N-Acetylglucosaminyltransferase V Expression Levels Regulate Cadherin-associated Homotypic Cell-Cell Adhesion and Intracellular Signaling Pathways*

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A common glycan alteration in transformed cells and human tumors is the highly elevated levels of N-linked β(1,6)glycans caused by increased transcription of N-acetylglucosaminyltransferase V (GnT-V). Here, we define the involvement of GnT-V in modulation of homotypic cell-cell adhesion in human fibrosarcoma HT1080 and mouse NIH3T3 cells. Increased GnT-V expression resulted in a significant decrease in the rates of calcium-dependent cell-cell adhesion. Reduced cell-cell adhesion was blocked by function-blocking antibody against N-cadherin and abrogated by pre-treatment of cells with swainsonine, demonstrating the involvement of N-cadherin in the cell-cell adhesion and that changes in swainsonine, demonstrating the involvement of N-cadherin and abrogated by pre-treatment of cells with GnT-V-deficient embryonic fibroblasts from GnT-V homozygous null mice (GnT-V−/−) express N-cadherin and showed significantly increased levels of N-cadherin-based cell-cell adhesion compared with those from GnT-V+/− mice. These levels of adhesion were inhibited significantly by transient expression of GnT-V, sensitized stimulation of tyrosine phosphorylation of catenins by growth factors and expression of v-src, which is consistent with its reduction of cell-cell adhesion. In vitro, GnT-V-overexpressing cells showed increased motility concomitant with increased phosphorylation of catenins. Moreover, GnT-V-deficient embryonic fibroblasts from GnT-V−/− embryos show increased motility, branching and invasiveness (1–3). Recent studies demonstrate that aberrant glycosylation of several types of cell surface receptors results in dysfunctional intracellular signaling and altered cellular behavior. For example, mutations in an N-acetylglucosaminyltransferase, POMGnT-I, cause aberrant glycosylation of skeletal muscle dystrophin, resulting in dysfunctional neuromuscular junctions (4). Mutations in POMGnT-I have been shown to result in multiple phenotypes, some of which have been classified as "muscle-eye-brain" disease, a form of muscular dystrophy. In addition, increased expression levels of another N-acetylglucosaminyltransferase, GnT-V, have been shown to inhibit αβ integrin adhesion to fibronectin, as well as stimulating cell migration through this matrix glycoprotein, most likely by inhibiting α5β1 receptor clustering in the plasma membrane (5).

Increased expression of GnT-V is observed during the oncogenesis of many cell types as a result of the stimulation of its transcription through the ras-ets signaling pathway. This enzyme synthesizes a multiantennary branch on N-linked glycans, the branch containing GlcNAc(1,6)Man. Studies have examined the expression of these branches N-linked oligosaccharides in various human and murine tumors and cell lines derived from them (6–10) and suggest that increased β(1,6) branching is associated positively with invasiveness. Mice that lack GnT-V expression due to targeted deletion (GnT-V−/− or Mgt5−/−) have been used to study the effects of eliminating GnT-V activity on tumor progression (11). When crossed with mice that express the polyoma middle T-antigen under control of the mouse mammary tumor virus promoter, the rate of invasion and metastasis of the mammary tumors was significantly reduced in the GnT-V−/− mice. This study provided

Changes in the expression of many cell surface adhesion receptors, including integrins, cadherins, CD44, and members of the immunoglobulin superfamily such as intercellular adhesion molecule, mediate cell-cell and cell-ECM1 interactions that are clearly critical as cells undergo oncogenesis and show changes in motility and invasiveness (1–3). Recent studies demonstrate that aberrant glycosylation of several types of cell surface receptors results in dysfunctional intracellular signaling and altered cellular behavior. For example, mutations in an N-acetylglucosaminyltransferase, POMGnT-I, cause aberrant glycosylation of skeletal muscle dystrophin, resulting in dysfunctional neuromuscular junctions (4). Mutations in POMGnT-I have been shown to result in multiple phenotypes, some of which have been classified as "muscle-eye-brain" disease, a form of muscular dystrophy. In addition, increased expression levels of another N-acetylglucosaminyltransferase, GnT-V, have been shown to inhibit αβ integrin adhesion to fibronectin, as well as stimulating cell migration through this matrix glycoprotein, most likely by inhibiting α5β1 receptor clustering in the plasma membrane (5).

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1 The abbreviations used are: ECM, extracellular matrix; POMGnT-I, protein O-mannose β-1,2-N-acetylglucosaminyltransferase; GnT-V, N-acetylglucosaminyltransferase V (Mgt5); GlcNAc, N-acetylglucosamine; N-glycan, asparagine-linked glycans; CAM, cell adhesion molecules; L-PHA, leucoagglutinating phytohemagglutinin; ConA, concanavalin A; SNA, S. nigra agglutinin; MAA, M. amurensis agglutinin; SW, swainsongin; MEF, mouse embryofibroblast; FCM, flow cytometry; BSA, bovine serum albumin; DME, Dulbecco’s modified Eagle’s medium; mAb, monoclonal antibody; HRP, horseradish peroxidase; ERK, extracellular signal-regulated kinase; PY, phosphorytrosine; PBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; MAPK, mitogen-activate protein kinase; PTP, protein-tyrosine phosphatase; EGF, epidermal growth factor; FGF, fibroblast growth factor; poly-HEMA, poly(2-hydroxyethyl methacrylate).
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strong evidence that changes in β(1,6) branching of N-linked glycans due to altered GnT-V activity affect carcinoma progression in vivo.

During the investigation of possible mechanisms by which GnT-V expression levels could regulate carcinoma invasiveness and motility, cells transfected with a retroviral construct encoding GnT-V that showed increased β(1,6) branching were observed to display altered calcium-dependent cell aggregation properties. Because calcium-dependent aggregation was likely due to altered cadherin function, and because reduced cell-adhesion in transformed cells can cause increased cell migration and lead to increased invasiveness and metastasis, we focused on testing the hypothesis that changes in GnT-V expression result in altered cadherin-based cell-cell adhesion and downstream intracellular signaling pathways. This hypothesis is relevant to the understanding of oncogenic transformation, because oncogenesis is often associated with decreased cell-cell adhesion, alterations in adhesion-mediated signaling pathways, and changes in organization of the cytoskeleton that can influence cell migration and invasiveness. Our results showed that increased expression of GnT-V activity in two fibroblastic cell types, HT1080 and NIH3T3, caused increased levels of N-linked β(1,6) branching and poly-N-acetyllactosamine on N-cadherin, a concomitant decrease in the rates of homophilic cell-cell adhesion mediated by N-cadherin, as well as a stimulation of cadherin-mediated cell migration. Induction of GnT-V expression inhibited clustering of cell surface N-cadherin and enhanced the susceptibility of β-catenin and p120ctn phosphorylation by growth factors and the src oncogene, consistent with the increased migratory phenotype of GnT-V-overexpressing cells. Mouse embryonic fibroblasts (MEF) from GnT-V−/− mice showed an increased rate of calcium-dependent cadherin-mediated cell-cell adhesion compared with MEF from GnT-V+/− littersmates; this increased rate of adhesion was decreased when the GnT-V−/− MEF were transfected with a construct encoding GnT-V, confirming that cadherin-based cell-cell adhesion is regulated by GnT-V expression levels.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—HT1080 human fibrosarcoma cells (CCL-121), NIH3T3 cells (CRL-1658), and MCF-7 cells (HTB-22) were obtained from ATCC; bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), swainsonine (SW), function-blocking monoclonal antibodies against N-cadherin (A-CAM, clone CC-4) and E-cadherin (DECMAC-1), phycoerythrin-conjugated streptavidin, and protein-A-agarose at 4°C were products of BD Falcon™. Protein A-agarose, polyclonal antibody against N-cadherin, β-catenin, and p120ctn (10 mg/ml swainsonine-added cell culture media for 24 h) were prepared for the aggregation assay. Cell aggregation was measured using an inverted phase-contrast microscope by counting the number of cells in aggregates (three or more cells) in each field over a total of five randomly chosen fields, calculating the mean and standard error of these measurements, and expressing the mean relative to the mean total number of cells per field as percent aggregation (13). Calcium-dependent aggregation was calculated by subtracting values obtained from aggregation assays in the presence of EGTA (2 mM) from the values of the total number of aggregating cells. The levels of aggregating cells in EGTA were typically <10% of the total number of aggregating cells.

Flow Cytometry Analysis—Cells were grown to subconfluence and detached with 2 mM EDTA in PBS. Cells (10⁶) were washed, resuspended in 100 μl of FCM buffer (PBS containing 1% BSA and 0.01% sodium azide), and then incubated with rabbit antibody against N-cadherin (1:100) at 4°C for 30 min, followed by incubation with biotinylated anti-rabbit IgG (1:100). After washing with FCM buffer, cells were labeled with phycoerythrin-conjugated streptavidin (10 μl) at 4°C for 30 min. Analysis was then performed using the FACScalibur (BD Biosciences) instrument.

Immunoblotting and Lectin Blotting—Cells were harvested and lysed in lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml leupeptin and leupeptin) were added into the culture media for 4°C for 2 h with agitation. After the membrane was washed with TBST, protein bands were developed with ECL reagents and exposed on x-ray film. Immunoreactive bands were quantitated using a Fluor-S (Bio-Rad) instrument.

Cell Surface Biotinylation and Immunoprecipitation—Cell surface protein labeling with biotin was performed as described before (5). Sub-confluent cells were washed and detached using 2 mM EDTA. Cells were then washed twice with ice-cold PBS and incubated with 1 mg/ml NHS-LC-biotin in PBS for 20 min at 4°C on a rocking platform. After washing with PBS, cells were lysed by incubation with lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and leupeptin) for 30 min at 4°C. For immunoprecipitation of N-cadherin, lysates (500 μg of protein) were cleared by centrifugation and incubated with protein-A-agarose at 4°C for 2 h with agitation for 1–2 h to remove nonspecific adsorption to the agarose beads. After the determination of total protein using a BCA assay, cell lysates were incubated with 2 μg of polyclonal antibody against N-cadherin for 3 h at 4°C under agitation, followed by incubation with protein-A-agarose at 4°C for 2 h with agitation. The pellets were then washed twice in lysis buffer subject to SDS-PAGE under reducing conditions. The gels were then transferred onto PVDF membrane and probed with streptavidin-HRP (1:5000). After stripping, the membrane was re-probed with secondary antibodies for appropriate antigen and incubated with 100 mM NaCl, 0.6 mM NaHPO4, 10 mM glucose, and 10 mM Hepes, pH 7.4) containing 0.02% trypsin and 2 mM CaCl2. Single cell suspensions were prepared, washed, and resuspended in HCMF buffer containing 2 mM CaCl2 at a concentration of 10⁶ cells/ml. 300-μl aliquots of cell suspension were added to the wells of 24-well plates pre-coated with 1% BSA and incubated for different times at 37°C with 80 rpm agitation. For some experiments, EDTA (2 mM), EGTA (2 mM), function blocking antibody against N-cadherin (A-CAM, 50 μg/ml), E-cadherin (DECMAC-1, 100), β3 integrin (Ab131, 50 μg/ml), α3 integrin (Ab161, 2 mg/ml), and non-immune IgG (50 μg/ml) were added into the cell suspension, respectively. For swainsonine-treated cells, 1 μg/ml swainsonine was added into the culture media for 24 h. Some experiments were performed using an inverted phase-contrast microscope by counting the number of cells in aggregates (three or more cells) in each field over a total of five randomly chosen fields, calculating the mean and standard error of these measurements, and expressing the mean relative to the mean total number of cells per field as percent aggregation (13). Calcium-dependent aggregation was calculated by subtracting values obtained from aggregation assays in the presence of EGTA (2 mM) from the values of the total number of aggregating cells. The levels of aggregating cells in EGTA were typically <10% of the total number of aggregating cells.

Cell Culture—HT1080 and NIH3T3 cells transfected with a retroviral plasmid encoding mouse GnT-V cDNA (pTJ66GnT-V) (5) were maintained and passaged routinely at 37°C in 5% CO2 in DMEM growth media containing 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), supplemented with 0.1 mM non-essential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg of streptomycin (Sigma).

Cell Aggregation Assay—Cell aggregation assays were performed as described previously (12) with minor modifications. Briefly, subconfluent cells were washed with PBS and then detached with HCMF buffer containing 150 mM NaCl, 0.6 mM NaHPO4, 10 mM glucose, and 10 mM Hepes, pH 7.4) containing 0.02% trypsin and 2 mM CaCl2. Single cell suspensions were prepared, washed, and resuspended in HCMF buffer containing 2 mM CaCl2 at a concentration of 10⁶ cells/ml. 300-μl aliquots of cell suspension were added to the wells of 24-well plates pre-coated with 1% BSA and incubated for different times at 37°C with 80 rpm agitation. For some experiments, EDTA (2 mM), EGTA (2 mM), function blocking antibody against N-cadherin (A-CAM, 50 μg/ml), E-cadherin (DECMAC-1, 100), β3 integrin (Ab131, 50 μg/ml), α3 integrin (Ab161, 2 mg/ml), and non-immune IgG (50 μg/ml) were added into the cell suspension, respectively. For swainsonine-treated cells, 1 μg/ml swainsonine was added into the culture media for 24 h. Single cell suspensions were prepared for the aggregation assay. Cell aggregation was measured using an inverted phase-contrast microscope by counting the number of cells in aggregates (three or more cells) in each field over a total of five randomly chosen fields, calculating the mean and standard error of these measurements, and expressing the mean relative to the mean total number of cells per field as percent aggregation (13). Calcium-dependent aggregation was calculated by subtracting values obtained from aggregation assays in the presence of EGTA (2 mM) from the values of the total number of aggregating cells. The levels of aggregating cells in EGTA were typically <10% of the total number of aggregating cells.
expressed as “lectin binding of N-cadherin.”

Antibody Immobilization and Clustering Assays—For antibody immobilization experiments (15, 16), 6-well plates were coated with goat anti-rabbit IgG (10 μg/ml) for 2 h at 37 °C. Plates were washed with PBS and then incubated with polyclonal antibody against N-cadherin (10 μg/ml) at 4 °C overnight and blocked with 1% BSA. Cells were grown to confluence, serum-starved for 18–24 h, and harvested with 2 mM EDTA treatment. After washing with PBS, cells were resuspended in serum-free DMEM and added into the antibody-coated plates at 37 °C for different times. After non-adherent cells were removed with washing, attached cells were lysed and used for the phospho-ERK1/2 assay. Poly-I-lysine-coated plates were used in control experiments. For antibody-clustering experiments (15), a single cell suspension was prepared above described, and 0.5 mM Na3VO4 and lysed for the phospho-ERK1/2 assay. Quantitative analyses were performed by densitometry and expressed as described previously (15). In brief, cells were grown to confluence and serum-starved overnight in DMEM culture media containing 10 mM Hepes. Cells were then incubated with DMEM containing 4 mM EGTA for 30 min at 37 °C. The EGTA chelates Ca2+ and disrupts cadherin-mediated cell-cell contacts. Then, Ca2+-free medium was removed, and CaCl2 was added back to 1.8 mM (Ca2+ switch) to induce cadherin-mediated intercellular interactions. Cells were harvested at different time points after the addition of Ca2+ and then lysed for assay of phospho-ERK1/2 by Western blotting using the monoclonal antibody against phospho-ERK1/2. Quantitative analyses were performed by densitometry and expressed as “relative ERK phosphorylation” by dividing the level of the phospho-ERK signal by the ERK protein signal at each time point.

Assay of MAPK Activation Mediated by Cell-Cell Adhesion—Cadherin-mediated cell-cell adhesion was performed as described previously (15). In brief, cells were grown to confluence and serum-starved overnight in DMEM culture media containing 10 mM Hepes. Cells were then incubated with DMEM containing 4 mM EGTA for 30 min at 37 °C. The EGTA chelates Ca2+ and disrupts cadherin-mediated cell-cell contacts. Then, Ca2+-free medium was removed, and CaCl2 was adjusted back to 1.8 mM (Ca2+ switch) to induce cadherin-mediated intercellular interactions. Cells were harvested at different time points after the addition of Ca2+ and then lysed for assay of phospho-ERK1/2 by Western blotting using the monoclonal antibody against phospho-ERK1/2. Quantitative analyses were performed by densitometry and expressed as “relative ERK phosphorylation,” as described above.

Tyrosine Phosphorylation of Catenins and Growth Factor Receptors—Cells were grown to confluence and serum-starved overnight. One hour before the treatment of cells with growth factors, 1 mM sodium orthovanadate, a specific inhibitor of PTP, was added to cultures to disrupt PTP-dependent tyrosine phosphorylation. Cells were then incubated with DMEM containing 4 mM EGTA for 30 min at 37 °C. After stripping, the membrane was subjected to Western blotting with mouse anti-phosphotyrosine (PY20). The membrane was counted in five different fields with a light microscope at room temperature for 5 min. The supernatant was transferred to a microcentrifuge tube, and genomic DNA was then precipitated by adding an equal volume of ice-cold 95% ethanol. The DNA pellet was rinsed with 70% ethanol, resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Primers specific for wild type GnT-V (5′-CC-TCTCCTCCACACAGGAAAT-3′, primer 5′-CCGTCGCTGGTGAAGTG-3′), yielding 0.26-kb PCR product and for LacZ (5′-TTACTGCGCGCTGTTTACAAAGGTCGTTA-3′, primer 5′-AT-GTAAACGGAATACACCCGTCTCGATTCT-3′), yielding 0.36-kb PCR product were used for PCR analysis. These primer pairs were developed and determined to allow accurate genotyping of MEF. Transient transfections of GnT-V-deficient MEF with GnT-V cDNA were performed with LipofectAMINE™ reagent according to the manufacturer’s instructions using 5 μg of DNA/60-mm dish. Cells were incubated for 48–72 h after transfection and then used for calcium-dependent cell-cell adhesion, as described above.

Fluorescent Staining—Cells were plated on coverslips and incubated at 37 °C overnight to allow cell spreading. Cells were then fixed with 3% formaldehyde in PBS for 15 min followed by three times washing with PBS, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 1% BSA. Coverslips were incubated with biotinylated α2-integrin (10 μg/ml) at 37 °C for 30 min. After washing three times with PBS, the bound α2-integrin was detected by incubation with rhodamine-conjugated streptavidin (5 μg/ml) at 37 °C for 30 min. After washing with PBS, the coverslips were mounted and photographed by using fluorescence microscopy.

RESULTS

Exogenous Expression of GnT-V Reduced Calcium-dependent Homotypic Cell-Cell Adhesion—Because increased transcription of GnT-V and N-linked β1,6-branched glycans are often observed in human tumors and carcinoma cell lines, to investigate in detail phenotypic changes that could result from this aberrant glycosylation, a retroviral expression system was constructed to produce a population of transfected HT1080 and NIH3T3 cells in which ~95% showed GnT-V overexpression, as evidenced by green fluorescent protein-reporter fluorescence (5). Because single-cell suspensions of these cells were prepared to examine cell-matrix adhesion, it became clear by vis-
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Involvement of N-cadherin in Reduced Cell-Cell Adhesion Caused by GnT-V Overexpression—To determine the relative expression levels of N- and E-cadherin in HT1080 and NIH3T3 cells, cell lysates were subjected to SDS-PAGE and Western blotting using antibodies that distinguished these two types of cadherin. The results (Fig. 2A) of this experiment showed that both cell types highly expressed N-cadherin but had negligible expression of E-cadherin, consistent with a previous report (21). The ability of several function-blocking antibodies to inhibit calcium-dependent, function-blocking antibody to N-cadherin (anti-A-CAM) inhibited almost completely calcium-dependent cell-cell adhesion of GnT-V-overexpressing cells and significantly reduced mock transfected cell-cell adhesion, demonstrating that the most significant calcium-dependent adhesion process measured in these aggregation assays was mediated by N-cadherin (19, 22). To provide additional evidence that changes in GnT-V expression and N-linked β(1,6)-branched glycans affected N-cadherin-mediated adhesion, swainsonine treatment was employed. Swainsonine treatment of cells (24 h) inhibits Golgi α-mannosidase II, which results in the lack of processing of the α(1,6)-linked mannose branch that is required for subsequent GnT-V action. Pre-treatment with swainsonine, therefore, should obviate effects of GnT-V overexpression, because the carbohydrate residues necessary for β(1,6) branching by GnT-V are not present. The results shown in Fig. 2C demonstrate that the inhibition of calcium-dependent cell-cell adhesion observed for the GnT-V-overexpressing cells was not present when the cells were grown in the presence of swainsonine, confirming that the inhibition resulted from increased GnT-V activity.

GnT-V Overexpression Had No Effect on N-cadherin Levels but Altered Glycosylation of N-cadherin—Because GnT-V overexpression caused reduction of N-cadherin-mediated cell-cell adhesion, it was important to determine if cell surface levels of N-cadherin were altered. To this end, flow cytometry (Fig. 3A) and Western blotting (Fig. 3B) were performed using an antibody against N-cadherin. Overexpression of GnT-V had no effect on the levels of cell surface expression of N-cadherin. These results were confirmed further by a cell surface labeling experiment where cell surfaces were biotinylated, followed by immunoprecipitation with antibody to N-cadherin and probing of Western blots with streptavidin-HRP (Fig. 3C). Equivalent levels of cell surface labeled N-cadherin were observed.

To determine if GnT-V overexpression caused changes in the N-linked glycosylation of N-cadherin, SDS-PAGE and blotting techniques were performed. The lectin l-phytohemagglutinin, l-PHA, binds to the glycan sequence α(1,6)Manβ(1,6) GlcNAcb(1,4)Gal and can reflect levels of β(1,6)-branched structures (23). Immunoprecipitation of cell lysates with antibody to N-cadherin, followed by blotting with biotinylated l-PHA, showed increased l-PHA binding to N-cadherin in HT1080 and NIH3T3 cells after GnT-V overexpression, as expected (Fig. 3D). To study further the alterations in N-linked glycosylation of N-cadherin caused by GnT-V, lectin precipitation (LP) using several lectins that bind to N-linked saccharides was employed. Similar expression patterns of N-glycans on N-cadherin were observed in both mock transfected HT1080 and NIH3T3 cells, and in both cases, increased l-PHA binding after overexpression of GnT-V was accompanied by increased D. stramonium agglutinin binding (Fig. 3E), suggesting enhanced expression of poly-N-acetyllactosamine on N-cadherin. This result confirms the observation that poly-N-acetyllac-
Tosamine is synthesized preferentially on N-glycans expressing the /H9252(1,6) branch (24). By contrast, little change in concanavalin A (ConA) and Sambucus nigra agglutinin (SNA) binding was observed after overexpression of GnT-V, indicating little effect on the expression of either high mannose or biantennary N-linked oligosaccharides (ConA ligands) and /H9251(2,6)-sialic acid (SNA ligand) on N-cadherin after GnT-V overexpression. In addition, no expression of /H9251(2,3)-sialylation was found on N-cadherin, as evidenced by the lack of Maackia ameurinsin agglutinin (MAA) binding (Fig. 3E), although this lectin does bind to other glycoconjugates in both cell types (data not shown). These results indicate that overexpression of GnT-V caused significant changes in N-glycan expression on N-cadherin and suggest that the effects of GnT-V overexpression on N-cadherin-mediated cell-cell adhesion most likely involve these glycan changes.

Altered N-Linked Glycan Expression Inhibits the N-cadherin-mediated Outside-in Signaling Pathways—To study the mechanisms by which aberrant N-glycosylation results in reduced cell-cell adhesion, cadherin signaling experiments were performed in HT1080 cells. Assays utilizing immobilized antibodies have been suggested to cluster adhesion receptors on the cell surface and thereby provoke downstream signaling pathways (16, 25). For example, immobilized E-cadherin and N-cadherin antibodies were shown to stimulate E-cadherin-mediated activation of MAPK (15) and N-cadherin-mediated Akt phosphorylation (16), respectively, and these effects were suggested to result from clustering the cadherins. To examine further the effect of increased N-glycosylation of N-cadherin on the clustering and signaling, a single-cell suspension of HT1080 cells in serum-free media were allowed to attach onto plates pre-coated with anti-N-cadherin antibody. As shown in Fig. 4A, contact of mock transfected cells onto immobilized antibody resulted in almost a 4-fold stimulation of ERK1/2 phosphorylation after 30 min, an effect that is known to activate MAPK (15). Stimulation of ERK1/2 phosphorylation in GnT-V-transfected cells, however, was inhibited by more than 30% at 30 min. Even more dramatic inhibition of ERK1/2 phosphorylation was observed, however, if the experiment was performed in a different manner. In this procedure, single cells were incubated first with rabbit anti-N-cadherin antibody on poly-HEMA-coated plates for 60 min at 4 °C, washed, and then incubated at 37 °C with goat-anti-rabbit IgG for various times. Fig. 4B shows that inhibition of ERK1/2 phosphorylation by increased GnT-V expression was over 70% at 30 min. In the experiments for which results are shown in Fig. 4 (A and B),

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omission of the anti-N-cadherin antibody resulted in no increased ERK1/2 phosphorylation (data not shown). In a third type of calcium switch experiment, adding back calcium to cells incubated in the absence of calcium resulted in the establishment of calcium-dependent cell-cell contacts that stimulated ERK1/2 phosphorylation, but the observed ERK1/2 phosphorylation was delayed in the GnT-V-overexpressing cells (Fig. 4C). The conclusion from these experiments was that altered β(1,6) branching most likely reduced the clustering ability of N-cadherin, which, in turn, inhibited N-cadherin-mediated

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**Fig. 3. Effects of GnT-V overexpression on cell surface N-cadherin levels and glycosylation of N-cadherin.**

A, flow cytometry analysis of HT1080 cell suspensions incubated with antibodies against N-cadherin, followed by incubation with biotinylated anti-rabbit IgG and PE-conjugated streptavidin. B, immunoblots of HT1080 or NIH3T3 cell lysates from mock and GnT-V-transfected cells probed with antibodies against N-cadherin (M, mock transfected; G, GnT-V-transfected). C, HT1080 cell surfaces were biotinylated with NHS-LC-biotin, followed by immunoprecipitation (IP) with anti-N-cadherin antibody, SDS-PAGE, blotting, and probing with streptavidin-conjugated horseradish peroxidase. D, N-cadherin was immunoprecipitated from mock and GnT-V-transfected cells, subjected to SDS-PAGE, and β(1,6) branching detected by lectin-blotting using L-PHA; the membrane was re-probed with anti-N-cadherin to confirm equal amounts of precipitated N-cadherin were used for L-PHA blotting. E, various lectins were used to precipitate glycoproteins from cell lysates, followed by SDS-PAGE, blotting, and detection using anti-N-cadherin (top panel). Lectin binding of N-cadherin was quantified by densitometric analysis and data represent the mean (±S.D.) of three independent experiments (bottom panel). LP, lectin precipitation.
GnT-V Overexpression Increased Tyrosine Phosphorylation of Catenins by Growth Factors and the v-src Oncogene—Cadherin-based adhesion is regulated by the formation of the cadherin-catenin complex (26, 27). Increased tyrosine phosphorylation of catenins has repeatedly been correlated with the disruption of cadherin-catenin cell-cell adhesion (28) and increased cellular migration (17, 29). β-Catenin, γ-catenin, and p120ctn have been shown to be tyrosine-phosphorylated by receptor tyrosine kinases such as the EGF receptor and by the src oncogene (27, 30–32). To explore the effect of GnT-V overexpression on phosphorylation of cadherin-catenin complex, the phosphotyrosine levels of β-catenin, γ-catenin, and p120ctn were examined by immunoprecipitation, blotting, and probing with anti-phosphotyrosine antibody during establishment of N-cadherin mediated cell-cell adhesion. Due to very low expression of γ-catenin in HT1080 and NIH3T3 cells, its phosphorylation could not be detected (data not shown). Much more marked enhancements in tyrosine phosphorylation of β-catenin and p120ctn, however, were observed in GnT-V-transfected HT1080 and NIH3T3 cells (Fig. 5A and B), after treatment of cells with either EGF or FGF, compared with the enhanced phosphorylation in mock transfected cells. These results indicated that overexpression of GnT-V increased the tyrosine phosphorylation of catenins by growth factor receptors, consistent with the reduced cell-cell adhesion and increased migratory phenotype of GnT-V-expressing cells (5). As a control, we also examined the phosphorylation levels of the EGFR and FGFR themselves. Tyrosine phosphorylation of EGFR or FGFR was increased to the same extent in both mock and GnT-V-transfected cells after stimulation of cells with EGF or FGF (Fig. 5A). These results indicated that identical signaling from EGFR or FGFR was transduced in mock and GnT-V-transfected cells, and increased tyrosine phosphorylation of catenins was not due to the altered function of the growth factor receptors caused by overexpression of GnT-V. Significant tyrosine phosphorylation of p120ctn was also observed in GnT-V-overexpressing HT1080 cells after transient expression of the Src protein than that observed in mock transfected cells (Fig. 5C). This result was consistent with those using growth factor treatment to stimulate catenin phosphorylation.

To confirm further the involvement of increased N-glycan β(1,6) branching in cadherin-mediated catenin phosphorylation, GnT-V-overexpressing cells were treated with swainsonine. Increased tyrosine phosphorylation of p120ctn was significantly abrogated by pre-treatment of cells with swainsonine (Fig. 5D), demonstrating that increased tyrosine phosphorylation of catenins was likely due to aberrant N-glycosylation caused by up-regulated GnT-V.

Overexpression of GnT-V Promoted Cadherin-related Cell Migration—Because N-cadherin has been implicated in the regulation of tumor cell migration and invasion (19, 21, 22) and increased tyrosine phosphorylation of catenins has also been correlated with increased cell migration (17, 29), we tested whether increased GnT-V activity could affect cadherin-related cell motility and phosphorylation of catenin during cell migration. Using Boyden-chamber (Transwell) migration assays, GnT-V-overexpressing HT1080 and NIH3T3 cells showed a significant increase in the rates of migration in response to incubated in wells at 37 °C, and shifted to calcium-containing media for various times, followed by processing for phospho-ERK1/2 and ERK1/2 detection by immunoblotting (top panel) and quantitative analysis (bottom panel). Data represent the mean (±S.D.) of three independent experiments. WB, Western blotting.
FIG. 5. Effects of GnT-V overexpression on tyrosine phosphorylation of catenins by growth factors and v-src oncoprotein expression. A, serum-starved HT 1080 cells were treated with EGF (100 ng/ml) or FGF (25 ng/ml) for 10 min at 37 °C and lysed in 1% Triton X-100 buffer containing PTP inhibitors. Immunoprecipitations were performed on lysed cells using antibodies against catenins or growth factor receptors, followed by separation by SDS-PAGE, blotting, and probing with anti-phospho-tyrosine antibody (PY) or other antibodies as noted (EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; M, mock transfected; G, GnT-V-transfected). B, serum-starved NIH3T3 cells were treated with EGF or FGF, and tyrosine phosphorylation of catenins was detected as described in A. C, HT1080 cells were transiently transfected with an expression plasmid encoding v-src and assayed for tyrosine phosphorylation of cell lysates (top panel) or p120 ctn (bottom panel) using immunoprecipitation, SDS-PAGE, and Western blotting with either anti-phospho-tyrosine (PY) or anti-p120 catenin, respectively. D, HT1080 cells were incubated with swainsonine (SW) for 24 h before treatment with EGF for 10 min at 37 °C, followed by detection of tyrosine phosphorylation of p120 ctn (top panel) and quantification analysis (bottom panel). Data represent the mean (±S.D.) of three independent experiments.
conditioned media containing chemotactic attractants, compared with mock transfected cells (Fig. 6A). Increased cell migration was inhibited by preincubation of cells with swainsonine, indicating that the increased cell motility was due, at least in part, to overexpression of β1(1,6) branching. As shown in Fig. 6A, cell migration was significantly increased by treatment of cells with function-blocking N-cadherin antibody, suggesting the involvement of N-cadherin in regulating cell migration. As expected, cell migration was diminished by different degrees by function-blocking antibody against β1 and α5 integrin, most likely due to the presence of fibronectin and/or collagen in the conditioned media used as an attractant, consistent with the results of our earlier study (5). To elucidate further the involvement of N-cadherin in cell migration, a monolayer of HT1080 cells was scratched multiple times using a sterile pipette tip, after which cells began to migrate into the “scratch wound.” At different times after wounding, whole cell lysates were collected for immunoprecipitation of p120ctn to study its phosphorylation status during active cell migration. As shown in Fig. 6B, scratch wounds were almost filled by GnT-V-overexpressing cells after 6-h incubation (top panel), suggesting increased cell migration of these cells. Increased cell migration of GnT-V-overexpressing cells was accompanied by significantly increased phosphorylation of p120ctn (bottom panel), indicating the direct involvement of the cadherin-catenin complex in cell migration. These results demonstrate the stimulation of cell migration by increased GnT-V activity and altered N-linked glycan expression and suggest strongly that the increased cell migration of GnT-V-overexpressing cells results, at least in part, from increased tyrosine phosphorylation of cadherin-catenin complexes and reduced cadherin-based cell-cell adhesion.

GnT-V-deficient Mouse Embryo Fibroblasts Show Increased N-cadherin-dependent Cell-Cell Adhesion That Could Be Reduced by Transient Expression of GnT-V—If overexpression of GnT-V does indeed inhibit N-cadherin-mediated cell-cell adhesion, then cells that lack GnT-V expression would be predicted to show increased cell-matrix adhesion. To test this hypothesis, we utilized mouse embryo fibroblasts (MEFs) from GnT-V homozogous null mice (GnT-V−/−) (11). As shown in Fig. 7A, MEF from heterozygous littermates (GnT-V+/−) stained very strongly with fluorescent L-PHA, whereas the staining of GnT-V−/− MEF with l-PHA was negligible, confirming the lack of N-linked β1(1,6) branches. Both GnT-V+/− and GnT-V−/− MEF expressed comparable levels of N-cadherin, and E-cadherin expression was not detectable by Western blotting (Fig. 7B). Compared with GnT-V+/− MEF, GnT-V−/− MEF showed dramatically higher levels of calcium-dependent cell-cell adhesion that were abrogated by function-blocking N-cadherin antibody, but not by E-cadherin and integrin antibody, as the hypothesis predicted (Fig. 7C). Furthermore, concomitant with increased cell-cell adhesion, decreased tyrosine phosphorylation of p120ctn was observed in GnT-V−/− MEF after treatment with EGF (Fig. 7D). As a further test of the hypothesis, GnT-V cDNA was transiently expressed in GnT-V−/− MEF; expression of GnT-V activity and β1(1,6) branching were demonstrated by l-PHA binding to the GnT-V−/− MEF after transfection. Calcium-dependent cell-cell adhesion was significantly inhibited after transient expression of GnT-V (Fig. 7E). These results strongly support the conclusion that increased expression of GnT-V and β1(1,6) branching that often occur during oncogenesis can reduce homotypic cell-cell adhesion mediated by cadherin and promote cell migration.

DISCUSSION

Studies have suggested the involvement of GnT-V levels in regulating cell-matrix adhesion, cell migration, and invasion...
(10, 33–35) by affecting the N-glycosylation of cell surface adhesion receptors, including the integrins (5). In the present study, evidence is presented that the relative levels of GnT-V activity expressed in a cell regulates homotypic cell-cell adhesion, migration, and intracellular signaling pathways by modulation of an additional cell surface adhesion receptor, N-cadherin. The cadherins constitute a family of single-pass transmembrane glycoproteins mediating calcium-dependent cell-cell adhesion that plays an essential role in regulating major cellular behaviors, including cell growth, motility, and differentiation (36, 37). Several cadherins, including E-cadherin and N-cadherin, have been shown to have in common a five-repeated extracellular domain and to regulate cell-cell adhesion in a homotypic manner through their amino-terminal extracellular domains, such as ECD1 (38, 39). The conserved cytoplasmic domain of cadherin interacts with various proteins, collectively termed catenins, that link cadherins to the actin-based cytoskeleton and promote strong cell-cell adhesion (40). Evidence from several sources suggests that the formation and tyrosine phosphorylation of the cadherin-catenin complex play important roles in the maintenance of the stabilization of cell-cell adhesion (17, 27, 29, 41). Human N-cadherin is widely present in mesenchymal and neural cells, endothelium, and skeletal myocytes and contains nine putative N-linked glycosylation sites, although it is not known which of these sites are utilized.

Our results show that overexpression of GnT-V caused altered N-linked glycosylation of N-cadherin in two fibroblastic cell types, human HT1080 and mouse NIH3T3 cells, and that this overexpression inhibited homotypic cell-cell adhesion, the majority of which was mediated by N-cadherin. It should be noted that other authors have also observed less than complete inhibition of cell-cell adhesion using function-blocking antibodies against N- and E-cadherin (19, 22, 42). Taniguchi's laboratory showed that overexpression of GnT-III in B16 mouse melanoma cells resulted in an altered glycosylation of E-cadherin.
(decreased expression of the N-linked β(1,6) branch and increased levels of the “bisected” N-linked glycan) (42). This result was expected, because GnT-III and GnT-V compete for biantennary substrates during glycoprotein biosynthesis in the Golgi. These changes in glycosylation were associated with a reduced E-cadherin turnover rate at the cell surface and reduced metastatic potential of the melanoma cells. This very important study showed that increased E-cadherin levels in melanoma cells, as a result of changes in glycosylation, increased cell-cell adhesion and inhibited levels of lung metastasis. It was essential, therefore, to determine in our experiments if overexpression of GnT-V and concomitant, altered glycosylation of N-cadherin influenced cell surface expression levels of N-cadherin.

Our results showed that by two separate types of experiments, flow cytometry and cell surface labeling/Western blotting, increased expression of GnT-V had no significant effect on N-cadherin cell surface levels. The regulation of cadherin-based cell-cell adhesion by changes in N-linked glycan expression, therefore, appears to occur by at least two distinct mechanisms; one based on changes in turnover of E-cadherin by GnT-III expression (42), and the other based on altered avidity of N-cadherin regulated by GnT-V expression levels, likely due to changes in receptor clustering, discussed below.

Overexpression of GnT-V altered the expression pattern of N-glycans on N-cadherin in both HT1080 and NIH3T3 cells, both of which expressed high levels of N-cadherin. Our study indicated that the expression of β(1,6)-branched oligosaccharides and poly-N-acetyllactosamine on N-cadherin was enhanced after expression of GnT-V. N-Linked β(1,6) branching and poly-N-acetyllactosamine have been associated, in some cases, with the increased invasive and metastatic potential of tumor cells (reviewed in Ref. 43); our results were, therefore, consistent with phenotypic changes in some transformed cells (44) and GnT-V-overexpressing cells (5). Moreover, our results also provided direct evidence that poly-N-acetyllactosamine shows concomitant increased expression when N-linked β(1,6) branches are increased by GnT-V overexpression.

Clustering of cell surface adhesion receptors in the plasma membrane plays an essential role in the regulation of cell-cell interactions and outside-in signaling pathways (15, 16, 25). Cell-cell adhesion initiates the clustering of the extracellular domain of cadherins that can be strengthened by the association between the cytoplasmic domain of cadherin with the actin cytoskeleton (45, 46). Immobilized anti-cadherin antibodies were shown to stimulate E-cadherin-mediated activation of MAPK (15) and N-cadherin-mediated Akt phosphorylation (16) by promoting the clustering of cadherin receptors. The results of our experiments suggest that in mock transfected cells, both immobilized anti-cadherin antibody or rabbit anti-cadherin antibody clustered by goat anti-rabbit IgG stimulated the phosphorylation of ERK1/2 (MAPK). This phosphorylation of ERK1/2, however, was significantly delayed temporally in GnT-V-transfected cells, suggesting a decreased clustering ability of N-cadherin after GnT-V overexpression. These results support the conclusions of our previous report (5) that GnT-V overexpression resulted in the inhibition of integrin clustering that led to lowered rates of cell-matrix adhesion, changes in integrin intracellular signaling pathways, and increased tumor cell invasion potential.

Cadherin-mediated cell-cell interactions also stimulate some outside-in signaling pathways. MAPK, small GTPases, Cdc42, and Rac were all activated upon the re-establishment of E-cadherin-mediated cell-cell contacts (15, 47–49), and this activation was dependent on functional cadherin (50, 51). The results from the “calcium switch” experiment showed that re-establishment of N-cadherin-mediated cell-cell contacts could also activate ERK1/2 and that this activation was delayed in GnT-V-overexpressing cells. Regulation of signal transduction pathways by the expression of specific carbohydrate structures on cell surface receptors has been documented during development; for example, the activity of the Notch receptor can be inhibited by the lack of a specific O-linked β(1,3)GlcNAc residue transferred by the N-acetylglucosaminyltransferase encoded by Fringe (52). The results of the present study are one of the first demonstrations that N-glycan changes on a specific adhesion receptor of tumor cells, in this case changes in β(1,6) branching levels on N-cadherin, may regulate an outside-in signal transduction pathway.

Homotypic cell-cell interactions are modulated by the formation of cadherin-catenin complexes and subsequent tyrosine phosphorylation events (53). β-Catenin or γ-catenin, in association with p120ctn, binds directly to the cytoplasmic domain of cadherin to form the cadherin-catenin complex, which is linked to the actin cytoskeleton via α-catenin (54). Increased phosphorylation of β-catenin and p120ctn are strongly associated with decreased cadherin-mediated cell-cell adhesion and increased cellular migration (5, 27, 30–32, 55, 56). To explore further the mechanisms of reduced cell-cell adhesion caused by changes in N-linked glycan expression, N-cadherin, and several catenins were immunoprecipitated from mock and GnT-V-transfected cells, either treated first by EGF, FGF, or transfected by v-src, to study tyrosine phosphorylation of cadherin-catenin complexes. Significantly increased tyrosine phosphorylation of both β-catenin and p120ctn was observed in GnT-V-overexpressing cells stimulated not only by EGF, but also by FGF, p120ctn was first identified as a prominent substrate of the Src oncoprotein, and its tyrosine phosphorylation by Src was intensively implicated in cadherin-mediated adhesion (reviewed in Ref. 57). Consistent with the results from the growth factor treatments, p120ctn showed markedly increased phosphorylation after transient expression of the Src oncoprotein in GnT-V-transfected cells compared with mock-transfected cells. These results indicated increased susceptibility of tyrosine phosphorylation of catenin in GnT-V-overexpressing cells, consistent with the lower rates of cell-cell adhesion seen in cells overexpressing GnT-V. Because phosphorylation of the EGF receptor or FGF receptor was not affected after GnT-V overexpression, the possibility that increased tyrosine phosphorylation of catenins resulted from aberrant signaling from the EGF or FGF receptor is unlikely. Increased phosphorylation of p120ctn could be suppressed by pre-treatment of GnT-V-expressing cells with swainsonine, further confirming the direct involvement of N-linked oligosaccharides in the enhanced phosphorylation of catenins. Our results are consistent with those of a previous report (56) that overexpression of GnT-III, which competes with GnT-V for biantennary substrates, enhanced cell-cell aggregation mediated by E-cadherin and down-regulated tyrosine phosphorylation of β-catenin. Taken together, these results indicate opposing effects exerted by GnT-V and GnT-III on cadherin-mediated cell-cell adhesion and catenin phosphorylation. Aberrant N-glycosylation of cadherin could result in conformational changes of the cadherin-catenin complex that would subsequently increase phosphorylation of catenin, which would, in turn, decrease the strength of cadherin-mediated cell-cell adhesion by influencing the organization of actin cytoskeleton (58, 59).

Cadherins have been well implicated in the process of tumor metastasis. Depending on the cell type, N-cadherin activity appears to be able to have either a positive or negative effect on migration and metastasis-related behaviors. For example, overexpression of N-cadherin was shown to stimulate MCF-7 tumor cell migration and invasion (19, 60) and forced expres-
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In the study of HT1080 human fibrosarcoma and mouse fibroblast NIH3T3 cells were used as experimental models, because both cell lines expressed high levels of N-cadherin, and in vivo embryo fibroblasts from GnT-V knock-out animals revealed significant increases in the rates of adhesion, compared with the rates for control mice. As a result, transfection of the GnT-V expression vectors and v-src expression vectors were used to investigate in detail the mechanism by which GnT-V activity is modulated. These studies demonstrated that GnT-V activity positively regulates cell-cell adhesion by modulating N-cadherin expression and function, and negatively regulates cell migration by modulating N-cadherin expression and function. This suggests a negative regulation by N-cadherin on the migration of HT1080 cells. Treatment with the function-blocking antibody resulted in decreased cell-cell adhesion, promoting cell motility. This result is consistent with the results from overexpressing N-cadherin in the mouse osteosarcoma cells (61) and confirms the dual role of N-cadherin in regulation of cell migration.

In our studies, HT1080 human fibrosarcoma and mouse fibroblast NIH3T3 cells were chosen as experimental models, because both cell lines expressed high levels of N-cadherin, and the results obtained with these cell types would be directly applicable to experiments using mouse embryo fibroblasts from GnT-V null mice. Our results showed altered N-linked glycans on N-cadherin from both HT1080 cells and NIH3T3 cells after overexpression of GnT-V and reduced rates homotypic cell-cell adhesion mediated by N-cadherin. The hypothesis generated from these conclusions states that GnT-V activity levels can, in effect, modulate cell-cell adhesion by effects on N-cadherin clustering, binding, and signaling. Examination of the calcium-dependent and N-cadherin-based cell-cell adhesion of mouse embryo fibroblasts from GnT-V–/– animals revealed significant increases in the rates of adhesion, compared with the rates for MEFs derived from GnT-V+/– littermates, thereby validating the hypothesis. Furthermore, transfection of the GnT-V–/– MEF with GnT-V cDNA caused the cells to express N-linked β1,6-branched glycans and decreased significantly calcium-dependent cell-cell adhesion mediated by N-cadherin. Taken together, these results most likely represent a common mechanism to regulate cell-cell adhesion whereby GnT-V expression levels, which commonly increase during oncogenesis, influence homotypic cell-cell adhesion via cadherin clustering and outside-in signaling which, in turn, promotes increased cell motility and potential invasiveness.

Although it is likely that the levels of β1,6 N-linked glycosylation on N-cadherin itself directly result in altered cell-cell adhesion and signaling, it is possible that this effect is indirect. To investigate in detail the mechanism by which β1,6 N-linked glycosylation regulates cadherin-mediated cell adhesion, experiments are in progress to determine the glycans structures at each potential N-linked site on N-cadherin and generate deletion mutants. Nevertheless, our results strongly support the conclusion that the up-regulation of GnT-V expression that often occurs during oncogenesis alters cadherin-mediated cell-cell adhesion and promotes a more motile and invasive phenotype.

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