INTRODUCTION:
It has been believed that the fully developed mammalian central nervous system (CNS) lacks significant regenerative capacity. However, recent discovery of neural progenitors including neural stem cells (NSCs) shows a potential of overcoming this limitation in repairing damaged CNS. During the course of neuronal differentiation from NSCs to neuron, the morphology and function of NSCs change drastically. Although a large number of studies have been attempted to clarify the mechanism of neuronal differentiation, it is still debated. Glycobiology has been recently applied to molecular-based works in biomedical field. The majority of glycans attached to proteins are classified into N-glycan and O-glycan. Among the 2 types of glycans, modifications of N-glycans on proteins are considered to contribute to embryogenesis and organogenesis. However, a full portrait of the glycome diversity and the effect of the structural variations of cellular glycoforms on individual cell stages in proliferation and differentiation remain unclear. The hypothesis of this study was that the alterations in N-glycan would occur during neuronal differentiation in mouse embryonic stem (ES) cell-derived NSCs. The objectives of this study were to identify alterations in N-glycans and associated genes of enzymes on the N-glycan biosynthesis during the course of neuronal differentiation in mice.

METHODS:
Cell Culture and Differentiation. NSCs were induced from mouse ES cells (1) according to Neural Stem Sphere method described previously (2,3). To induce neuronal differentiation, NSCs were cultured in the neuron differentiation medium (NDM). The NDM consisted of NSCs medium supplemented with 50 ng/ml brain derived neurotrophic factor, 0.1 μM retinoic acid, 10 μM forskolin and 100 μM ascorbic acid. For differentiation into glial cells, the culture medium of NSCs was changed to DMEM supplemented with 10% FBS (astrocyte differentiation medium, ADM). The present experiments were approved by the institutional animal care committee.

RT-PCR Analysis. Total RNA was extracted from cultured cells with Trizol reagent. Aliquots of total RNA were then subjected to cDNA synthesis using Invitrogen’s Revert AidTM reverse transcriptase and a random primer. A quantitative analysis of the mRNA levels was performed on Opticon Real-Time PCR using sequence specific primer pairs. PCR was carried out on duplicate cDNA samples using SYBR Green Master Mix according to the manufacturer’s protocol. Expression of each mRNA were normalized to that of GAPDH-mRNA. The normalized values are presented relative to the maximum value of each mRNA.

Basic Protocol of Glycoblottting-based Quantitative Cellular N-glycomics. The enzymatically released N-glycans in cell lysate were captured onto the Bioglyco TM beads chemo-selectively. Other contents in lysate, such as peptides and lipids, were able to be removed by washing, allowing of purification and enrichment of the captured sugars. Alkaline sugars were methyl-esterified on the beads, and then the enriched sugars were released from the beads by tagged with a reagent which enhances the sensitivity in MALDI-TOF/MS. The obtained N-glycans were analyzed by MALDI-TOF/MS and TOF/TOF. Each glycan was quantified by normalization of the peak area to that of an internal standard mixed with the samples before applying glycoblottting.

Identification of N-glycogenes Related to the Altered N-glycan. The mRNA expression of N-glycogenes related to the biosynthetic pathway of the altered N-glycan was measured by quantitative RT-PCR analysis.

Statistical Analysis. Quantitative data are presented as means ± SD. Statistical analysis was performed by one-way ANOVA with Bonferroni’s multiple comparison test using GraphPad Prism software.

RESULTS:
Expression of the Specific Marker Genes during Differentiation of ES Cells into NSCs, Neurons, and Astrocytes. Abundant mRNA level of Oct4, a typical marker gene for the ES cells, was observed only in the ES cells. The expression level of nestin-mRNA was at its highest in the ES-derived NSCs and decreased significantly upon differentiation into either neuron or astrocytes. Concomitantly, the expressions of βIII- and GFAP-mRNAs were induced accordingly during the neuronal and glial differentiations from the NSCs, respectively (Fig.1.2.).

Alterations in N-glycans and the Associated Genes of Enzymes on the N-glycan Biosynthesis During Neuronal Differentiation in Mouse Embryonic Stem Cell-Derived Neural Stem Cells

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*Total N-glycan level per 200 μg proteins

Alterations in N-glycan Structures. Bar-coding analysis by 3 glycosyltransferase5. The obtained results suggest the possibility of bisecting type glycoproteins which play an important role in regulating the biosynthesis of Asn-linked oligosaccharides on glycoproteins. The obtained results revealed that expression of GnT-III significantly increased during the course of neuronal differentiation from mouse ES cells. The glycosylation patterns of cell surface glycoproteins play important roles in multiple molecular interactions in cellular adhesion that contribute to embryogenesis and organogenesis. The regulatory role of GnT-III is based on effects of a reaction product, namely the bisecting GlcNAc which is known as a key glycoltransferase which plays an important role in regulating the biosynthesis of Asn-linked oligosaccharides on glycoproteins. This unique structure is not tolerated by other enzymes involved in the formation of the core structures, and, as a result, prevents further reactions which are catalyzed by these enzymes. This inhibitory regulation is the rules of the broad specificity of GnT-III, as well as the properties of the bisecting GlcNAc. GnT-III appears to be normally expressed at high levels in mammalian CNS, and an overexpression of GnT-III impairs the functions for epidermal growth factor and nerve growth factors. The obtained results suggest the possibility of bisecting type N-glycans as new class biomarkers for identifying and monitoring processes of neuronal differentiation, though the effect of feeder cells and other various factors of individual culture conditions.

REFERENCES:

Fig.1. Establishment of ES-derived NSCs and their differentiation into neurons and astrocytes. A:ES cells, B:NSCs, C:Neuron, D:Astrocyte

Fig.2. Quantitative RT-PCR analysis of marker gene expression (n=5)

Fig.3. Bar-coding analysis during differentiation of NSCs into neuron. (n=3) HM: high mannose type, BS: bisecting type, Others (from 34 % to 46 %) (Fig.3.).

NSCs Neuron day 1 Neuron day 3 Neuron day 7
51.2 pmol* 65.8 pmol* 80.7 pmol* 94.9 pmol*
61 % 56 % 46 % Others 46 %


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