Regulatory roles of ganglioside GQ1b in neuronal cell differentiation of mouse embryonic stem cells

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Gangliosides play an important role in neuronal differentiation processes. The regulation of ganglioside levels is related to the induction of neuronal cell differentiation. In this study, the ST8Sia5 gene was transfected into mESCs and then differentiated into neuronal cells. Interestingly, ST8Sia5 gene transfected mESCs expressed GQ1b by HPTLC and immunofluorescence analysis. To investigate the effects of GQ1b over-expression in neurogenesis, neuronal cells were differentiated from GQ1b expressing mESCs in the presence of retinoic acid. In GQ1b expressing mESCs, increased EBs formation was observed. After 4 days, EBs were co-localized with GQ1b and nestin, and GFAP. Moreover, GQ1b co-localized with MAP-2 expressing cells in GQ1b expressing mESCs in 7-day-old EBs. Furthermore, GQ1b expressing mESCs increased the ERK1/2 MAP kinase pathway. These results suggest that the ST8Sia5 gene increases ganglioside GQ1b and improves neuronal differentiation via the ERK1/2 MAP kinase pathway. [BMB reports 2011; 44(12): 799-804]

INTRODUCTION

Gangliosides are a family of glycosphingolipids and are ubiquitous components of eukaryotic cell membranes (1-3). They contain an amphipathic ceramide moiety, which acts as a membrane anchor, and a hydrophilic oligosaccharide chain, which carries one or more sialic acid residues. Ganglioside synthesis is regulated by specific ceramides bound to the Golgi apparatus where diverse forms of gangliosides, such as GM3, GD3 and GT3 with the a-, b-, and c-series, are assembled. They have regulatory roles in biological processes such as differentiation, cell to cell interaction, proliferation, migration, adhesion, and apoptosis (4, 5).

The expression patterns of gangliosides change during neuronal differentiation and development (2, 3, 6). Several studies have reported that the expression of gangliosides is regulated during nervous system development and is closely associated with neurogenesis, neural repair, and synaptic function (7-11). Moreover, the expression level of gangliosides increases during retinoic acid (RA)-induced neuronal differentiation in embryonic carcinoma cells (12). Tsuji et al. reported that ganglioside GQ1b promotes neurite outgrowth during early neuronal differentiation (13). Furthermore, the co-localization of ganglioside-GQ1b with neuronal markers was demonstrated in an in vitro model of neuronal differentiation of mESCs and neuronal differentiation was found to be facilitated by the increased levels of gangliosides due to the presence of daunorubicin (1). However, the mechanism whereby ganglioside GQ1b controls the neuronal differentiation process in mESCs is still not clear.

During early neural development in vivo, related signaling pathways and signal molecules may play important roles in the differentiation of mESCs. A previous study showed that mitogen-activated protein (MAP) kinase signaling plays an important role in the differentiation of stem cells. For example, the extracellular signal-regulated kinase 1/2 (ERK1/2) MAP kinase pathway is related to neuronal differentiation of ESCs or leukemia inhibitory factor (LIF) signaling independently of ERK1/2 MAP kinase (14). In addition, this pathway has various functions in the development of the nervous system (15). However, the mechanism by which these pathways regulate downstream transcription factors during development is not yet clear. Other studies have reported that gangliosides regu-
late various signal molecules, including receptor tyrosine kinases, of various growth factor receptors, and the ERK1/2 MAP kinase pathway and function as transducers of extracellular signaling during differentiation (16-19). We recently evaluated the roles of glycosphingolipids during the suppression of the Ugcg gene expression in mESCs and human mesenchymal stem cells (hMSCs) and found that they influence neuronal differentiation of mESCs and hMSCs (20, 21).

In this study, we investigated the roles of ganglioside GQ1b via over-expression of the ST8 alpha-N-acetyl-neuraminidase alpha-2,8-sialyltransferase 5 (ST8Sia5) gene in mESCs and neuronal cell differentiation.

RESULTS

Analysis of GQ1b expression in in ST8Sia5 gene transfected mESCs

The mESCs exhibited characteristics including expression of AP, SSEA-1, Oct4, and Sox-2 (Supplementary Fig. 1).

To induce an increase in GQ1b expression, mESCs were transfected with the ST8Sia5 gene using pEGFP-C2 vectors (Supplementary Fig. 2). The transfection efficiency was examined 48 hrs after transfection by GFP fluorescence. Additionally, transfected cells were confirmed by selection using G418 (neomycin resistance) for 2 weeks (Fig. 1A). Quantification of the ST8Sia5 gene mRNA level by PCR showed that the ST8Sia5 gene expression level increased in the ST8Sia5 gene transfected mESCs when compared to the mESCs (Fig. 1B).

Ganglioside expression patterns were analyzed by HPTLC (Fig. 1C). Control mESCs, mock vector transfected mESCs, and ST8Sia5 gene transfected mESCs expressed GM3 and GD1a, but GM2 and GQ1b only expressed in mESCs and ST8Sia5 gene transfected mESCs, respectively. Therefore, these results indicated that ST8Sia5 gene transfected mESCs induced expression of GQ1b.

Beating EBs derived from GQ1b expressing mESCs

To determine the differentiation capability of GQ1b expressing mESCs, control mESCs and mock vector transfected mESCs and GQ1b expressing mESCs were cultured in suspension culture conditions in bacterial-grade dishes. After 4 days of culture, the mESCs aggregated and formed smooth circular clumps, which indicated that the cells were in the EB (4-) stage (Fig. 2A). EBs derived from GQ1b expressing mESCs maintained GFP fluorescence due to the presence of the GFP expression vector that (Supplementary Fig. 2). The transfection efficiency was examined 48 hrs after transfection by GFP fluorescence. Additionally, transfected cells were confirmed by selection using G418 (neomycin resistance) for 2 weeks (Fig. 1A). Quantification of the ST8Sia5 gene mRNA level by PCR showed that the ST8Sia5 gene expression level increased in the ST8Sia5 gene transfected mESCs when compared to the mESCs (Fig. 1B).

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Fig. 3. Influence of GQ1b expression during the neuronal differentiation of mESCs. (A) Morphology of neural cells derived from mESCs induced by RA. (B, C) and (D) are immunofluorescence stained to confirm the expression of nestin, GFAP, and MAP-2, respectively. The scale bar represents 10 μm.

Fig. 4. Effect of GQ1b expression on MAP kinase activation in mESCs. (A) MAP kinase activation of control mESCs was examined by western blot analysis with an anti-phospho MAP kinase antibody. 1d, 1 day in culture; 3d, 3 days in culture. ***P < 0.001 (B) Control mESCs, mock vector transfected mESC, and GQ1b expressing by ST8Sia5 gene transfected mESCs were grown for 6 hrs in serum-free medium and then stimulated with serum for 10 min. The cells were lysed 10 min after stimulation and the lysates were examined by western blot analysis. ***P < 0.001

The effects of GQ1b on neuronal differentiation of mESCs
To examine the role of GQ1b on neuronal differentiation, we induced neuronal differentiation of GQ1b expressing mESCs. The EBs derived from GQ1b expressing mESCs adhered to poly-D-lysine/laminin-coated tissue culture dishes during early neuronal differentiation (Fig. 3A). After 4 days, the effects of GQ1b on the differentiation of mESCs into neuronal cells were determined by immunofluorescence staining for the neuronal precursor cell specific marker, nestin, and the glial marker, GFAP (Fig. 3B and 3C). These results showed that GQ1b expressing mESCs differentiated into neuronal precursor cell and glial cells. Therefore, GQ1b might have a positive effect in the differentiation to nestin and GFAP. Furthermore, GQ1b co-localized with microtubule-associated protein (MAP-2) expressing cells in 7-day-old EBs (Fig. 3D). These results indicated that GQ1b may play an important role in neuronal differentiation of mESCs.

Increase of MAPK activation by ST8Sia5 gene expression
Differentiation of mESCs depends on the MAP kinase pathway (22, 23). Therefore, we examined whether ganglioside GQ1b regulates neuronal differentiation of mESCs via activation of ERK 1/2 MAP kinase. The MAP kinase pathway in mESCs was activated during maintenance under normal culture conditions (Fig. 4A). The total amount of ERK 1/2 MAP kinase (Fig. 4B) did not differ between the neuronal differentiated cells and the control mESCs and mock vector transfected mESCs. In contrast, the increase of GQ1b expressing mESCs by ST8Sia5 gene transfection significantly increased phosphorylation of ERK 1/2 MAP kinase.

DISCUSSION
The results of this study show that the GQ1b is related to differentiation of mESCs. Numerous studies have indicated that gangliosides play an important role in the differentiation and development of neuronal cells. In a previous study, we demonstrated that daunorubicin-induced GQ1b expression in undifferentiated mESCs occurred during neuronal differentiation and enhanced neurite formation (13). Additionally, we demonstrated that gangliosides play a role in neuronal differentiation in mESCs by shRNA (20). However, it is still not clear if ganglioside GQ1b controls the neuronal differentiation process in mESCs. We used an expression vector to induce ST8Sia5 gene over-expression and then GQ1b expressed in mESCs. RT-PCR revealed that transfection with the over-expression vector led to a significant increase in ST8Sia5 gene expression levels. In addition, the expression of ganglioside GQ1b in undifferentiated mESCs also increased in response to transfection with the ST8Sia5 over-expression vector. Taken together, these results suggest that over-expression of the ST8Sia5 gene induced the expression of ganglioside GQ1b in mESCs.
press GD3, GD1a, GT1b and GQ1b (1). In addition, expression of neuronal cell markers and gangliosides is related to the formation of neuritis in cultured neuroblastoma cells (24). The relationship between expression of gangliosides and neuronal differentiation of P19 cells agrees with changes in the expression of gangliosides that occur during mouse brain development (25). Interestingly, GQ1b co-localizes with synaptophysin and neural cell adhesion molecules (NCAMs) on synaptic boutons or dendritic spines in RA-induced neurons (11) and neuronal differentiation can be induced by exogenous ganglioside GQ1b (13, 26). Significant expression of GQ1b also occurs during neuronal differentiation of mESCs transfected with ST8Sia5 gene. These results indicate that the expression of ganglioside GQ1b may play a role in the neuronal differentiation of mESCs.

The roles of gangliosides include alteration of adhesion, proliferation, and differentiation in cells (22, 23). Over-expression of ganglioside GQ1b occurs during neuronal differentiation in mESCs (27). Our results showed that the effects of over-expression of the GQ1b in cellular differentiation of mESCs occurred in the MAP kinase pathway. Thus, MAP kinase might act as a primary signal in the regulation of differentiation of mESCs. MAP kinase activation leads to the differentiation of PC12 cells and activation of MEK/ERK1/2 MAP kinase occurs during early neuronal differentiation of mouse P19 cells (28, 29). These studies support our results that GQ1b was over-expressed by the ST8Sia5 gene during neuronal differentiation of mESCs via the MAP kinase pathway. Activation of the MAP kinase upstream activator, MEK, occurred coincidentally with ERK1/2 MAP kinase activation. This indicates that ERK1/2 MAP kinase phosphorylation plays an important role in early neuronal differentiation of mESCs. This is supported by other studies which showed that ERK1/2 MAP kinase signaling is involved in the differentiation and development of mammalian cells. For example, ERK1/2 MAP kinase activation plays a crucial role in neuronal differentiation of ES P19 cells (29). Also, insulin-like growth factors (IGFs) modulated the differentiation and growth of neural stem cells derived from ES (30). Thus, MAP kinase might act as a primary signal in the regulation of differentiation of mESCs. To determine the neuronal differentiation capability of GQ1b expression in mESCs, we evaluated neuronal differentiation of mESCs induced by RA treatment of the EBs (27). ERK1/2 MAP kinase phosphorylation plays an important role in the early neuronal differentiation. We found that the spontaneous beating activity of EBs derived from GQ1b expressing mESCs was greater than that of the control mESCs. These results indicate that the rates of differentiation increased in response to the over-expression of ganglioside GQ1b via the ERK1/2 MAP kinase pathway. Therefore, ganglioside GQ1b is dependent on cell type and the signal molecules involved in the MAPK pathway in the differentiation of mESCs.

The results of this study suggest that ganglioside GQ1b may be involved in the neuronal differentiation of mESCs via the ERK1/2 MAP kinase pathway. Taken together, these results provide evidence of the important role that ganglioside GQ1b plays in the neuronal differentiation of mESCs.

**MATERIALS AND METHODS**

**mESCs cell culture and neuronal differentiation**

mES cell line J1 was proliferated and maintained on mitomyacin treated embryo fibroblast feeder cell layers in mES cell medium containing DMEM (Gibco) supplemented with non-essential amino acids (NEAA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and 15% fetal bovine serum (FBS) (Hyclone), at 37°C in 5% CO2. In vitro neuronal cell differentiation was induced from mES cells using the 4/4+ protocol. The mES cells were grown for 4 days to form unattached EB(4+) in differentiation medium containing DMEM supplemented with NEAA, 2 μM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FBS. The EBs(4+) were then cultured to generate EBs(4+ for an additional 4 days culturing in the presence of trans-retinoic acid (2 μM). The EBs(4+) were rinsed and transferred to poly-D-lysine (Sigma)/laminin (Sigma)-coated tissue culture dishes. The transferred EBs (4 +) were incubated overnight in knockout DMEM with 10% heat-inactivated FBS to attach the EBs(4+) to the dish. The EBs(4+) were rinsed and grown to induce neuronal differentiation in DMEM/F12 medium for seven more days.

**Plasmid preparation and gene transfection**

For transfection, both cell types were harvested using Tryple solution (Gibco) and then pelleted via centrifugation. A total of 1 × 10⁵ cells were seeded into each well of a 0.1% gelatin-coated well in 6-well plates 12 hrs prior to transfection. The pEGRFP-C2 expression vector (Clontech) was grown in DH5α competent cells, after which the plasmid DNA was isolated. For transfection, Lipofectamin (Invitrogen) was used and cells were grown in OPTIMEM (Invitrogen) according to the manufacturer’s instructions. To select antibiotic-resistant eGFP + J1 (1-eGFP) mESC clones, transfected cells were treated with G418 (200 μg/ml) for 2 weeks. Cloned cells were then individually plated on gelatin-coated culture dishes in ES culture medium.

**High-performance thin-layer chromatography (HPTLC)**

HPTLC analysis of the gangliosides was performed using a 10 × 10 cm HPTLC 5651 plate (Merck), which is described in a previous study (31). The purified gangliosides (1 mg protein/ lane) were loaded onto the HPTLC 5651 plates, which were then developed in chloroform: MeOH: 0.25% CaCl₂ · 2H₂O (50 : 40 : 10, v/v/v). Next, the gangliosides fraction on the HPTLC plate was visualized using 0.2% resorcinol stain and the density of the ganglioside bands was quantified by HPTLC densitometry analysis (Image J). Rat brain and bovine brain gangliosides were used as standards to categorize the individual ganglioside species.
Immunofluorescence analysis
Cells were permeabilized with 0.25% Triton X-100 for 10 min at 37°C and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were blocked for 20 min with PBS containing 5% BSA. Next, the cells were incubated for 16 hrs at 4°C with mixtures containing primary antibodies specific to neuronal cell markers such as, MAP-2 (Sigma), nestin (Chemicon), and GQ1b (Seikagaku, Tokyo, Japan) in PBS containing 1% BSA. The cells were washed with PBS containing 1% BSA, after which secondary antibodies conjugated to fluorescent markers (goat anti-mouse IgG-TRITC for neuronal cell specific markers and goat anti-mouse IgG- alexa 350 for gangliosides) (Sigma) were applied at dilutions of 1:500. The Hoechst33342 reagent (Sigma) was used to detect the nuclei in cells. After washing with PBS, the cells were analyzed using a confocal scanning laser fluorescence microscope (Model FV300, Olympus).

Western blot analysis
Cells were homogenized with lysis buffer and then centrifuged at 15,000×g for 30 min. The protein concentration was measured according to the Bradford method. Next, equal amounts of proteins (40 μg) were separated on a SDS/7%-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The blots were blocked for 2 hrs with 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS). The membrane was then incubated for 16 hrs at 4°C with rabbit IgG-specific antibodies to ERK1/2 and phospho-ERK1/2 (Promega). The blot was then incubated with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma). Finally, the samples were incubated in BCIT/NBT (Sigma), to detect the alkaline phosphatase activity, and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were blocked for 15 min at room temperature. The fixed cells were incubated for 16 hrs at 4°C with mixtures containing primary antibodies specific to neuronal cell markers and goat anti-mouse IgG- conjugated to fluorescent markers (goat anti-mouse IgG-TRITC (Promega) with a Takara PCR Thermal Cycler DICE Gradient (Takara) under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 10 min. The PCRs were conducted using the primer pairs listed in supplementary Table 1.

RNA extraction and reverse transcription-PCR mRNA analysis
Total RNA from mESCs was isolated using the TRizol reagent (Invitrogen). RT-PCR was conducted to examine the expression mRNA of Oct4, Sox2, and ST8Sia5 using a one-step RT-PCR kit (Promega) with a Takara PCR Thermal Cycler DICE Gradient (Takara) under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 10 min. The PCRs were conducted using the primer pairs listed in supplementary Table 1.

Statistical analysis
Comparisons of multiple groups were analyzed by one-way analysis of variance (ANOVA) and two-way ANOVA followed by Bonferroni post-hoc pair-wise comparisons of each group. All statistical analyses for this study were performed using GraphPad Prism (ver. 5.00, GraphPad software).

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