

Suppression of lung metastasis of B16 mouse melanoma by *N*-acetylglucosaminyltransferase III gene transfection

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Communicated by Tadamiyu Kishimoto, Osaka University Medical School, Osaka, Japan, May 5, 1995

ABSTRACT The β 1-6 structure of *N*-linked oligosaccharides, formed by β -1,6-*N*-acetylglucosaminyltransferase (GnT-V), is associated with metastatic potential. We established a highly metastatic subclone, B16-hm, from low metastatic B16-F1 murine melanoma cells. The gene encoding β -1,4-*N*-acetylglucosaminyltransferase (GnT-III) was introduced into the B16-hm cells, and three clones that stably expressed high GnT-III activity were obtained. In these transfectants, the affinity to leucoagglutinating phytohemagglutinin was reduced, whereas the binding to erythroagglutinating phytohemagglutinin was increased, indicating that the level of β 1-6 structure was decreased due to competition for substrate between intrinsic GnT-V and ectopically expressed GnT-III. Lung metastasis after intravenous injection of the transfectants into syngeneic and nude mice was significantly suppressed, suggesting that the decrease in β 1-6 structure suppressed metastasis via a mechanism independent of the murine system. These transfectants also displayed decreased invasiveness into Matrigel and inhibited cell attachment to collagen and laminin. Cell growth was not affected. Our results demonstrate a causative role for β 1-6 branches in invasion and cell attachment in the extravasation stage of metastasis.

Malignant transformation is highly associated with alterations of *N*-linked oligosaccharides. The extensive and persistent changes in the population of protein-bound oligosaccharides in malignant cells have been proposed to disturb cell division, decrease intercellular adhesiveness, and mask immunogenicity (1). The glycopeptides in polyoma-transformed baby hamster kidney cells have unusually high molecular weights due to the presence of external sialic acid as well as galactose and GlcNAc (2). The predominant surface glycopeptide of baby hamster kidney cells transformed by Rous sarcoma virus was determined to be a triantennary, completely sialylated, complex glycopeptide containing a core region of Man, GlcNAc, and Fuc (3). Synthesis of these elongated branches is initiated by β -1,6-*N*-acetylglucosaminyltransferase (GnT-V; EC 2.4.1.155), which catalyzes the formation of the β 1-6 branch (4). GnT-V activity correlated well with the metastatic potential of *ras*-transformed Rat2 fibroblasts, SP1 mammary carcinoma cells, the MDAY-D2 lymphoma cell line (5, 6), and human colon cancer cells (7). Rat2 fibroblasts transfected with *H-ras* or *v-fps* exhibited metastatic potential and had elevated GnT-V activity and increased β 1-6 branches (8), whereas a mutant with decreased GnT-V activity from a highly metastatic tumor cell line had a decreased potential for metastasis in mice (6). These observations have suggested a positive correlation between β 1-6 branching and metastatic capacity.

One approach to analyzing the role of GnT-V and its product, β 1-6 branches, in metastasis is to reduce GnT-V

activity in malignant cells with a high tendency to metastasize. As shown in Fig. 1, both β -1,4-*N*-acetylglucosaminyltransferase (GnT-III; EC 2.4.1.144) and GnT-V use the triantennary structure of *N*-linked oligosaccharides as substrate, and once a bisecting GlcNAc residue is added to the core mannose by GnT-III, GnT-V is not able to form any further triantennary structure (9).

The present investigation was undertaken to determine whether introduction of the GnT-III gene into malignant cells would result in decreased production of β 1-6 branches by GnT-V and lead to reduced metastatic potential in experimental models of lung metastasis. We have demonstrated that artificially expressed GnT-III suppressed the formation of β 1-6 branches in positive transfectants of B16 mouse melanoma. Lung colonization after intravenous administration of the GnT-III transfectants was decreased, as was the *in vitro* invasiveness of these transfectants.

MATERIALS AND METHODS

Reagents. Swine collagen type I solution was purchased from Iwaki (Chiba, Japan), and mouse laminin produced by Engelbreth-Holm-Swarm sarcoma and mouse plasma fibronectin were obtained from GIBCO/BRL. Biotinylated erythroagglutinating phytohemagglutinin (E-PHA) and biotinylated leucoagglutinating phytohemagglutinin (L-PHA) were obtained from the Honen (Tokyo, Japan).

Cell Lines. Murine melanoma cell variant B16-F1, a low metastatic cell subclone derived from the B16 murine melanoma cell line (10), was provided by the Japanese Cancer Research Resources Bank (Tokyo). For this investigation, B16-hm, a highly metastatic subclone of B16-F1, was recloned from metastatic lung colony by repeating an intravenous injection eight times. B16-hm cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum and antibiotics. Cells in exponential growth and within five passages were used throughout this investigation.

Construction of the GnT-III Expression Vector. An expression vector, pCAGGS, with an actin promoter was kindly donated by K. Yamamura and M. Suzuki (Kumamoto University, Kumamoto, Japan). The pSV2Neo vector with a neomycin-resistance gene was supplied by the Japanese Cancer Research Resources Bank. For the construction of the GnT-III expression vector, a rat GnT-III cDNA, C4 (11), which contains the entire coding sequence, was deleted at the 5' noncoding region by nested deletion using *Exo* III and mung bean nuclease, resulting in removal of 42 residues. The shortened C4 fragment was excised with *Eco*RI, blunt-ended by treatment with the Klenow fragment of DNA polymerase I, and subcloned into

Abbreviations: GnT-V, β -1,6-*N*-acetylglucosaminyltransferase; GnT-III, β -1,4-*N*-acetylglucosaminyltransferase; Gal-T, β -1,4 *N*-galactosyltransferase; L-PHA, leucoagglutinating phytohemagglutinin; E-PHA, erythroagglutinating phytohemagglutinin.

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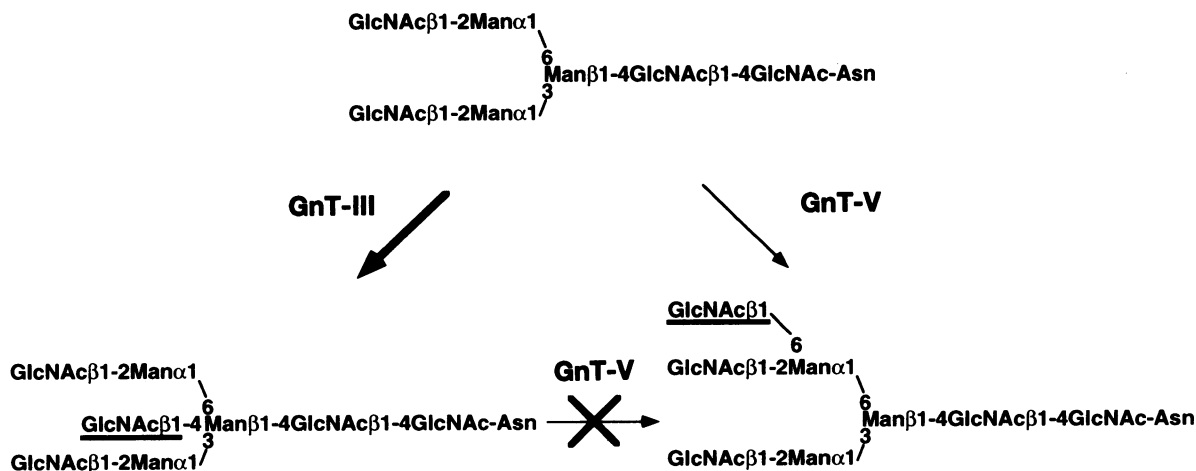


FIG. 1. Reactions catalyzed by GnT-III and GnT-V. The transfer of a bisecting GlcNAc to an N-linked sugar chain by GnT-III inhibits the transfer of GlcNAc by GnT-V to form a β 1-6 branch.

the *Sma* I site of pSVK3 vector SV-7 (Clontech). The *Sac* I fragment was then cut out and treated with T4 DNA polymerase, and the blunt-ended fragment was subcloned into the pCAGGS *Eco*RI site. GnT-III gene expression in this construct, Act-3, was controlled by the actin promoter.

Selection of Transfectants Stably Expressing GnT-III. Act3 and pSV2Neo were linearized by digestion with *Sal* I and *Bam*HI, respectively. Following this, 20 μ g of Act-3 expression vector and 2 μ g of pSV2Neo were cotransfected by the electroporation method (250 V/0.4 cm and 960 μ F). Infected B16-hm cells were incubated in DMEM containing G418 (1 mg/ml; GIBCO/BRL). After a 2-week incubation, neomycin-resistant colonies were isolated and recloned by serial dilution to ensure clonality.

Enzyme Activity Assays for GnT-III, GnT-V, and β -1,4-N-Galactosyltransferase (Gal-T). GnT-III (12), GnT-V (13), and Gal-T (EC 2.4.1.38) (14) activities were determined, as described, using the pyridylaminated biantennary sugar chain as a substrate (15).

Lectin Blot. Cells were harvested and lysed for 20 min at 4°C in Tris-HCl-buffered saline (pH 7.4) containing 1% Nonidet P-40, 5 mM EDTA, 10% (wt/vol) glycerol, and protease inhibitors. After centrifugation to remove insoluble material, the protein concentration of the lysate was measured using a BCA kit (Pierce). Cell lysates (20 μ g) were subjected to 8% SDS/PAGE under nonreducing conditions and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 3% (wt/vol) bovine serum albumin and incubated for 4 h with biotinylated L-PHA or biotinylated E-PHA at 2 μ g/ml. After washing, the blots were incubated with horse radish peroxidase-avidin complex (Vector) and developed using an ECL detection system (Amersham).

Experimental Metastasis. The metastatic potency of B16-hm cells and GnT-III transfectants was determined using an "experimental metastasis assay" (16). Female C57BL/6J mice and female athymic BALB/c nude mice (6–8 weeks old) were obtained from SLC Japan (Shizuoka, Japan). The mice were determined to be free from pathogens and caged in groups of four in facilities meeting National Institutes of Health guidelines. The nude mice were housed in laminar flow cabinets. Cells were harvested by a brief exposure to PBS containing 5 mM EDTA, and cell number and viability were examined using trypan blue. Samples containing only single-cell suspensions of >90% viability were resuspended at 3×10^5 cells in 50 μ l of Hanks' balanced salt solution and were injected into the tail veins of seven mice. Three weeks after injection, the mice were

sacrificed, and their lungs were fixed in Bounin's solution. Metastasized colonies were counted using a dissecting microscope.

Invasion Assay. The invasiveness of B16-hm melanoma cells and transfectants was evaluated by the Matrigel assay (17). Boyden chambers (Biocoat Matrigel; Collaborative Biomedical Products–Becton Dickinson) containing a polycarbonate membrane, the upper surface of which was already coated with Matrigel (10 μ m thickness and 8 μ m pore size), were put on 12-well plates. B16-hm and GnT-III transfectants, which were detached from the dish by a brief treatment with PBS containing 5 mM EDTA, were added to the upper chamber and allowed to invade through the Matrigel for 24 h at 37°C. After the incubation, the chamber was removed, and invading cells on the bottom of the well were fixed in 4% glutaraldehyde in PBS and stained with 0.1% crystal violet. The dye was eluted, and its absorbance was determined at 600 nm. The absorbance paralleled the cell number, as previously described (18).

Adhesion Assay. Cell adhesion was assayed by crystal violet staining, as described (18). Briefly, 24-well culture plates were coated overnight at 4°C with 10 μ g of collagen type I, fibronectin, or laminin per ml. After washing, nonspecific binding sites were blocked with 3% bovine serum albumin in PBS. Cells (1×10^5) were added to each matrix-coated well and incubated for 30, 60, 120, 180, and 240 min at 37°C. Cells adhering after two washings with PBS were stained, and the absorbance at 600 nm (A_{600}) was measured. As a positive control representing total cell adhesion, cells in DMEM supplemented with 10% fetal bovine serum were planted on uncoated wells. As a negative control for the spontaneous adhesion, cells in serum-free DMEM were seeded on bovine serum albumin-coated wells. The percentage of adhering cells after various incubation times was calculated as follows:

$$\frac{A_{600}(\text{matrix}) - A_{600}(\text{spontaneous})}{A_{600}(\text{total}) - A_{600}(\text{spontaneous})} \times 100\%.$$

Cell Growth. Cell growth of parental B16-hm cells and GnT-III transfectants was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (19).

RESULTS

Establishment of B16-hm Clones Stably Expressing High Levels of GnT-III. An expression vector, Act-3, that carried a rat GnT-III cDNA under the control of the actin gene promoter in the pCAGGS plasmid was prepared. Act-3 and the pSV2-neo plasmid were then cotransfected into B16-hm cells

Table 1. GnT-III, GnT-V, and Gal-T activities of B16-hm melanoma cells and GnT-III-positive and GnT-III-negative transfectants

Cells	Enzyme activity, pmol per h per mg		
	GnT-III	GnT-V	Gal-T
B16-hm	ND	758 ± 35	2150 ± 188
B16-hm-neo1	ND	743 ± 222	1760 ± 189
B16-hm-neo2	ND	861 ± 74	1410 ± 267
B16-hm-III1	125,000 ± 12,000*	1080 ± 61†	2720 ± 632
B16-hm-III2	164,000 ± 5,200*	1350 ± 112†	2840 ± 329
B16-hm-III3	92,100 ± 3,070*	1280 ± 158†	1990 ± 293

Enzyme activities of GnT-III, GnT-V, and Gal-T were measured using fluorescence-labeled sugar chains as substrates. Each value was expressed as mean ± SD of three or four experiments. ND, not detected. Statistical analysis was carried out according to Student's *t* test.

**P* < 0.001 vs. B16-hm.

†*P* < 0.05 vs. B16-hm.

by electroporation. Parental B16-hm cells showed no detectable GnT-III activity (Table 1). A total of 12 G418-resistant clones, 9 of which had elevated GnT-III activity (positive transfectants) and 3 of which displayed no detectable GnT-III activity (negative transfectants), were isolated. Three positive clones were selected at random and designated as B16-hm-III1, -III2, and -III3. Two of the negative transfectants were used as negative controls and designated as B16-hm-neo1 and -neo2. The enzyme activities of GnT-III, GnT-V, and Gal-T in the cells are given in Table 1. The intrinsic activities of GnT-V and Gal-T were assayed in the presence of EDTA, which inhibits the competitive effect of GnT-III. Under these conditions, GnT-V and Gal-T activity did not differ significantly among the parental cells and transfectants, which indicated that introduction of the GnT-III gene did not decrease the intrinsic activity of GnT-V and Gal-T. For an unknown reason, the intrinsic GnT-V activity was slightly higher in the positive transfectants. GnT-III activity in the three positive transfectants was elevated to between 92,100 and 164,000 pmol per h per mg of protein.

Decreased β 1-6 Branching in Whole-Cell Lysates, as Measured by L-PHA and E-PHA Binding. Lectin blotting was performed to analyze the alterations of carbohydrate structures on the cell surface of parental and transfectant cells. The two lectins used were L-PHA and E-PHA. L-PHA binds specifically to GlcNAc residues on β 1-6 branches of tri- or tetraantennary sugar chains, and the binding is interrupted in the presence of bisected biantennary glycopeptide (20). E-PHA has high affinity for bisected oligosaccharides but does not interact strongly with β 1-6 structures (21).

Whole-cell lysates from B16-hm cells and negative transfectants were highly reactive to L-PHA (Fig. 2 Upper). Two proteins of 95 kDa and 80 kDa showed particularly strong L-PHA binding. The proteins from positive transfectants showed reduced reactivity to L-PHA. In contrast, staining with E-PHA (Fig. 2 Lower) yielded only a few faint signals for proteins from B16-hm cells and negative transfectants, whereas strong signals (especially at 95 kDa and 80 kDa) were seen for proteins from positive transfectants. These data showed that expression of GnT-III led to increased synthesis of bisecting GlcNAc, which in turn suppressed formation of β 1-6 tri- and tetraantennary N-linked oligosaccharides.

Lung Colonization in Experimental Metastasis Was Decreased in GnT-III Transfectants. When positive transfectants were injected into syngeneic C57BL/6 mice, significantly fewer metastatic nodules were observed than with the parent cells and negative transfectants (Fig. 3). The number of metastatic colonies with negative transfectants was similar to that with the parent cells. These results demonstrated that GnT-III expression decreased the metastatic potential of B16-hm melanoma

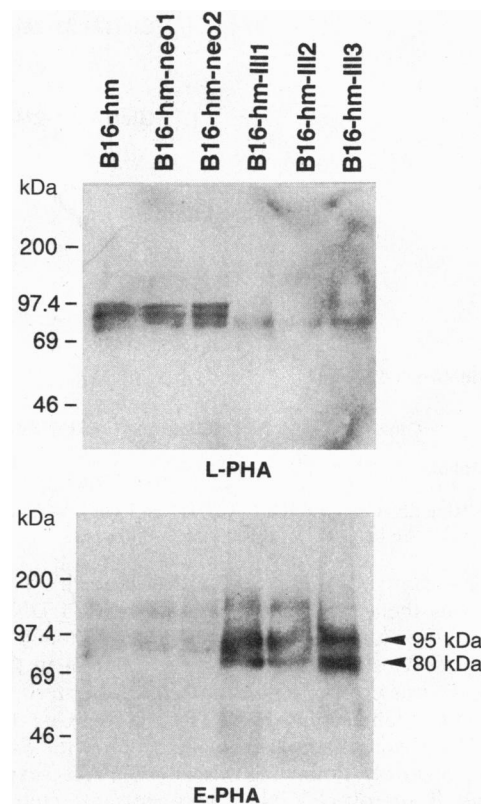


FIG. 2. Lectin blot analysis of whole-cell lysates from the parental B16-hm cells and GnT-III transfectants. Cell lysates from B16-hm cells and GnT-III transfectants were separated on SDS/PAGE and transferred onto a nitrocellulose membrane. The blots were probed with L-PHA (Upper) or E-PHA (Lower). The positions of molecular size markers are indicated to the left.

cells *in vivo*. To determine whether this was due to increased susceptibility of the GnT-III-transfected cells to capture by the immune system, the B16-hm cells and GnT-III transfectants were also injected into athymic BALB/c nude mice intravenously. The positive transfectants produced fewer metastatic

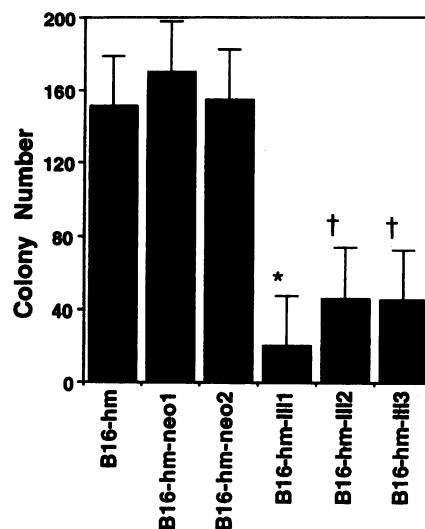


FIG. 3. Experimental metastasis assay in C57BL/6 mice. Viable cells (3×10^5 cells per mouse) were injected via the tail vein of seven female C57BL/6 mice. The mice were sacrificed 21 days after inoculation, and the metastasized colonies were counted. Bars indicate the standard error of the mean for three independent experiments. Statistical analysis was carried out according to the Student's *t* test. *, *P* < 0.01; †, *P* < 0.05 vs. B16-hm.

nodules than the parent cells and produced negative transfectants to the same extent observed in syngeneic C57BL/6 mice, suggesting that the immune system did not affect the metastatic process (data not shown). Subcutaneous injection of B16-hm cells and GnT-III transfectants into C57BL/6 mice yielded fewer than 20 lung colonies even 8 weeks later, too few to evaluate the metastatic potential in the "spontaneous metastasis assay," which is in agreement with previous reports (16, 19).

In Vitro Invasiveness and Cell Attachment Were Decreased in GnT-III Transfectants Without Affecting Cell Growth. In this experimental metastasis, the number of metastasized lung colonies is dependent on tumor invasiveness, adhesion on the endothelial cells, attachment to the extracellular matrix, and cell growth. Invasive potential was tested *in vitro* using a Boyden chamber coated with Matrigel, an extract prepared from mouse Engelbreth-Holm-Swarm sarcoma (20). The ability of GnT-III-positive transfectants to reach the bottom of the well through the Matrigel was suppressed significantly compared to that of B16-hm cells and negative transfectants (Fig. 4A). Decreased affinity for the extracellular matrix would reduce cell adherence to the lung, resulting in decreased lung metastasis. As shown in Fig. 4B, cell attachment to collagen and laminin was significantly delayed for the GnT-III-positive transfectants than for the B16-hm cells and negative transfectants for the first 30 and 60 min during the incubation. At the end of the 4-h incubation, these cells attached equally to collagen and laminin. Cell attachment to fibronectin did not differ significantly among the B16-hm cells and GnT-III transfectants. No difference in cell morphology was observed among parental B16-hm cells and GnT-III-positive and -negative transfectants when these cells were planted and spread on collagen, laminin, or fibronectin. The doubling times (days), as judged by cell growth kinetics (19), were 3.5 ± 0.2 for parental B16-hm cells, 3.3 ± 0.3 for GnT-III-negative transfectants, and 3.5 ± 0.3 for GnT-III-positive transfectants, suggesting that ectopic expression of the GnT-III gene did not affect the proliferation of the cells. It is difficult to evaluate the adhesion of the GnT-III-positive transfectants to the murine lung capillary endothelium *in vitro*, because the capillary endothelial cells isolated from the murine lung are not available at present. So there remains a possibility that the expression of GnT-III may also affect the adhesion to the endothelial cells, which is mediated in part by sialyl-Lewis-X structure and selectin receptors (22).

DISCUSSION

The process of tumor metastasis is very complex and is made up of many biological events (reviewed in ref. 23). Tumor cells must detach from the primary lesion, invade the surrounding extracellular matrix, and intravasate into the bloodstream, where they must dodge the host's immune system until they reach the secondary site. There the tumor cells adhere to endothelial cells and invade through the endothelium into the matrix (extravasation). Finally, the tumor cells begin to grow at the secondary site. In this investigation, metastatic potential was evaluated after direct injection of melanoma cells into the bloodstream via the tail vein. This pulmonary colonization assay did not address the early steps of metastasis (i.e., the ability of the tumor cells to release from the primary lesion and to invade the blood vessels).

Many of the protein molecules reported to participate in the metastatic process have N- and/or O-linked sugar chains, and alteration of these sugar chains could modify the biological function of these molecules (1). Two kinds of approaches have been employed to evaluate the role of sugar chains in metastasis. One is to use monoclonal antibodies against sugar chains. Such antibodies have been found to inhibit cell motility and metastasis (24). The other approach is to modify the oligo-

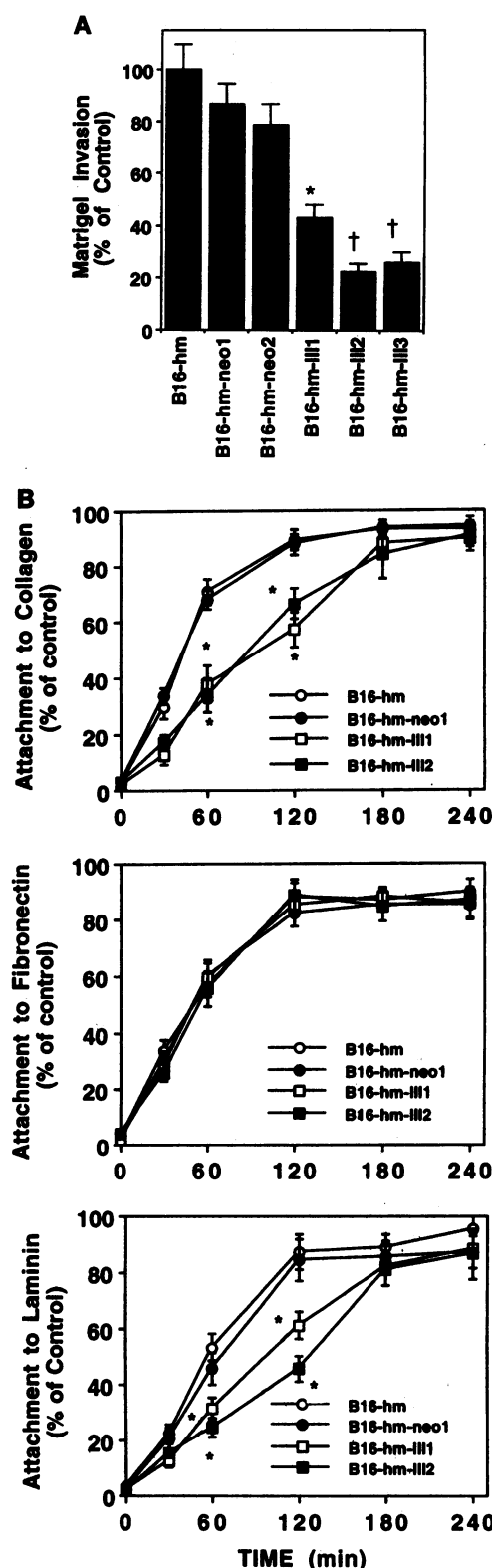


FIG. 4. (A) The *in vitro* invasiveness of B16-hm melanoma cells and transfectants was evaluated using a Matrigel-coated Boyden chamber. Results were expressed as the percent absorbance compared to the absorbance with B16-hm cells as a control (mean \pm SD). Bars indicate the standard error of the mean for three independent experiments. Statistical analysis was carried out according to the Student's *t* test. *, $P < 0.05$; †, $P < 0.02$ vs. B16-hm. (B) Cell adhesion to collagen (Top), fibronectin (Middle), and laminin (Bottom) was examined. Bars indicate the standard error of the mean. Cell adhesion was calculated as described in *Materials and Methods*. Statistical analysis was carried out according to Student's *t* test. *, $P < 0.05$ vs. B16-hm.

saccharides by using reagents that inhibit the synthesis of specific types of sugar chains. For example, treatment with swainsonine, an inhibitor of Golgi α -mannosidase II, the enzyme responsible for formation of hybrid-type oligosaccharides, suppressed lung colonization by B16-hm mouse melanoma cells (25). In the current investigation, we employed modification of oligosaccharide structure by transfection of the gene for a glycosyltransferase. The competition study and lectin blotting with L-PHA and E-PHA confirmed that transfection of the GnT-III gene into B16-hm melanoma cells reduced β 1-6 branching due to the inhibitory effect of GnT-III on GnT-V.

GnT-III transfection suppressed formation of metastatic colonies in the lung, invasion into Matrigel, and cell attachment to collagen and laminin without affecting cell growth. Cell attachment to the extracellular matrix is mainly mediated by β 1-integrin (reviewed in ref. 26). Previous studies showed the functional role of N-glycosylation for the integrin receptor. When human fibroblasts were cultured in the presence of 1-deoxymannojirimycin, an inhibitor of Golgi α -mannosidase IA and IB, an immature fibronectin receptor appeared and its binding function to fibronectin was greatly reduced (27). Another study showed that enzymatic deglycosylation of both α 5 and β 1 subunits of the α 5 β 1-integrin caused the dissociation of both subunits and resulted in the reduced α 5 β 1-mediated cell attachment to fibronectin in K562 human leukemia cells (28). In this investigation, we demonstrated that cell attachment to collagen and laminin was also affected by the ectopically expressed enzyme responsible for N-glycosylation as well as by the inhibitors for the process of N-glycosylation or enzymatic N-deglycosylations. The suppressive effect of GnT-III transfection on cell attachment to collagen and laminin suggests that the alteration of N-linked sugar chains by GnT-III expression significantly affected integrin function. The inhibition of cell attachment in the GnT-III-positive transfectants was observed as early as 60 min during the incubation, as assayed under nonflow conditions. Under physiological flow, such as the blood stream, cell attachment of GnT-III transfectants is more likely suppressed, which might be implicated as the reduced experimental metastasis in mice.

In conclusion, we have demonstrated that introduction of the GnT-III gene decreased synthesis of β 1-6 branches through the competition for substrate between GnT-V and the overexpressed GnT-III. The GnT-III-transfected B16-hm cells displayed reduced capacities for metastasis *in vivo* and invasion *in vitro*. N-linked oligosaccharides of the cell-surface-glycoproteins were modified by overexpressed GnT-III. To our knowledge, this is the first demonstration that the introduction of a gene encoding a glycosyltransferase resulted in significant suppression of metastatic growth.

M.Y. and A.N. contributed equally to this work. We thank Drs. K. Yamamura and M. Suzuki for kindly providing expression vectors;

Drs. H. Go, K. Miyado, E. Miyoshi, M. Ogawa, and M. Koketsu and Ms. M. Tabata and Ms. K. Horiguchi for technical support; and Ms. S. House for editing the manuscript. This work was supported in part by grants-in-aid for cancer research and scientific research on priority areas from the Ministry of Education, Science, and Culture, Japan and the Noda Cancer Fund.

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