

A Disaccharide Precursor of Sialyl Lewis X Inhibits Metastatic Potential of Tumor Cells¹

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ABSTRACT

Clustered presentation of sialyl Lewis X (sLe^X) on tumor cell mucins is thought to facilitate metastasis through binding to selectin adhesion receptors expressed on platelets, leukocytes and endothelial cells. Thus, interfering with sLe^X assembly might provide a chemotherapeutic method for treating metastatic disease. Prior studies have shown that peracetylated disaccharides can act in cells as substrates for the assembly of oligosaccharides related to sLe^X synthesis, and the assembly of oligosaccharides on the disaccharides diverts the assembly of sLe^X from endogenous cell surface glycoconjugates. Here, we show that treatment of cultured human adenocarcinoma cells with micromolar concentrations of the peracetylated disaccharide, (Ac)₆GlcNAcβ1,3Galβ-O-naphthalenemethanol (AcGnG-NM) reduces the expression of sLe^X and diminishes binding *in vitro* to selectin-coated dishes, thrombin-activated platelets, and tumor necrosis factor α-activated endothelial cells. Altering glycosylation in this way significantly reduced the ability of tumor cells to distribute to the lungs of wild-type mice over a 3-h period after *i.v.* injection. No significant difference in biodistribution was noted after the injection of AcGnG-NM-treated tumor cells into P-selectin deficient mice, although the extent of lung seeding was reduced compared with that in wild-type mice. *In vitro*, we demonstrate that normal mouse platelets, but not P-selectin-deficient platelets, bound to control tumor cells and protected them from leukocyte-mediated cytotoxicity. Conversely, treatment of tumor cells with disaccharide markedly reduced the ability of normal platelets to protect them from cytotoxicity. Finally, in an experimental metastasis model, we show that treatment of tumor cells with the disaccharide markedly reduced their lung colonization potential after injection into severe combined immunodeficient mice. These findings suggest that this compound may represent a novel class of chemotherapeutic agents for prevention and treatment of metastatic disease.

INTRODUCTION

Tumor metastasis is thought to depend on cell adhesion between blood-borne tumor cells, circulating platelets (facilitating platelet-tumor emboli), and endothelia, promoting arrest in the vasculature, growth, and extravasation (1–3). Several types of adhesion receptors and ligands have been described as important elements in this process, including selectins, chemokines, and integrins (1, 4, 5). Overall, these features of tumor cell adhesion resemble characteristics of leukocyte extravasation during inflammation. In both cases, expression of the oligosaccharides sLe^X [Siaα2,3Galβ1,4(Fucα1,3)GlcNAc]³ and sLe^a [Siaα2,3Galβ1,3(Fucα1,4)GlcNAc] on cell-surface glycoconjugates endows cells with the ability to adhere to E-, P-, and L-selectins present on endothelia, platelets, or leukocytes. Studies of human

tumors and mice bearing genetic alterations in one or more selectins underscore the importance of these interactions in hematogenous spread of cancer cells (6–10).

The carbohydrate ligands for the selectins are predominantly O-linked glycoprotein mucins and glycolipids that display sLe^X or sLe^a in clustered arrangements (11–13). Several aggressive solid tumors display significant reactivity to anti-sLe^X mAbs and to E- and P-selectins. These include a relatively large proportion of tumors from the lung, colon, and breast (4, 7, 13–17). Adhesion interactions involving sLe^X constitute important early steps in the pathophysiology of metastasis (9, 10, 18–21) possibly by stabilizing “neoplastic emboli” via P-selectins on platelets or L-selectin on leukocytes, or by facilitating adhesion to, and possible extravasation through, the endothelium. The importance of these interactions derives from studies in patients postresection from colon, lung, gastric, and other carcinomas that show that survival correlates inversely with tumor expression of sLe^X (22–24).

With this information in mind, we have focused on the development of small molecule inhibitors that might block the expression of Lewis carbohydrate antigens on cells. Per-*O*-acetylated disaccharides (acetylated forms of Galβ1,4GlcNAcβ-*O*-naphthalenemethanol (AcGnG-NM) or GlcNAcβ1,3Galβ-*O*-naphthalenemethanol (AcGnG-NM)) are taken up by cells, deacetylated, and acted on as substrates by relevant glycosyltransferases located in the Golgi. Assembly of oligosaccharides on the disaccharides takes place, resulting in the diversion of glycan biosynthesis from endogenous glycoconjugates (25–27). The result is a concomitant reduction of sLe^X expression on the cell surface. The monosaccharide GalNAcα-*O*-benzyl behaves in a similar fashion, altering the expression of O-linked chains on mucins of colon and leukemia cell lines *in vitro* and altering cell adhesion to platelets and endothelia (28–30). However, much higher concentrations of the monosaccharide are needed to achieve a similar level of inhibition as the disaccharide (1–5 mM *versus* 10–50 μM, respectively). This report examines the ability of the more potent disaccharide, AcGnG-NM, to inhibit adhesion of adenocarcinoma cells to both immobilized recombinant selectins and to selectins on activated human platelets and endothelia. We show that inhibiting tumor cell glycosylation in this way leads to decreased interactions with selectins, increased susceptibility to leukocyte-mediated lysis, and reduction in organ colonization in an experimental model of metastasis.

MATERIALS AND METHODS

Cell Culture. Tumor cell lines derived from human colon (LS180, CCL187) or lung (A549, CCL185; A427, CCLHTB53) adenocarcinomas were purchased from American Type Culture Collection (Manassas, VA). HAL-8 human lung adenocarcinoma cells were a kind gift from O. Matsuo (Kinki University, Osaka, Japan). Cells were grown in α-MEM medium (LS180), F12 (A549), F12/DMEM (A427), or RPMI 1640 (HAL-8). All of the media (Life Technologies, Inc.) were supplemented with 10% (v/v) FBS (HyClone Laboratories), L-glutamine (0.3 g/L), streptomycin sulfate (100 μg/ml), and penicillin (100 units/ml). Cells were passaged every 4–6 days using ATV trypsin

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³ The abbreviations used are: sLe^X, sialyl Lewis X; sLe^a, sialyl Lewis a; AcGnG-NM, per-*O*-acetylated GlcNAcβ1,3Galβ-*O*-naphthalenemethanol; AcGnG-NM, per-*O*-acetylated Galβ1,3Galβ-*O*-naphthalenemethanol; Gal, galactose; Fuc, fucose; GlcNAc,

N-acetylglucosamine; Sia, sialic acid; AUS, *Arthrobacter ureafaciens* sialidase; FBS, fetal bovine serum; HMVEC, human microvascular endothelial cell; TNF, tumor necrosis factor; mAb, monoclonal antibody; SCID, severe combined immunodeficient.

solution (Life Technologies, Inc.). HMVECs were grown in EBM-2 media (Clonetics) supplemented with 10% FBS, subcultured using a solution of 0.025% trypsin/0.01% EDTA, and harvested on first or second passage for adhesion assays. All of the cell lines were maintained at 37°C in a humidified incubator under an atmosphere of 5% CO₂ and 95% air.

Peracetylated forms of GlcNAc β 1,3Gal β -*O*-naphthalenemethanol (AcGnG-NM) and Gal β 1,3Gal β -*O*-naphthalenemethanol (AcGG-NM) were prepared as described previously (27). The compounds were dissolved in DMSO and were added to growth medium to achieve the concentrations indicated in the figures. The supplemented medium was then exchanged for the medium in established cultures of cells to avoid lysis caused by adding concentrated DMSO directly to the plates. The final concentration of DMSO was adjusted to $\leq 0.5\%$ (v/v). After the specified number of days, the cells were harvested with 2 mM EDTA in PBS (20 min) and were used for experiments.

Cell Sorting. To detect the presence or absence of the relevant carbohydrate determinants, cells were stained with CSLEX-1 (anti-sLe^x, 5 μ g/ml; Becton-Dickinson) or CA-19-9 (anti-Le^a, 14.5 μ g/ml; Chemicon) and analyzed by flow cytometry (FACScan Becton-Dickinson, Franklin Lakes, NJ). Approximately 5×10^5 cells were incubated for 1 h at 4°C in 100 μ l PBS/1% BSA containing CSLEX-1 or CA-19-9, followed by phycoerythrin-conjugated rabbit antimouse IgG (2 μ g/ml). As a negative control, cells were treated with nonspecific mouse isotype-matched antibody (0.5 μ g/ml; Sigma) for 1 h at 4°C in 100 μ l PBS/1% BSA followed by phycoerythrin-conjugated rabbit antimouse IgG (2 μ g/ml).

Cell Adhesion to Immobilized Selectins. We coated 96-well plates overnight at 4°C with recombinant E- (4 μ g/ml) or P-selectin (2 μ g/ml; R & D Systems) and blocked with 1% BSA/PBS. LS180 cells were grown for 5 days with various amounts of acetylated disaccharide and were harvested and labeled with calcein AM (5 μ M; Molecular Probes) in DMEM/1% FBS, and were allowed to settle at room temperature on selectin-coated wells (5×10^4 cells/well). Plates were then stirred at 75 rpm for 30 min (Orbit shaker; Lab-Line Instruments) followed by immersion upside-down in a vessel filled with HBSS (Sigma), which allowed nonadherent cells to fall under gravity (31). The wells were then washed by aspiration using HBSS. LS180 cells were less adherent to P-selectin and the immersion step was not necessary before washing. Controls included treating tumor cells for 1 h at 37°C with AUS (Calbiochem; 20 mU/ 1×10^6 cells) in 0.05 M HEPES buffer (pH 6.9), pretreating selectin-coated wells with anti-E- or anti-P-selectin mAbs (1 μ g/well; PharMingen), or growing tumor cells in 50 μ M of the inactive disaccharide primer, peracetylated Gal β 1,3Gal β -*O*-NM. Fluorescence was measured using a 96-well fluorimeter (CytoFluor II), and the average of triplicate measurements was determined \pm SE. Cell viability was judged to be $>90\%$ by trypan blue exclusion at the end of each experiment.

Adhesion of Tumor Cells to Activated Human Endothelial Cells. HMVECs were added to 96-well plates (1×10^4 cells/well) in EBM-2 medium (Clonetics) and were allowed to grow to confluence over 2 days. The cells were activated with TNF- α (20 ng/ml; R&D Systems) for 4 h at 37°C. Calcein-labeled tumor cells, harvested after growth for 5 days in various levels of AcGnG-NM, were added to HMVECs at 2.5×10^4 cells/well in 100 μ l of DMEM and were allowed to settle for 20 min. The wells were washed twice with cold PBS, and the extent of binding was determined by fluorimetry. In some experiments, anti-E-selectin mAb (2 μ g/well) was added before the addition of tumor cells, cells were treated with sialidase (20 mU/ 10^6 cells), or TNF- α was omitted.

Adhesion of Activated Human Platelets to Tumor Cells. Tumor cells were seeded into 6-well plates (5×10^4 /well), and allowed to grow into colonies in the presence or absence of 50 μ M AcGnG-NM. After 3 days, platelets were isolated from 15 ml of normal human blood collected into 20% (v/v) Acid-Citrate-Dextrose (ACD) anticoagulant. A platelet-rich plasma was prepared by centrifugation and repeated washing using 5 mM HEPES (pH 6.8), 145 mM NaCl, 4 mM KCl, 0.5 mM sodium phosphate, 5.5 mM glucose, 0.5% BSA, and 25 nM prostaglandin E1 (Sigma). The platelets were then labeled with calcein AM (5 μ M), and counted with a hemocytometer. Wells containing tumor cells were washed with HBSS, and 3×10^6 platelets were added in 1 ml of HBSS, followed by activation with human thrombin (0.8 IU/well; Sigma). The plates were rocked for 10 min, and the wells were washed twice with HBSS, fixed with 5% formalin in HBSS, and analyzed by fluorescence microscopy. Controls included pretreatment of tumor cells with AUS [20

mU/well in 0.05 N HEPES (pH 6.9), 1 mM CaCl₂, and 1 mM MgCl₂], pretreatment of tumor cells with *O*-sialoglycoproteinase (2.4 μ g/ml; Cedarlane) before the addition of platelets, addition of anti-P-selectin mAb (10 μ g/ml in HBSS) to the platelet suspension before thrombin activation and addition to tumor cells, or omission of thrombin.

Platelets bound to tumor cells were visualized by fluorescence microscopy (NikonDiaphot) with a digital camera (Nikon) linked to an Apple Macintosh computer running Adobe Photoshop. The fluorescence image showing the platelets was superimposed on a phase-contrast picture of the cells, and the number of attached platelets was quantified. A "Platelet Association Index" was generated for each well by dividing the number of tumor-associated platelets by the area occupied by tumor cells.

Biodistribution Studies in Mice. LS180 cells were grown for 3 days in the presence or absence of 50 μ M AcGnG-NM. Methyl-[³H]thymidine (10 μ Ci/ml; NEN Life Sciences Products) was added to the medium, and cells were incubated for another 3 days. The cells were then harvested with EDTA, resuspended in sterile 0.9% saline, and injected (1×10^5 cells/100 μ l) into the lateral tail vein of anesthetized (inhaled methoxyflurane, Janssen Pharmaceuticals) 6–8 week-old wild-type C57BL/6 mice or P-selectin-deficient mice bred on the same background (Jackson Laboratory; Ref. 32). On awakening, mice were observed for 3 h, anesthetized, bled (~ 200 μ l each), sacrificed via cervical dislocation, and dissected for collection of lungs, liver, kidney/adrenals, spleen, and brain. The organs were digested at 55°C overnight with proteinase K (0.15 μ g/ml; Boehringer Mannheim) in 2 ml of PBS containing 1% SDS, and homogenized by repeated passage through an 18-gauge needle. The amount of radioactive DNA in the blood and the organ extracts was then determined using an Easy DNA kit (Invitrogen) and liquid scintillation spectrometry. The total counts in blood were estimated by assuming a total blood volume of 2 ml/mouse. Proper attention has been given to experimental ethical considerations toward animals as prescribed by the Animal Subjects Program at the University of California.

Tumor Formation. LS180 cells were grown in the presence or absence of 50 μ M AcGnG-NM for 6 days, harvested with EDTA, and resuspended in sterile PBS. Approximately 3×10^5 tumor cells in 150 μ l of PBS were injected into the lateral tail vein of anesthetized 7-week-old immunodeficient mice (Fox Chase SCID; Charles River, MA). The mice were then maintained in microb-free housing with free access to standard laboratory chow and water, and inspected regularly for any signs of distress. After 4 weeks, mice were euthanized by CO₂ asphyxiation under anesthesia, and lungs, livers, brains, kidneys/adrenals, and spleens were fixed in Bouin's solution (Sigma) for 6 h, followed by transfer to 70% ethanol. Lungs for each animal were inspected under a dissecting microscope for the total number of surface tumors. Histological sections (H&E) were examined for tumor foci, and representative photomicrographs were taken. The other organs were also reviewed histologically for any tumor foci.

Cytolytic Assays. LS180 cells were grown to near-confluence in the presence or absence of 50 μ M AcGnG-NM for 6 days, harvested using 2 mM EDTA/PBS, washed, and resuspended in 2 ml RPMI 1640 containing 10% FBS and 15 μ Ci Na₂⁵¹CrO₄ (435 mCi/mg, DuPont NEN). After 2 h, at 37°C, the cells were then washed twice with medium and placed into a conical 96-well plate (1500 cells/well). Effector cells were prepared from normal C57BL/6 mouse spleens by mincing the tissue and sieving the cells over a fine screen. Red cells were lysed by resuspension in 0.83% NH₄Cl in PBS, and the enriched leukocytes were resuspended in RPMI medium containing 10% FBS and 200 units/ml of recombinant human IL-2 (Life Technologies, Inc.). After 3 days of culture, the leukocytes were added to the wells containing tumor cells. Some wells also received platelets (10^4 platelets/tumor cell) isolated from pooled whole blood (2–4 mice/group) prepared by the same procedures described above for the isolation of human platelets (33). After 3 h at 37°C, the amount of ⁵¹Cr released was measured by centrifuging the plate at 1500 rpm and taking an aliquot of the supernatant. Spontaneous release (R_{spont}) of radioactivity was measured by incubating target cells in RPMI medium only. Maximum release (R_{max}) was measured after complete lysis of targets in 2% SDS. Specific lysis was determined according to the equation: % specific lysis = $(R_{\text{exp}} - R_{\text{spont}}) \times 100 / (R_{\text{max}} - R_{\text{spont}})$, where R_{exp} = counts released in the presence of effector cells. In some experiments, labeled tumor cells were added to whole human blood (1.6 ml mixed with 0.4 ml of Acid-Citrate-Dextrose anticoagulant) and incubated with stirring at 37°C for 3 h.

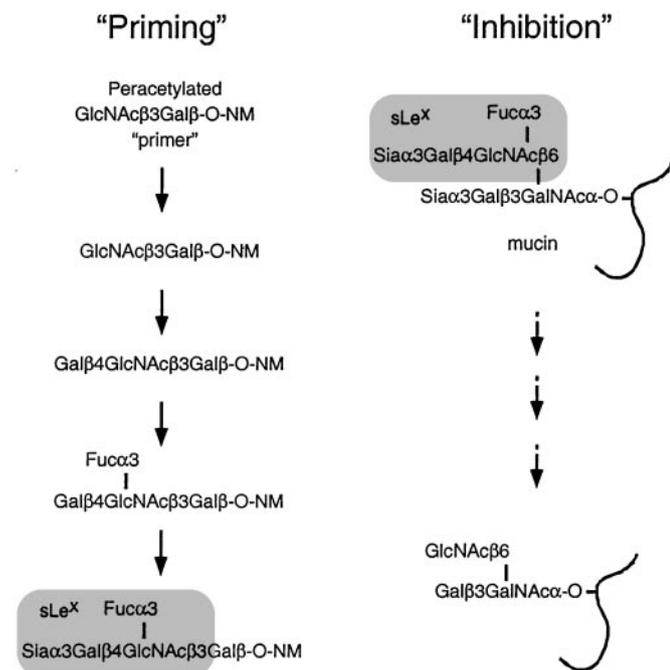


Fig. 1. Inhibiting tumor cell-surface sLe^X using a disaccharide primer. On the left, AcGnG-NM passively enters cells by diffusion, undergoes rapid deacetylation, and acts as a substrate for the assembly of oligosaccharides related to Lewis-type antigens. "Priming" in this way inhibits terminal glycosylation on endogenous glycoprotein substrates as shown on the right, resulting in a reduction in cell-surface sLe^X.

RESULTS

Previous studies showed that cells take up and rapidly deacetylate peracetylated disaccharides (25, 26) and assemble oligosaccharides onto the deacetylated disaccharide. The result is reduced cell-surface levels of the relevant endogenous terminal oligosaccharide (e.g., sLe^X), as illustrated in Fig. 1. Several disaccharides related to mucin-like oligosaccharides that carry sLe^X determinants were shown to be effective as primers, with peracetylated GlcNAcβ1,3Galβ-O-NM (AcGnG-NM) exhibiting the highest potency (27). Priming of oligosaccharides in this way inhibited the expression of sLe^X by HL-60 and U937 cell lines. In this study, we examined the effect of this disaccharide on selectin-binding and tumor-forming properties of LS180 human colon adenocarcinoma cells. These cells were chosen because they express carbohydrate ligands known to bind to E- and P-selectins, and they form lung tumors in an experimental murine model of hematogenous metastasis (9, 10, 13, 16, 34).

Treatment of LS180 Cells with AcGnG-NM Reduces Cell-Surface sLe^X but not sLe^a. Tumors of the gastrointestinal tract generally express relatively high levels of both sLe^X and sLe^a. Because either of these oligosaccharides may mediate binding to selectins, we examined whether treatment of LS180 cells with AcGnG-NM could inhibit expression of either oligosaccharide on the cell surface. Treatment with 50 μM AcGnG-NM resulted in significant inhibition of cell-surface sLe^X, whereas it had no effect on cell-surface sLe^a (Fig. 2).

Adhesion to Selectins Is Altered in Disaccharide-treated Tumor Cells. Treatment of LS180 cells with AcGnG-NM was not toxic to the cells up to 100 μM, based on growth curves and exclusion of trypan blue. However, treatment with the disaccharide inhibited expression of sLe^X on the cell surface in a dose-dependent manner, as measured by ELISA using CSLEX-1 mAbs to probe the cell surface.⁴

When the disaccharide was removed from the culture medium, the ligand reappeared on the cell surface with a $t_{1/2}$ of ~6 h, indicating that no permanent damage to the cells had occurred. Inhibiting the expression of sLe^X in this way reduced the ability of LS180 cells to adhere to recombinant E- and P-selectin immobilized on plastic dishes (Fig. 3). Adhesion to P-selectin was more sensitive to the inhibitor than was adhesion to E-selectin under these conditions. The inhibitory effect of AcGnG-NM was specific because the incubation of cells with acetylated Galβ1,3Galβ-O-NM (AcGG-NM) had no effect on expression of sLe^X (data not shown) or adhesion to either selectin conjugate (Fig. 3). The maximum extent of inhibition approached the values obtained when the cells were pretreated with sialidase or blocking antibody to the corresponding selectin.

AcGnG-NM Inhibits Adhesion to Activated Endothelia and Platelets. In the circulation, tumor cells can encounter E- and P-selectins expressed on endothelial cells, P-selectin on platelets, and L-selectin on leukocytes. Because the presentation of receptors on cells may differ from their arrangement when immobilized on plastic surfaces, we challenged LS180 cells to bind to E-selectin expressed on TNF-α-activated HMVECs (Fig. 4). In this system, adhesion was mostly dependent on E-selectin expression because blocking antibody or absence of TNF-α stimulation dramatically lowered the extent of adhesion. AcGnG-NM inhibited adhesion with a dose response sim-

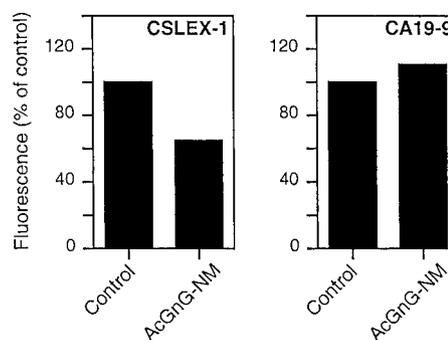


Fig. 2. AcGnG-NM alters cell-surface sLe^X in LS180 cells. LS180 cells were grown in the presence of 50 μM AcGnG-NM, harvested with EDTA, stained with mAbs (CSLEX-1, anti-sLe^X; CA19-9, anti-sLe^a) as indicated and analyzed by flow cytometry ("Materials and Methods"). The average fluorescence value for each sample was normalized to the value obtained from a sample of cells that had not been treated with inhibitor. The value obtained for nonspecific isotype-matched antibody in each case was <10% of the value obtained with CSLEX-1 or CA19-9.

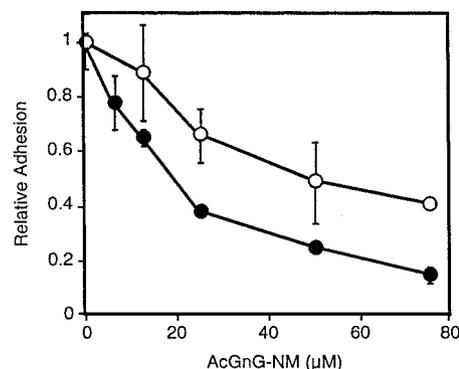


Fig. 3. Altered adhesion of AcGnG-NM treated tumor cells to immobilized selectins. LS180 colon carcinoma cells were "panned" onto wells precoated with recombinant E- or P-selectin as indicated. ○, adhesion to E-selectin; ●, adhesion to P-selectin. The extent of adhesion was normalized to the value obtained for cells not treated with disaccharide. Samples treated with sialidase, anti-E- or anti-P selectin mAb, or 50 μM of the inactive disaccharide primer acetylated-Galβ1,3Galβ-O-NM gave values of 0.28–0.32, 0.05–0.33, and 0.95–1.1, respectively. Each experimental condition was done in quadruplicate, and the average values ± SEs are given.

⁴ J. R. Brown, M. Fuster, T. Whisenant, and J. D. Esko, in press.

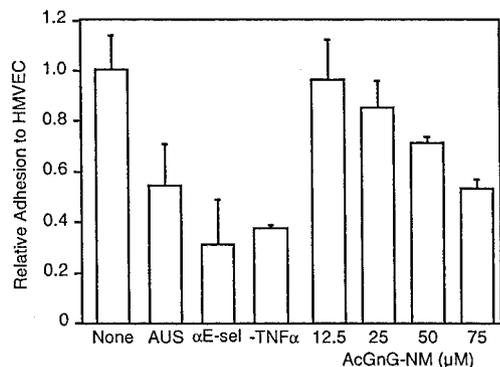


Fig. 4. Altered adhesion of AcGnG-NM treated tumor cells to cultured HMVECs. HMVECs were activated with TNF- α and overlaid with calcein-loaded LS180 cells. The extent of adhesion was normalized to the value obtained for cells not treated with disaccharide. Some samples were treated with sialidase (AUS) or anti-E-selectin antibody, or the HMVECs were not activated with TNF- α . Each condition was done in triplicate, and the values were averaged.

ilar to that observed using immobilized receptor (Fig. 3). The maximum extent of inhibition was similar to that obtained after treatment of the tumor cells with sialidase, which destroys sLe^x, or by using a blocking antibody to E-selectin.

We next examined how the compounds affected adhesion of platelets, which is mediated by P-selectin. Platelets were loaded with fluorescent calcein dye and the number of platelets adhering to islands of cultured LS180 cells was quantified by fluorescence microscopy. AcGnG-NM caused a dose-dependent inhibition of platelet adhesion, with 60% reduction achieved after treatment of the tumor cells with 50 μ M of disaccharide (Fig. 5A). The extent of inhibition was comparable with that achieved by treating tumor cells with sialidase and O-sialoglycoproteinase, which requires clustered oligosaccharide chains for cleavage of the underlying protein core (16, 35). The extent of inhibition was not as great as that achieved by blocking antibody or by omitting thrombin activation, suggesting that the disaccharide did not fully suppress expression of sLe^x or, alternatively, that non-Sia-containing ligands for P-selectin exist. Similar effects were observed in studies of two lung adenocarcinoma cell lines, A549 and A427 (Figs. 5, B and C, respectively). These cells also express selectin ligands and sLe^x determinants,⁴ but they varied in parallel in their response to sialidase and AcGnG-NM treatment.

Altered Biodistribution of AcGnG-NM-treated Tumor Cells. The lung is the major "first-pass" adhesion target for tumor cells introduced into the venous circulation. Ten min after injection of radiolabeled LS180 cells into the lateral tail vein of mice, >90% of the recovered counts were found in the lung (data not shown). After 3 h, ~60% of recovered counts remained in the lung, ~20% in the

liver, ~15% in the blood, and lesser amounts in other organs (Fig. 6A). Inhibitor-treated cells exhibited a different biodistribution after injection. Seeding of the lungs was substantially reduced and accompanied by a corresponding increase in counts recovered in the blood compartment, without significant differences in seeding of other tissues. When treated and untreated cells were injected into P-selectin-deficient mice, no difference was observed in the distribution of the cells (Fig. 6B), although the extent of seeding was reduced compared with wild-type mice. Thus altering either P-selectin or its carbohydrate ligand had similar effects, suggesting that the interaction of the tumor cell glycans with host cellular elements expressing P-selectin affected the fate of the cells.

Impairment of Metastatic Tumor Formation. Prior studies have shown that the deletion of P-selectin in mice alters the tumorigenicity of hematogenously distributed LS180 cells (9). To test whether altering carbohydrate ligands on the tumor cells had a similar effect, SCID

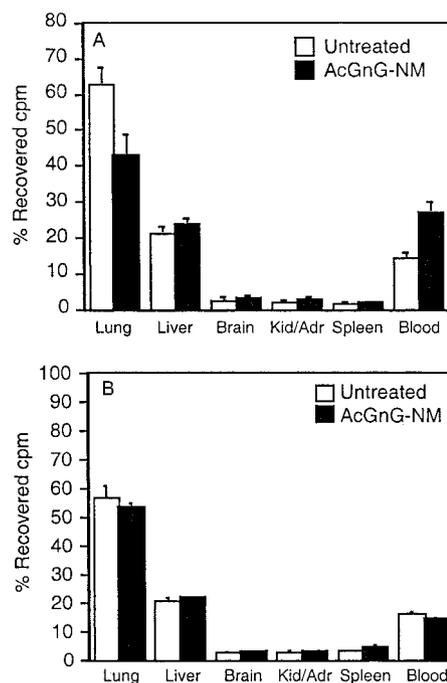
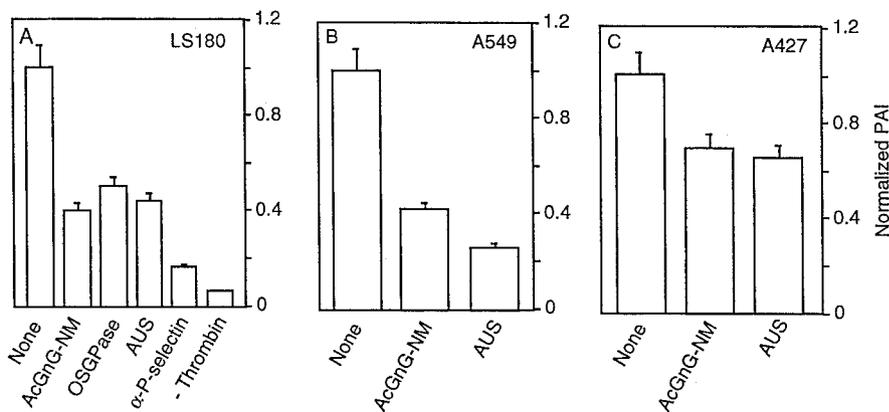


Fig. 6. Altered biodistribution of inhibitor-treated tumor cells in mice. Radiolabeled cells were injected into the lateral tail vein of C57BL/6 mice and were allowed to circulate for 3 h. Mice were sacrificed, and DNA was extracted from organ homogenates and whole blood. The counts were normalized to the total recovered counts, which typically represented 80–90% of the injected samples. A, each value represents the average recoveries from four wild-type mice \pm SD from the mean. B, the experiment was repeated in P-selectin-deficient C57BL/6 mice that were injected either with control or with AcGnG-NM-treated LS180 cells.

Fig. 5. Platelet adhesion to cultured tumor cells is reduced after treatment with AcGnG-NM. LS180 cells were grown on 6-well plates as multicell "islands" in the presence or absence of 50 μ M AcGnG-NM for 3 days. Human platelets were labeled with calcein, activated with human thrombin, and allowed to adhere to the tumor cells. The number of adherent platelets/area occupied by tumor cells was determined [platelet adhesion index (PAI)]. PAI values for all wells were normalized to that for platelet adhesion to untreated tumor cells. Some samples of tumor cells were treated with sialidase (AUS), O-sialoglycoproteinase (OSGPase), anti-P-selectin antibody, or with platelets that had not been activated with thrombin. A, LS180 colon carcinoma cells; B, A549 lung adenocarcinoma cells; C, A427 lung adenocarcinoma cells.



mice were injected with normal or disaccharide-treated LS180 cells via the tail vein. After 4 weeks, the animals were sacrificed, and formation of lung tumor foci was assessed at necropsy by counting nodules on the lung surface and in histological sections (Fig. 7). Numerous foci were present on lungs from animals that had been given injections of untreated cells, whereas foci were less numerous in animals receiving disaccharide-treated cells ($P < 0.0002$, Student's t test; Fig. 7C). A similar trend was noted on examination of foci in histological sections ($P < 0.02$). No foci were found in other organs by surface and histological surveys. A human metastatic lung adenocarcinoma cell line (HAL8) was also examined. These cells behaved similarly, although the absolute number of tumor foci was much lower (data not shown).

Cytolysis of Tumor Cells Is Affected by Altered Platelet Protection. Platelet adhesion to tumor cells may play an important mechanistic role in the *in vivo* findings reported above, possibly by protecting tumor cells from immune-mediated lysis (10, 33, 36). To examine this possibility, LS180 tumor cells were loaded with ^{51}Cr and mixed with various numbers of cytolytic immune effector cells (Fig. 8). The extent of cell lysis was proportional to the E:T ratio. Adding platelets to the incubation significantly reduced cytolysis, although some lysis was noted at very high E:T values outside the range of values that would occur in a typical experimental metastasis assay performed in mice (indicated by the broken vertical lines in

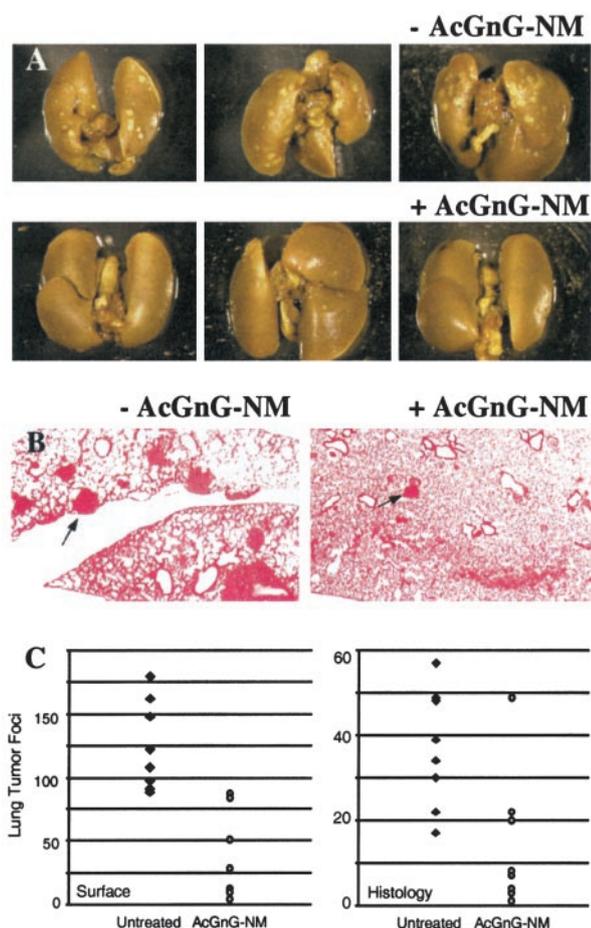


Fig. 7. Metastatic lung tumor formation is inhibited by treatment with AcGnG-NM. LS180 cells were grown with or without $50 \mu\text{M}$ AcGnG-NM for 6 days before injection into the tail-vein of SCID mice. After 4 weeks, the mice were sacrificed, and their lungs were analyzed for surface tumor foci (A) and internal tumor foci (B). C, surface foci and histological foci present in a representative section were counted for each animal ($n = 8$) in each group. Injection of treated cells resulted in significantly fewer tumor foci ($P < 0.0002$, Student's t test).

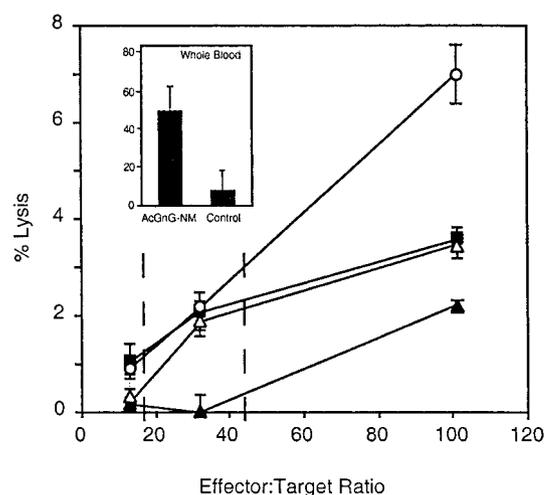


Fig. 8. Cytolysis in the presence of platelets is increased after treatment of tumor cells with AcGnG-NM. LS180 cells, labeled with ^{51}Cr , were exposed to different numbers of murine effector leukocytes in the presence or absence of platelets in a 96-well plate for 3 h at 37°C . After centrifugation, equal aliquots of medium were taken from each well to measure radioactivity as a measure of lysis. Vertical dashed lines, an estimate of the range of E:T ratios (peripheral-blood mononuclear cells: tumor cells) that occur *in vivo* during biodistribution experiments. ○, no platelets were added; △, P-selectin-negative platelets were added; ■, tumor cells were treated with peracetylated GnG-NM and mixed with P-selectin-positive platelets. Inset, the effect of whole human blood on the lysis of ^{51}Cr loaded tumor cells with and without treatment with AcGnG-NM. The experiment was performed in triplicate, and average values \pm SDs are shown.

Fig. 8). P-selectin-deficient platelets showed a marked reduction in their ability to protect tumor cells over the same range. Platelet protection was also markedly reduced after treatment of the tumor cells with AcGnG-NM. Exposure of disaccharide-treated and untreated LS180 cells to whole human blood gave comparable results (Fig. 8, inset), although the overall effect and extent of lysis was significantly greater. Together, these findings demonstrate that P-selectin on platelets binding to sLe^x determinants on tumor cells provides protection against leukocyte-mediated cytolysis. Moreover, they show that treatment with AcGnG-NM inhibits platelet-mediated protection of the cells.

DISCUSSION

In this report, we showed that treatment of human adenocarcinoma cells with a disaccharide-based primer of sLe^x can markedly inhibit their metastatic potential *in vivo*. Mechanistically, the compound appears to work by (a) priming the synthesis of oligosaccharides related to Lewis antigens, (b) blocking the function of sLe^x on cell surface glycoconjugates, and (c) inhibiting selectin-dependent events that promote hematogenous metastasis, including platelet adhesion and attachment to endothelial cells. Platelet adhesion seems to confer protection from immune cytolytic responses. Our findings complement recent studies in mice that showed a profound effect of altering host selectin expression on the metastatic potential of tumor cells in the circulation (6, 8–10). Loss of tumor-cell sLe^x also resulted in a concomitant and equally potent reduction in interactions with E-selectin *in vitro*, which may significantly interfere with adhesion to activated endothelia. One might predict that L-selectin ligands expressed on tumor cells would also be affected, which would prevent leukocyte interactions that facilitate tumor growth (37). Together, these findings imply that AcGnG-NM and related compounds may inhibit multiple interactions between tumor cells and selectin-bearing host elements (platelets, endothelia, and leukocytes) during hematogenous metastasis.

The treatment of tumor cells with AcGnG-NM has a particularly important effect on metastatic potential as a result of altered platelet adhesion. As shown in Fig. 8, selectin-mediated platelet adhesion endows tumor cells with significant protection from immune-mediated cytotoxicity, which may explain the higher tumorigenicity of untreated cells compared with cells treated with the disaccharide inhibitor (Fig. 7). Because these experiments were performed in SCID mice, humoral factors and T-cell mediated responses should not be involved, but elements of innate immunity (e.g., innate cytotoxic responses, natural killer cells, and so forth) may play a role (38). The apparent protection of tumor cells by platelets critically depends on P-selectin-carbohydrate interactions because P-selectin deficiency dampens the effect both *in vivo* and *in vitro*. On injection of treated cells, sLe^x begins to reappear on the cell surface with a $t_{1/2}$ of ~6 h. This suggests that cytotoxicity occurs relatively rapidly and that interfering with platelet adhesion to tumor cells soon after their release into the circulation will render the cells more sensitive to killing. Other inhibitory agents, such as heparin or mucin fragments, also transiently block selectin-dependent adhesion and block tumor formation (10). These agents are rapidly cleared from the circulation but nevertheless have profound effects on ultimate colonization of the lungs by metastatic cells. Thus, antimetastatic agents that target selectin-carbohydrate interactions need only to act in a narrow time frame to be effective.

A final consideration is the ability of selectins to participate in "arresting" newly circulating tumor cells in organ capillary beds. Entrapment of emboli may have important consequences on the eventual uptake and growth of tumor "seeds" into large metastatic tumor foci. AcGnG-NM-treated LS180 cells showed a limited ability to eventually grow as tumors in the lungs of immunodeficient mice harboring the cells for a 4-week period after tail-vein delivery (Fig. 7). Whereas alterations in tumor sLe^x may have an as-yet-unexplained effect on tumor growth (including apoptosis), an initial inhibition of selectin-mediated capillary arrest may be critical to the survival of metastases. Additional evidence supporting this view includes: (a) treatment of adenocarcinoma cells with AcGnG-NM (up to 50 μ M) has minimal effects on LS180 growth in culture; (b) experimental mice were not maintained on pharmacological AcGnG-NM after tumor cell injection, indicating that the effects are rapid and independent of continuous inhibition; and (c) whereas tumors that grew in experimental mice were markedly fewer in number, tumor size in the two groups was approximately the same. A recent study highlights the importance of an early period of intravascular tumor residence and proliferation before extravasation and uptake (39). The probability of securing a prolonged intravascular period of arrest should increase after selectin-mediated formation of platelet-tumor emboli and direct contact of tumor cells with endothelial selectins. Inhibition of sLe^x-mediated adhesion would be expected to decrease these parameters. A recent study demonstrates that the inhibition of β 3 integrins also interferes with hematogenous metastasis in a platelet-dependent fashion, consistent with this idea (40). These findings imply a potential role for AcGnG-NM or related compounds as possible antimetastasis agents for treating human cancer.

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