S. G. (1987) Blood 70, 1284–1290.
15. Lawrence, M. B., and Springer, T. A. (1991) Cell 65, 859–873.
16. Rice, G. E. and Blevins, M. P. (1989) Science 246, 1303–1306.
17. Walz, G., Aruffo, A., Kolanus, W., Beviloquio, M. F., and Seed, B. (1990) Science 250, 1132–1135.
18. Fuller, J. J. (1975) Cancer Res. 35, 219–224.
19. Hart, J. R. (1979) Am. J. Pathol. 97, 587–600.
20. Tao, T.-W., and Burger, M. M. (1977) Nature 267, 437–438.
21. Laufacher, L. (1987) Histochem. J. 19, 225–234.
22. Sebagh, G., Moore, S. A., Fabo, Z., Schepfer, R. L., and Hart, M. N. (1988) Am. J. Pathol. 134, 1227–1232.
23. Hakomori, S. (1983) in Sphingolipid Biochemistry (Kanfer, J. N., and Hakomori, S., eds) pp. 1–165, Plenum Press, New York.
24. Gilboa-Barber, N., and Mizrahi, L. (1981) Can. J. Biochem. 59, 315–320.
25. Raz, S., and Luton, R. (1985) Cancer Metast. Rev. 6, 435–452.
26. Gahius, H.-J., Engelhardt, R., Cramer, F., Batge, R., and Nagel, G. A. (1985) Cancer Res. 45, 253–257.
27. Young, W. W., Jr., MacDonald, E. M. S., Nowinski, R. C., and Hakomori, S. (1979) J. Exp. Med. 150, 1008–1019.
28. Muroi, K., Suda, T., Nogita, H., Ema, H., Amemura, Y., Miura, Y., Nakauchi, H., Singhal, A. K., and Hakomori, S. (1982) Blood 79, 713–719.
29. Miyake, M., and Hakomori, S. (1981) Biochim. Biophys. Acta 660, 332–334.
30. Gahium, C. G., and Hakomori, S. (1975) J. Biol. Chem. 239, 4311–4317.
31. Saito, T., and Hakomori, S. (1971) J. Lipid Res. 12, 257–259.
32. Gillard, H. K., Jones, M. A., Turner, A. A., Lewis, D. E., and Marcus, D. M. (1990) Arch. Biochem. Biophys. 279, 122–129.
33. Kojima, N., and Hakomori, S. (1991) Glycobiochemistry (Nicolson, G. L., and Milas, L., eds) pp. 5–26, Raven Press, New York.
34. Muroi, K., Suda, T., Nogita, H., Ema, H., Amemura, Y., Miura, Y., Nakauchi, H., Singhal, A. K., and Hakomori, S. (1982) Blood 79, 713–719.
35. Miyake, M., and Hakomori, S. (1981) Biochim. Biophys. Acta 660, 332–334.
36. Gahium, C. G., and Hakomori, S. (1975) J. Biol. Chem. 239, 4311–4317.
37. Saito, T., and Hakomori, S. (1971) J. Lipid Res. 12, 257–259.
38. Gillard, H. K., Jones, M. A., Turner, A. A., Lewis, D. E., and Marcus, D. M. (1990) Arch. Biochem. Biophys. 279, 122–129.
39. Kojima, N., and Hakomori, S. (1991) Glycobiochemistry (Nicolson, G. L., and Milas, L., eds) pp. 5–26, Raven Press, New York.
40. Muroi, K., Suda, T., Nogita, H., Ema, H., Amemura, Y., Miura, Y., Nakauchi, H., Singhal, A. K., and Hakomori, S. (1982) Blood 79, 713–719.
41. Kojima, N., and Hakomori, S. (1991) Glycobiochemistry (Nicolson, G. L., and Milas, L., eds) pp. 5–26, Raven Press, New York.