Ganglioside GT1b Mediates Neuronal Differentiation of Mouse Embryonic Stem Cells

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ABSTRACT : It has been reported that ganglioside GT1b is expressed during neuronal cell differentiation from undifferentiated mouse embryonic stem cells (mESCs), which suggests that ganglioside GT1b has a direct effect on neuronal cell differentiation. Therefore, this study was conducted to evaluate the effect of exogenous addition of ganglioside GT1b to an *in vitro* model of neuronal cell differentiation from undifferentiated mESCs. The results revealed that a significant increase in the expression of ganglioside GT1b occurred during neuronal differentiation of undifferentiated mESCs. Next, we evaluated the effect of retinoic acid (RA) on GT1b-treated undifferentiated mESCs, which was found to lead to increased neuronal differentiation. Taken together, the results of this study suggest that ganglioside GT1b plays a crucial role in neuronal differentiation of mESCs.

Key words : Mouse embryonic stem cells (mESCs), Neuronal differentiation, Ganglioside GT1b, Retinoic acid (RA).

INTRODUCTION

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of the blastocyst of 3.5 day old embryonic mice (Evans and Kaufman, 1981). Pluripotency is a unique quality of ES cells that enables them to develop into other cell types or produce mammalian cells (Martin, 1981). mESC lines, which can be used in the creation of mouse models, are the tool of choice for determining the function of a sequence of interest in the context of a whole organism, its role in disease and possible roles in the generation and testing of therapeutics (Yu et al., 2006). Gangliosides are a family of amphipathic constituent ceramid moietys and hydrophilic oligosaccharide chains that contain one or several sialic acid residues. Gangliosides are biosynthesized stepwise from ceramides by glycosyltransferase in the endoplasmic reticulum and Golgi apparatus and degraded by glycosidase in late endosome and lysosome (van Meer, 1993). In addition, gangliosides are involved in the regulation of a variety of biological processes, including neuritogenesis, neural repair and cell-cell recognition, proliferation migration and adhesion (Roisen et al., 1981). Gangliosides exist in high concentrations in the central nervous system (CNS) and their expression is regulated during nervous system development and closely associated with neurogenesis (Yu, 1994). Additionally, gangliosides are thought to play important roles in neural differentiation by influencing neurite outgrowth and cell-to-cell interactions. The levels and types of gangliosides change during neural differentiation and development (Ledeen et al., 1998). In embryonic carcinoma cells, the expression of gangliosides is enhanced during RA induced neural differentiation. In addition, RA has been shown to be a useful tool for studying the early steps of neural differentiation *in vitro* (McBurney et al., 1982). Furthermore, the absence of ganglioside GD3, GD1b and GT1b caused by the disruption of GD3 synthase was found to have no effect on RA induced neural differentiation in mESCs (H. Kawai et al., 1998). A previous report indicated that GT1b was a potential mechanism for the inhibitory effect of substances

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on neurite outgrowth (Vinson et al., 2001). In this study, we attempted to elucidate the relationship between exogenous ganglioside GT1b expression and early neuronal differentiation (Kwak et al., 2006). To accomplish this, we investigated the exogenous addition of ganglioside GT1b to determine if it enhanced RA induced neural differentiation of mESCs. Expression of neuronal cell markers and morphological characteristics of differentiated neuronal cells were examined in GT1b-treated mouse embryonic stem cells. Taken together, the results of this study provide evidence of the important role that ganglioside GT1b plays on RA induced early neuronal cell differentiation of mESCs.

MATERIALS AND METHODS

1. mESCs Culture

The mESCs J1 line was proliferated and maintained on mitomycin treated embryo fibroblast feeder cell layers in mESCs medium containing DMEM (Gibco, Rockville, MD) supplemented with non-essential amino acids, 2mM L-glutamine, 0.1 mM B-mercaptoethanol, penicillin (100 U/㎖), streptomycin (100 ㎍/㎖) and 15% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, USA) at 37℃ in 5% CO_2. The cells were induced using the 4/4+ protocol (Marone et al., 1995; Jason et al., 2004). Briefly, ES cells were grown as unattached embryoid bodies (EB) for 4 days in non-adherent Petri dishes and then cultured in ES differentiation medium containing DMEM supplemented with non-essential amino acids, 2 mM L-glutamine, 100 units/㎖ penicillin, 100 g/㎖ streptomycin and 10% heat-inactivated ES-qualified FBS to promote the formation of embryoid bodies. The EB were then cultured for an additional 4 days in the presence of retinoic acid (all-trans retinoic acid, 2uM, Sigma Cat.#R2625). This study refers to this stage as EB4. In addition, serum-deprived EB were trypsinized or treated with a non-enzymatic cell dissociation solution, plated on poly-D-lysine (Sigma)/laminin (Sigma)-coated tissue culture dishes, and grown for 3 days in DMEM/F12 (Gibco, Rockville, MD).

2. RNA Extraction and Reverse Transcripton-PCR mRNA Analysis

RT-PCR was conducted to confirm expression of the Oct-4, Sox-2 and GAPDH genes using a one-step RT-PCR kit (Promega, USA) with a Takara PCR Thermal Cycler DICE Gradient (Takara Biomedicals, Shiga, Japan). The total RNA from the mESCs was isolated using a Trizol Kit (MRC Inc., Cincinnati, OH), after which it was subjected to PCR under the following conditions: 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 sec, 58℃ for 30 sec and 72℃ for 30 sec, with a final extension at 72℃ for 10 min. PCR was conducted using the following primer sets: forward (5'-ggcgttctctttggaaaggtgttc-3') and reverse (5'-ctc gaaccacatccttctct-3') for Oct3/4, and forward (5'-agacag tgaggcagatgagt-3') and reverse (5'-atgagaggtcagagtcatgg-3') for Sox-2. The RT-PCR products were separated by agarose gel electrophoresis using a 1% agarose gel stained with ethidium bromide and then visualized with UV light.

3. Cell Viability Assay

The mESCs (4×10^4 cell/well) were cultured in 96-well microtiter plates under CO_2 in a humidified atmosphere for 48 h at 37℃. After 24 hours of culture to allow the cells to adhere to the plate, GT1b (Matreyallc) was added to each well at various concentrations (0, 2, 5, 10, 20 ㎛/㎖). Each well was then incubated in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma) solution for 4 h. The absorbance of each well was determined at 490 nm using a spectrophotometer. The cell viability was then calculated as follows: (absorbance of GT1b-treated cells/absorbance of non-treated cells) × 100. All experiments were repeated six times and the cell viability data were expressed as the mean ± standard deviation. The DNA content in each sample was analyzed by staining with propidium iodide (PI; Sigma) (Perry et al., 2000). After the mESCs were fixed with 50% ethanol for 30 min at 4℃, the cultured cells were permeabilized with 0.25% Triton X-100 for 10 min at 37℃ and RNase A (Sigma) at 0.1 ㎎/㎖ for 30 min at 37℃. Next, PI was

added to the sample at 0.1 ㎎/㎖ and then incubated for 15 min at room temperature. The samples were subjected to immunofluoresce microscopes for cell death analysis.

4. Immunofluorescence Staining

The cells were permeabilized with 0.25% Triton X-100 for 10 min at 37℃ and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Next, the fixed cells were blocked for 20 min with PBS containing 4% bovine serum albumin (BSA). Double-label immunostaining was carried out as described. The cells were then incubated with mixtures of primary antibodies specific to neuronal cell markers [nestin (Chemicon International Inc., Temecula, CA) and MAP-2 (Sigma)] and ganglisoside GT1b (Seikagaku Corp., Tokyo, Japan) in PBS (1:1000) containing 5% BSA at 4℃ overnight. Next, the cells were washed in PBS containing 1% BSA, after which secondary antibodies conjugated to fluorescent tags (goat anti-rabbit IgG-Texas Red for neuronal cell markers, and goat anti-mouse IgM conjugated to FITC for gangliosides) (Molecular Probe) were applied at dilutions of 1:500 and 1:800, respectively. Hoechst 33342 reagent (Sigma) was used to detect nuclei in cells. After being washed with PBS, the cells were observed by confocal scanning laser fluorescence microscopy (Model FV300, Olympus Co., Tokyo, Japan). The mESCs were washed with PBS between every step.

5. Statistical Analysis

Each experiment was replicated at least six times. The cell viability among groups was compared by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A $p<0.05$ was considered to indicate statistical significance.

RESULTS

1. Characterization of mESCs using Immunohistochemical and Specific Molecular Markers

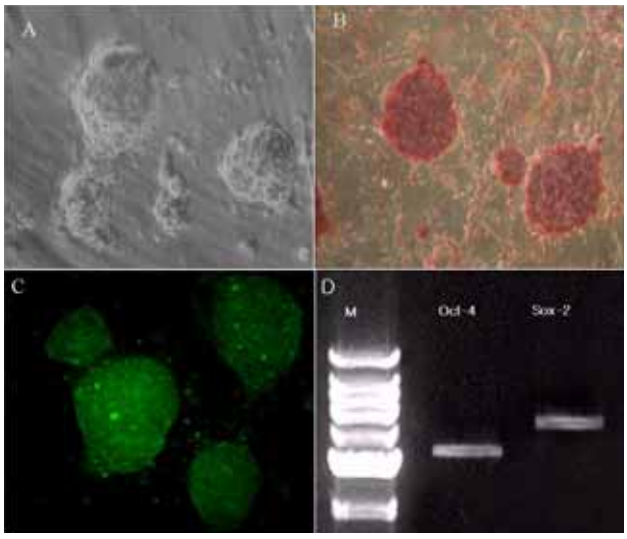

Fig. 1. Specific marker expression of mESCs. (A) Undifferentiated mESCs normal morphology, (B) Alkaline phosphatase staining of undifferentated mESCs, (C) SSEA-1 stained positively with antibody against SSEA-1, (D) Specific marker gene expression analysis by RT-PCR (577bp and 488bp for Sox-2 and Oct-4, respectively).

Undifferentiated mES cells formed the round-shaped colonies with well-defined edges, which are common morphological characteristics (Fig. 1A). To confirm the undifferentiated state of the mESCs, we first examined the alkaline phosphatase (AP) activity as an undifferentiated marker in the mESCs (Fig. 1B). Immunofluorescence staining also revealed that mESCs expressed stage specific embryonic antigen-1 (SSEA-1), which is a cell surface marker protein that indicates that the cell is in an undifferentiated state (Fig. 1C). Furthermore, RT-PCR analysis using the pluripotent marker, SRY-related HMG-box gene 2 (SOX-2) and Octamer-4 (Oct-4) specific primers showed amplified gene fragments of the expected sizes (577 bp and 488 bp for Sox-2 and Oct-4, respectively) (Fig. 1D).

2. Changes in GT1b-induced Cell Viability in mESCs

The effects of GT1b on cell growth and death in mESCs were evaluated. The ratio (%) of apoptotic cell death assessed by PI staining was found to increase in response to GT1b, and this effect was found to be dose-dependent.

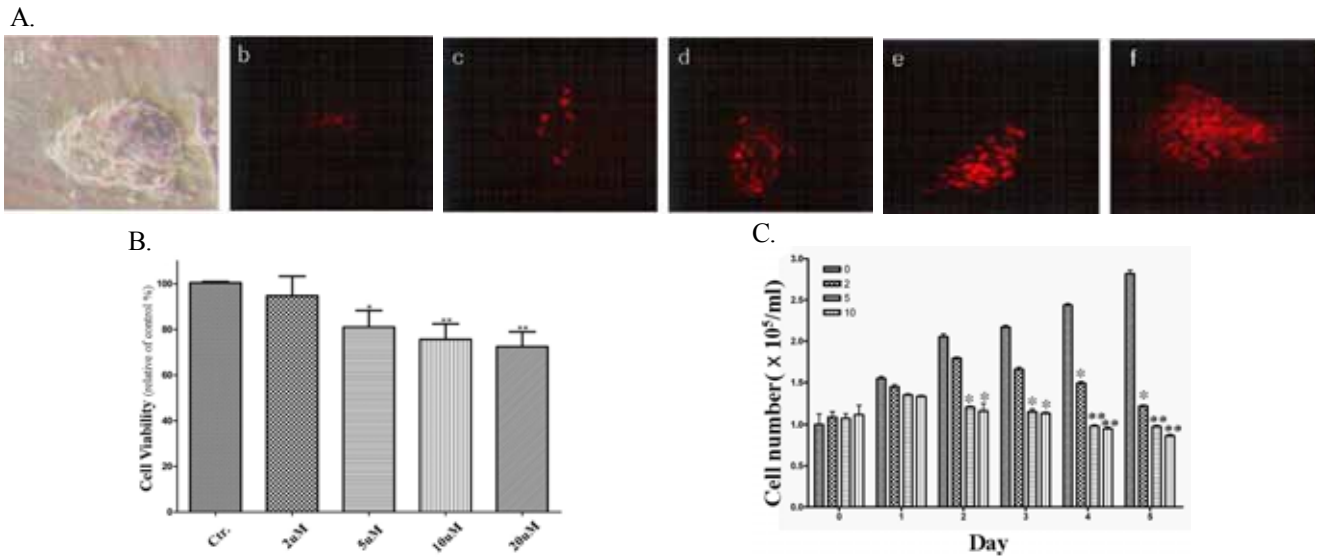

Fig. 2. Changes in GT1b-induced cell viability in mESCs. (A) Effects of GT1b on cell death in mES cells. Normal morphology panel (a), non-treated mES cells with an initial density of 1×10^5 cell/㎖ (b). mES cells treated with (c-f) GT1b (2 ㎛/㎖), GT1b (5 ㎛/㎖), GT1b (10 ㎛/㎖), GT1b (20 ㎛/㎖) for 24 hours. (B) Dose-dependent effects of GT1b on cell growth and cell death in mESCs. The cell were seeded at an initial density of 1×10^5 cell/㎖ and then treated with or without GT1b (2 ㎛/㎖), GT1b (5 ㎛/㎖), GT1b (10 ㎛/㎖) or GT1b (20 ㎛/㎖) for the indicated times. Data are presented as the mean ± SD from six separate experiments. Groups were compared by one-way ANOVA followed by Tukey's Multiple Comparison Test (*p<0.05, **p<0.01). (C) Time-dependent effects of GT1b on cell growth and cell death in mESCs. The cells were seeded at an initial density of 1×10^5 cell/㎖ and then treated with or without each GT1b (2 ㎛/㎖), GT1b (5 ㎛/㎖), GT1b (10 ㎛/㎖) or GT1b (20 ㎛/㎖) for the indicated days. Data are presented as the mean ± SEM from three separate experiments. Groups were compared by one-way ANOVA followed by Tukey's Multiple Comparison Test (*p<0.05, **p<0.01).

It was believed that the GT1b-induced morphological changes in the cells that were apparent upon PI nucleic acid staining indicated an apparent cell death pattern (Fig. 2A). Therefore, mESCs (1×10^5 cell/㎖) were incubated with different concentrations of GT1b for 24 hr to further evaluate the pattern of death. The number of viable mESCs decreased in response to treatment with 10 ㎛/㎖ and 20 ㎛/㎖ GT1b, but treatment with GT1b at concentrations of 2 ㎛/㎖ and 5 ㎛/㎖ GT1b for 24 hours had very little effect (Fig. 2B). However, in the presence of 10 ㎛/㎖, 20 ㎛/㎖ GT1b, this study observed a significantly decrease in viability as compared with the control value. A decrease in cell number was detected within 1 to 5 days when compared with the untreated control (Fig. 2C). Additionally, a dose-dependent inhibition of cell growth was detected in mESCs when compared with controls at the analogous point. Therefore, no significant difference was observed between the control and GT1b (2 ㎛). However, after all the viable mESCs during the 3 days, the suitable concentration of 2 ㎛/㎖ GT1b was equivalent to that of the untreated initial controls. Taken together, these results indicate that differentiation of mESCs occurred in response to treatment with 2 ㎛/㎖ GT1b.

3. Effect of GT1b on Maturation of Differentiated Neuronal Cells from EB (4+):3.

The effects of ganglioside GT1b on the differentiation of neuronal-like EB cells (4+) were evaluated after 3 days of treatment. Fig. 3A shows the expression of the neuron dendrite marker, MAP-2, in response to treatment with GT1b. Fig. 3-B shows the expression of the early neural cell marker, nestin, following GT1b treatment. The results

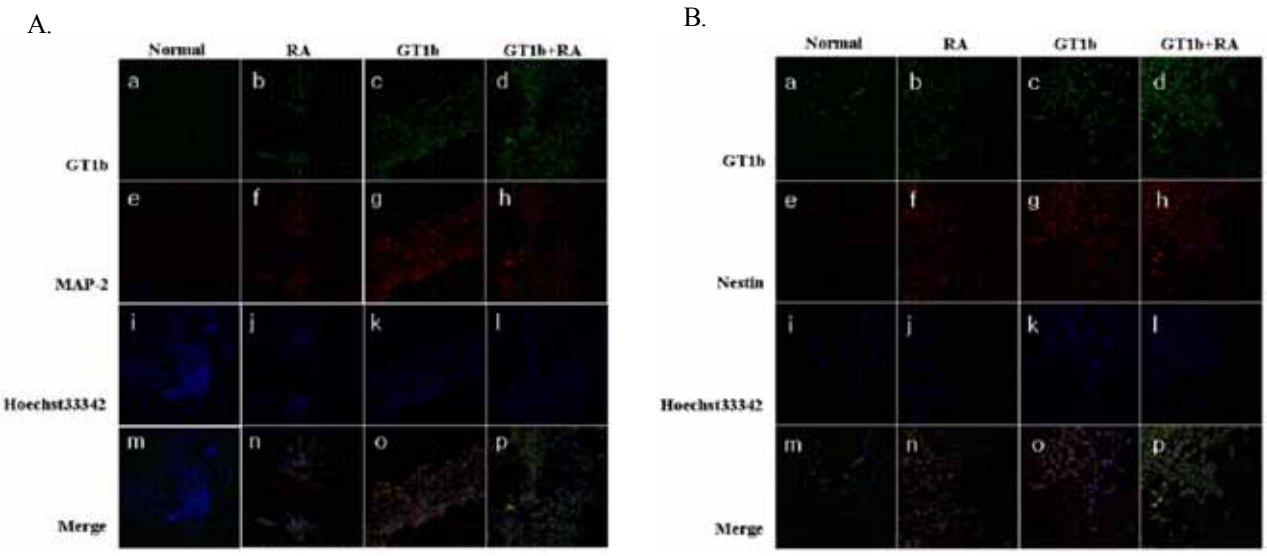

Fig. 3. Immunofluorescence staining of GT1b, MAP-2 and Nestin expressed in EB (4+): 3. EB cells were treated with GT1b for 7 days (4+). (A) GT1b and MAP-2. (B) GT1b and Nestin, ganglioside GT1b expression were detected by FITC (green) (a, b, c and d), while MAP-2 and Nestin expression were detected by TRITC (red) (e, f, g and h) and Hoechst 33342 (blue) (i, j, k and l). Merge is the merged image of the four individual images (m, n, o and p).

revealed that ganglioside GT1b was slightly expressed in the differentiated neuronal-like EB cells (4+) after 3 days of culture. mES cells treated with both RA and GT1b had better maturation rate than each RA and GT1b alone. However, GT1b induced more expression of both nestin and MAP-2 than RA. Therefore, the expression of nestin and MAP-2 were also affected by GT1b.

DISCUSSION

Numerous studies have suggested that there is a close relationship between the regulation of ganglioside levels through exogenous drug analogues and the induction of neuronal differentiation. For example, a previous study indicated that the levels and types of gangliosides change during neural differentiation, and that GD3, GT1b and GQ1b were enhanced when neural differentiation of embryonic carcinoma cells was induced by RA (Osanai et al., 2003). Therefore, to determine if ganglioside GT1b has a relationship with neuronal differentiation, we exogenously added ganglioside GT1b to RA-induced mESc. Specifically, we investigated the effects of various concentrations of ganglioside GT1b on the differentiation of neuronal cells from mESCs. The results revealed that the clinically optimal concentration of GT1b for the mediation of cell death was 0.2 ㎛ (Fig. 2). Previous studies have suggested that ganglioside GT1b is necessary for the differentiation of mESCs and MSCs into neuronal cells (Kwak et al., 2006). Additionally, we previously found that DNR-induced overexpression of ganglioside GQ1b and enhanced neurite formation in mESCs (Lee et al., 2007). In this study, the expression of nestin, an early neural cell marker, and MAP-2, a neuron dendrite marker, were found to be affected by GT1b (Fig. 3). There is accumulating evidence that ganglioside GT1b may regulate neuronal cell differentiation. Immunohistochemical examinations have provided evidence that ganglioside GT1b is markedly upregulated in synapses in the brain (Kotani et al., 1993), and GT1b oligosaccharide has been shown to facilitate the elongation and branch formation of dendrites of the hippocampal neurons and cerebella Purkinje neurons grown *in vitro* (Higashi and Chen, 2004). The results of this study

suggest that ganglioside GT1b facilitates the differentiation of neuronal cells. These observations validate the advantage of using mESCs as an *in vitro* model for the study of GT1b function in neuronal differentiation. The results of this study also suggest that regulation of ganglioside GT1b expression has been used as a marker for differentiated neuronal cells from mESCs. Accordingly, additional studies should be conducted to better understand the role that ganglioside GT1b plays in the neuronal differentiation of mESCs.

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