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Expression of glycogenes in differentiating human NT2N neurons. Downregulation of fucosyltransferase 9 leads to decreased Lewis^x levels and impaired neurite outgrowth

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ABSTRACT

Background: Several glycan structures are functionally relevant in biological events associated with differentiation and regeneration which occur in the central nervous system. Here we have analysed the glycogene expression and glycosylation patterns during human NT2N neuron differentiation. We have further studied the impact of downregulating fucosyltransferase 9 (FUT9) on neurite outgrowth.
Methods: The expression of glycogenes in human NT2N neurons differentiating from teratocarcinoma NTERA-2/cl.D1 cells has been analysed using the GlycoV4 GeneChip expression microarray. Changes in glycosylation have been monitored by immunoblot, immunofluorescence microscopy, HPLC and MALDI-TOF MS. Peptide mass fingerprinting and immunoprecipitation have been used for protein identification. FUT9 was downregulated using silencing RNA.
Results and conclusions: One hundred twelve mRNA transcripts showed statistically significant up-regulation, including the genes coding for proteins involved in the synthesis of the Lewis^x motif (*FUT9*), polysialic acid (*ST8SIA2* and *ST8SIA4*) and HNK-1 (*B3GAT2*). Accordingly, increased levels of the corresponding carbohydrate epitopes have been observed. The Lewis^x structure was found in a carrier glycoprotein that was identified as the CRA-a isoform of human neural cell adhesion molecule 1. Downregulation of FUT9 caused significant decreases in the levels of Lewis^x, as well as GAP-43, a marker of neurite outgrowth. Concomitantly, a reduction in neurite formation and outgrowth has been observed that was reversed by FUT9 overexpression.
General Significance: These results provided information about the regulation of glycogenes during neuron differentiation and they showed that the Lewis^x motif plays a functional role in neurite outgrowth from human neurons.

1. Introduction

Glycosylation is a common post-translational modification of proteins, and glycans are constituents of glycolipids and proteoglycans. Specific glycosylation structures associated with the neuronal tissue have been shown to be implicated in diverse functions such as neuron and glial cell migration, neurite outgrowth and fasciculation, and synapse formation and plasticity [1].

Several traits have been ascribed as characteristic for nervous system tissue glycosylation. For example, the Lewis^x (Le^x) determinant (Galβ4(Fucα3)GlcNAc) is abundant in brain [2–11], where it is synthesised by fucosyltransferase 9 (FUT9), an enzyme coded by the *FUT9* gene [12]. Le^x is implicated in neurite outgrowth in several neuron types [9,13,14].

Other glycan epitopes have been implicated in specific functions of the adult brain. For example, polysialic acid, which is composed by sialic acid residues in α2,8-linkage and carried by a restricted set of glycoproteins, namely NCAM [15] and SynCAM1 [16], is involved in several cellular events of the central nervous system including neurogenesis, outgrowth and sprouting of axons and synaptic plasticity [1]. The sulphoglucuronyl-containing HNK-1 epitope is expressed on glycoproteins and glycolipids, and it has been associated with cell adhesion and migration, neurite outgrowth and synaptic plasticity [17]. Several other features of protein glycosylation in the brain have been shown

Abbreviations: BSA, bovine serum albumin; dHex, deoxyhexose; EDTA, etylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HNK-1, human natural killer-1; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed-amperometric detection; HRP, horseradish peroxidase; Le^x, Lewis^x; MALDI-TOF MS, matrix-assisted laser desorption ionization with time-of-flight mass spectrometry; Man, mannose; PBS, phosphate-buffered saline; PNGase F, peptide *N*-glycosidase F; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline

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to be functionally relevant, such as the bisecting GlcNAc motif [18], *O*-mannosylation [19], and peripheral α2-linked fucose [20].

We have used NTERA-2/cl.D1 (NT2) cells as a model for some features of the human central nervous system in this study. The NT2 cell line is derived from a human embryonic carcinoma, and is characterized by undifferentiated neuronally committed progenitor cells ($NT2^-$) that, after exposure to retinoic acid, differentiate into postmitotic neurons (NT2N) [21] with characteristics of primary human neurons [22]. Differentiation of NT2N neurons in culture follows a pattern of differential gene expression similar to that of the neuronal precursors during neurogenesis [23] and they have been used to study differential gene expression [24–27].

In the present study, the expression of glycogenes involved in the synthesis of glycan structures functionally relevant in neuronal cells, as well as the corresponding carbohydrate epitopes, namely, Le^x, polysialic acid and HNK-1, were found at increased levels in NT2N neurons. Furthermore, the Le^x carrier detected in NT2N neurons has been identified as NCAM. Finally, downregulation of FUT9 caused decreased Le^x levels concomitantly with impaired neurite initiation and elongation.

2. Material and methods

2.1. Cell culture and transfection

Human NTERA-2/cl.D1 cells were cultured and differentiated into postmitotic NT2N neurons essentially as described previously [9,21]. Briefly, undifferentiated NTERA-2/cl.D1 ($NT2^-$) cells were maintained for 5 weeks in Dulbecco's modified Eagle's medium with high glucose medium supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific, New York, NY), 1% penicillin/streptomycin, and 10 μM retinoic acid, at 37 °C and 5% CO_2. Cells were then plated into new flasks and maintained for 2 weeks in Dulbecco's modified Eagle's medium with high glucose medium (DMEM-HG) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and mitotic inhibitors (1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM uridine). NT2N neurons were then obtained and kept in DMEM-HG conditioned medium with mitotic inhibitors, and were further used for the various assays. Human $NT2^-$ cells were grown at 37 °C in 5% CO_2 in OPTI-MEM medium containing 5% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin.

NT2N neurons were seeded onto 6-well plates coated with poly-D-lysine at 1×10^5 cells.cm^{-2}, and cultured for 3 days in DMEM-HG conditioned medium. Transfection was performed by incubation with serum-free OPTI-MEM containing 0.2 mM scrambled or FUT9 siRNAs (set of three oligos, Stealth RNAi Ref. 1299003; Invitrogen) pre-mixed with 6 μL of Lipofectamine 2000 reagent (Invitrogen) for 4 h at room temperature, with rocking. Transfection medium was exchanged and cells were maintained in conditioned medium at 37 °C in 5% CO_2 for 20 h. A second transfection was then performed using 0.2 mM of pcDNA3.1-LacZ-V5 or pcDNA3.1-FUT9-V5 plasmids [28] under the same conditions described above. After 4 h incubation, neurons were detached using trypsin-EDTA (Invitrogen), counted, and cultured for 24 h in wells coated with poly-D-lysine and Matrigel at densities of 1×10^5 or 1×10^4 cells.cm^{-2} for immunoblotting or microscopic analysis, respectively.

2.2. Microarray analysis

The GlycoV4 oligonucleotide array is a custom Affymetrix GeneChip (Affymetrix, Santa Clara, CA) designed for the consortium for Functional Glycomics (http://www.functionalglycomics.org/) [29]. A complete description and annotation for the GlycoV4 array is available at http://www.functionalglycomics.org/static/consortium/resources.shtml. The GlycoV4 focused array includes probes for approximately 1260 human and 1200 mouse probe-IDs related to glycogenes. Data normalization was performed using RMA Express 1.0 with quantile normalization, median polish and background adjustment. The sample clustering was done using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html), with centered correlation and average linkage. For the analysis of variance, fold changes and standard errors were estimated by fitting a linear model for each gene and empirical Bayes smoothing was applied to the standard errors. The linear modeling approach and the empirical Bayes statistics as implemented in the Limma package in the R software were employed for ANOVA. Results are presented between two or more experimental conditions as a fold change in expression level, the moderated *t*-statistic, the *p*-value, and the adjusted *p*-value. The adjusted *p*-value is the *p*-value adjusted for multiple testing using the Benjamini and Hochberg's method to control the false discovery rate of 0.1 or less. The number of differentially expressed transcripts found for NT2N versus $NT2^-$ was 261 using the cut-offs of fold change > 1.4 and adjusted *p*-value < 0.10.

2.3. Immunoblotting, lectin blotting and immunoprecipitation

Total cell protein from NT2N neurons and $NT2^-$ cells was solubilized with 50 mM Tris–HCl buffer pH 7.4, containing 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Complete Cocktail; Roche, Manheim, Germany). Extracts or immunoprecipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked for 1 h using 0.1% Tween 20 with variable blocking agents and buffers, depending on the primary antibody used: 5% nonfat dry milk (Nestlé, Portugal) in phosphate-buffered saline pH 7.2 (PBS) for mouse IgM anti-HNK-1 (C0678; Sigma-Aldrich, St. Louis, MO), mouse IgM anti-Le^x monoclonal antibody (L5; [30]), and goat IgG anti-FUT9 (C-17; Santa Cruz Biotechnology, CA), or Tris-buffered saline pH 7.5 (TBS) for mouse IgG anti-L1CAM (L1-11A) and rabbit IgG anti-NCAM (sc-10735; Santa Cruz Biotechnology), and 5% BSA (Sigma-Aldrich) in TBS for mouse IgM anti-polysialic acid-NCAM (2-2B, Miltenyi Biotech, Germany) and rabbit IgG anti-GAP-43 (AB5220; Millipore, Billerica, MA). HRP-labeled secondary antibodies anti-goat IgG, anti-mouse IgM (A-8786, Sigma-Aldrich), and anti-mouse and anti-rabbit IgG (GE Healthcare, Waukesha, WI) were diluted in blocking solution. Detection was performed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

The pull-down of NCAM and Le^x was performed by diluting the Le^x-enriched ammonium sulphate fraction in denaturing buffer (50 mM Tris, pH 7.5, 70 mM β-mercaptoethanol), 10 min boiling at 100 °C, and adding to four volumes of IP buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.5, 1% Triton X-100, 4% Complete protease inhibitors cocktail). Samples were incubated overnight with 3 μg anti-NCAM or anti-Le^x L5 antibody. Immunoprecipitates were concentrated with Protein A/G beads and further analysed by immunoblotting.

2.4. Purification of hL1 protein and Le^x carriers

Membrane-bound native human L1CAM (hL1) was purified from whole cell lysates of NT2N neurons and $NT2^-$ undifferentiated cells. The purification was performed by affinity chromatography using the anti-L1 (L1-11A) antibody coupled to Affi-Gel 10 resin (Bio-Rad, Hercules, CA). Cells were extracted in 50 mM Tris–HCl buffer pH 7.4, containing 5 mM EDTA, 1% Triton X-100, and 2% protease inhibitors (Complete Cocktail). Elution was performed with 0.1 M glycine–HCl, 1% Triton X-100, pH 2.5.

Protein carriers for Le^x were isolated from NT2N neurons extracts using ammonium sulphate precipitation. Cell extracts were done with 50 mM Tris–HCl buffer pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 2% protease inhibitors and proteins were precipitated with ammonium sulphate (Sigma-Aldrich). Pellets from 20, 40, 60 and 85% ammonium sulphate fractions were analyzed by 8%