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Loss of expression of *N*-acetylglucosaminyltransferase Va results in altered gene expression of glycosyltransferases and galectins

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Abstract **We isolated mouse embryo fibroblasts (MEFs) from *N*-acetylglucosaminyltransferase Va (GnT-Va) knockout mice and studied the effects of loss of expression of GnT-Va on asparagine-linked glycans (*N*-glycan) synthesis and the gene expression of groups of glycosyltransferases and galectins. Loss of GnT-Va expression caused aberrant expression of several *N*-glycan structures, including *N*-linked β(1,6) branching, poly-*N*-lactosamine, bisecting *N*-acetylglucosamine (GlcNAc) and sialic acid. Using quantitative reverse transcriptase-PCR (qRT-PCR), altered gene expression of several groups of glycosyltransferases and galectins was observed in GnT-Va null MEFs, supporting the observed changes in *N*-glycan structures. These results suggest that genetic disruption of GnT-Va ultimately resulted in altered MEFs gene expression and decreased tumor progression associated with loss of GnT-Va observed may result in part from a combination of effects from these altered gene expressions.**

Keywords: Mouse embryo fibroblasts (MEFs); GnT-Va (Mgat5); Knockout; Glycosyltransferase; Galectin; qRT-PCR

1. Introduction

Many studies show that alterations in *N*-linked oligosaccharides of tumor cells are associated with tumorigenesis, progression and metastasis. *N*-linked β(1,6)-*N*-acetylglucosamine (GlcNAc) synthesized by *N*-acetylglucosaminyltransferase Va (GnT-Va or Mgat5) is one of the more common glycans up-regulated during malignant transformation [1]. Studies have proposed a close relationship between increased GnT-Va activity and its glycan products with enhanced cell invasiveness, and in some cases metastatic potential [2–4]. In GnT-Va deficient mice, the progression of mammary tumors induced by polyoma middle T oncoprotein (PyMT) expression was significantly suppressed [5].

It is well-documented that modification of cell surface adhesion molecules and growth factor receptors by GnT-Va is implicated in GnT-Va mediated, invasiveness-related phenotypes [2,6–8]. A recent report showed that aberrant expression of GnT-Va caused altered expression of surface sialyl Lewis X (Sle^x), a sialylated fucose-containing antigen related to tumor metastasis, by changing the expression of Sle^x biosynthesis-related glycosyltransferases [9]. We found that deletion of GnT-Va in mouse embryo fibroblasts (MEFs) causes up-regulation of β1 integrin transcripts, which consequently increased fibronectin-induced cell adhesion and reduced cell motility [10]. These results suggest that aberrant glycosylation caused by GnT-Va inhibition might regulate the gene expression of other proteins.

To further elucidate the molecular mechanisms whereby GnT-Va-modified asparagine-linked glycans (*N*-glycans) may affect tumor invasiveness-related phenotypes in MEFs and mammary tumor cells [7,10], we explored the effect of genetic disruption of GnT-Va on the expression of other glycosyltransferase and galectin transcripts. We report here, for the first time, that the genetically targeted deletion of GnT-Va causes the altered gene expression of several glycosyltransferases and galectins in MEFs using a newly developed qRT-PCR transcript analysis platform [11]. We speculate that some of the inhibited invasiveness-related phenotypes in MEFs caused by deletion of GnT-Va may result, at least in part, from the combination of altered expression of these glycosyltansferases and/or galectins.

2. Materials and methods

2.1. Antibodies and chemicals

Antibodies against galectin-1 and galectin-3, ERK1/2, and HRP-labeled anti-rabbit IgG and anti-mouse IgG were from Santa Cruz Biotechnology. Monoclonal antibody against β1 integrin was a product of BD Biosciences. OL.28 anti-polysialic acid antibody was kind gift from Dr. Karen Colley. Streptavidin–HRP and streptavidin–agarose were obtained from Rockland. WGA–agarose and biotinylated lectins were products of Vector Laboratories.

2.2. Isolation of MEFs

Mouse embryo fibroblasts (MEFs) were isolated from E13.5 embryos from heterozygous GnT-Va knock-out mice mating, and the genotype of MEFs was identified by PCR analysis using genomic DNA as described earlier [6].

2.3. GnT-V activity assay

The assay of GnT-V activity was performed using a synthetic trisaccharide acceptor as previously described [2].

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Abbreviations: MEFs, mouse embryo fibroblasts; GnT-Va, *N*-acetylglucosaminyltransferase Va; GlcNAc, *N*-acetylglucosamine; *N*-glycan, asparagine-linked glycans; L-PHA, leucoagglutinating phytohemagglutinin; qRT-PCR, quantitative reverse transcriptase-PCR

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2.4. Assay of cell growth and survival

MEFs were harvested, re-suspended in DMEM growth medium and seeded into six-well plates with $2–3 \times 10^5$ cells/well. Assay of cell growth and survival was performed as previously described [12].

2.5. N-Glycan analysis by HPLC

About 100 mg liver tissue were homogenized and *N*-glycans were released from glycoproteins by digestion of the sample with 10 U PNGase-F at 37 °C overnight. Released glycans were fluorescently labeled with 2-aminopyridine (2-AP) as previously reported [13]. Excess 2-AP was removed by gel filtration using a Sephadex G-15 column (1×50 cm) and the 2-AP labeled glycans were fractionated on a 4.6×250 mm TSK Amide-80 column as described previously [14].

2.6. Immuno- and lectin-blotting and lectin pull-down

Cells were harvested, lysed, and 20 μg of protein was used for immuno- and lectin-blotting [10]. For some experiments, cell lysates were boiled in denaturing buffer containing 0.1% SDS and 1 mM DTT for 5 min and treated with PNGase-F at 37 °C overnight before they were subjected to lectin-blotting. For lectin pull-down experiments, 500 μg of protein was incubated with 5 μg of a biotinylated lectin or lectin–agarose at 4 °C overnight, followed by addition of agarose–streptavidin.

2.7. Quantitative RT-PCR analysis

Primer pairs for investigated genes and control genes were designed within a single exon using conditions described in Nairn et al. [11] and listed in Table 1. Primers were validated with respect to primer efficiency and single product detection [11].

The RNeasy kit (Qiagen) was used to isolate total RNA from MEFs. One microgram of total RNA was used for a 20 μl cDNA synthesis reaction using the SuperScript III First-Strand Synthesis System (Invitrogen). Reactions were assembled as described [11]. The control gene, Ribosomal Protein L4 (RPL4, NM_024212) was included on each plate to control for run variation and to normalize individual gene expression.

Triplicate cycle threshold (Ct) values for each gene were averaged and the standard deviation was calculated. Samples that resulted in a standard deviation of >0.5 Ct units were re-run until values with standard deviations within an acceptable range were acquired. The logarithmic average Ct value for each gene and the control gene was converted to a linear value using the conversion: 2^{-Ct}. Converted values were normalized to RPL4 by dividing the individual gene value by the control gene value. Normalized values were scaled so that genes that were below the level of detection were given a value of 1×10^{-6} and this value was used as the lower limit on histograms. Following normalization and scaling, expression data is reported as the relative transcript abundance for each gene assayed. Error bars represent one standard deviation from the mean value.

3. Results

3.1. Characterization of MEFs from embryos

Compared with wild-type, GnT-Va activity was absent in major tissues from GnT-Va null mice (Fig. 1A). Analysis of 2-AP labeled *N*-linked glycans by HPLC indicated a complete disappearance of the peak representing the asialo tetra-antennary *N*-glycan in liver (Fig. 1B), indicating the disappearance of *N*-linked β(1,6) branching caused by the absence of GnT-Va. We have previously observed that GnT-Va null MEFs displayed undetectable GnT-Va activity and lost the typical morphology of a fibroblast when compared to wild-type cells [10]. GnT-Va deficient (KO) and wild-type (WT) MEFs were isolated from 13.5 days embryos. Cell growth in serum-containing medium (Fig. 1C, top) and cell viability in serum-free medium (Fig. 1C, bottom) were measured. GnT-Va null MEFs showed significant decreases in both cell growth and cell survival indicating that the rate of proliferation of MEFs was affected by deletion of GnT-Va.

3.2. Aberrant N-glycan expressions in GnT-Va deficient MEFs

To further study *N*-glycans changes caused by deletion of GnT-Va in MEFs, lectin-blotting and -precipitation experiments were performed. The binding of not only leucoagglutinating phytohemagglutinin (L-PHA), but also DSA was extensively suppressed in GnT-Va null MEFs (Fig. 2A), indicating the suppression of both β(1,6) branched and poly-*N*-acetyllactosamine structures, respectively. This result confirmed our observation that poly-*N*-acetyllactosamine is synthesized preferentially on *N*-glycans expressing the β(1,6) branch [6]. After treatment of cell lysates with PNGase-F, most binding of L-PHA and DSA were abolished, confirming these bound *N*-linked glycans. The expression of either high mannose or biantennary *N*-linked oligosaccharides was not significantly affected by deletion of GnT-Va, as detected by ConA binding (Fig. 2A). Increased E-PHA binding was observed for a few, but not all proteins from GnT-Va knockout cells (indicated as arrows, Fig. 2A), indicating selective increased expression of bisecting *N*-linked GlcNAc. α(1,3) fucosylation did not appear to be affected, as detected by *Lotus* agglutinin (LTA) binding (data not shown). To study the effect of GnT-Va knockout on sialylation, we used WGA–agarose first to pull-down sialic acid-containing glycoproteins, followed by blotting with MAA, SNA and anti-polysialic acid antibody (OL.28), respectively. Surprisingly, α(2,6)-sialylation detected by *Sambucus nigra* agglutinin (SNA) was increased, while α(2,3)-sialylation and α(2,8)-polysialylation, detected by *Maakia ameurinsin* agglutinin (MAA) binding and OL.28, respectively, were dramatically decreased in GnT-Va null MEFs (Fig. 2B). Consistent with these findings, β1 integrin, an abundant surface proteins on MEFs, showed undetectable expression of β(1,6) branching in GnT-Va null MEFs, but a significant increase of α(2,6)-sialylation, compared to wild-type cells (Fig. 2C). These results indicate that deletion of GnT-Va caused significant changes in not only β(1,6) branching, but also other *N*-glycan expression in MEFs.

3.3. Altered gene expression of glycosyltransferses in GnT-Va null MEFs

To begin a study of the mechanisms by which the different *N*-glycan structures were changed in GnT-Va deficient MEFs, transcript levels of relevant groups of glycosyltransferases, listed in Table 2, were detected by qRT-PCR using total RNA isolated from MEFs. As shown in Fig. 3 and Table 2, altered gene expression patterns were observed for several groups of glycosyltransferases after deletion of GnT-Va. The expression of 7*N*-acetylglucosaminyltransferase (GnT) transcripts was determined (Fig. 3A and Table 2). The expression of GnT-I, GnT-III and GnT-IVb was significantly increased in GnT-Va null cells. GnT-Va was detected in wild-type but not in GnT-Va null cells. GnT-Vb was not detected in either wild-type or GnT-Va null cells. Two other groups of glycosyltransferases that regulate the initiation and extension of *N*-acetyllactosamine chains on glycoproteins and glycolipids, β(1,3)-*N*-acetylglucosaminyltransferases (β3GnT) and β(1,4)galactosyltransferases (β4GalT), were affected by either up- or down-regulation after deletion of GnT-Va. Among them, β3GnT-III, V and VII were extremely reduced in GnT-Va null MEFs, while β3GnT-I and II and β4GalT-I, II, VI, were increased (Fig. 3B and Table 2).