Embryonic Stem Cells with a Disrupted GD3 Synthase Gene Undergo Neuronal Differentiation in the Absence of b-Series Gangliosides*

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The dramatic changes in the expression of GD3 and other b-series gangliosides during neuronal development and morphogenesis have led to a widely held belief that these gangliosides may be necessary for neuronal differentiation. To determine directly if GD3 and b-series gangliosides are required for neuronal differentiation, we have produced embryonic stem (ES) cells with both alleles of the GD3 synthase gene (GD3S) disrupted by successive rounds of gene targeting. The double-targeted ES cells were deficient in GD3 synthase activity and did not synthesize b-series gangliosides. Despite this deficit, the GD3S(−/−) ES cells could be induced to undergo neuronal differentiation. Neuronally differentiated wild-type and GD3S(−/−) ES cells formed a complex neurite network around the embryoid bodies. Both types of neuronal cells expressed the axon-specific cytoskeletal proteins, neurofilament-M, and growth-associated protein-43 as well as the dendrite-specific marker, microtubule-associated protein-2. Our results indicate that GD3 synthase and b-series gangliosides are not necessary for the neuronal differentiation of uncommitted precursor cells.

Gangliosides are a diverse series of sialic acid-containing glycosphingolipids present on the outer leaflet of the plasma membrane of most vertebrate cells (1). They are particularly abundant in the central nervous system. The structural diversity of the ganglioside oligosaccharide chains is determined by biosynthetic glycosyltransferases residing in the Golgi apparatus. Ceramide is modified sequentially by glycosyltransferases to produce the simple ganglioside GM3 (Fig. 1), a key structural feature in the regulation of the biosynthetic pathway. GM3 ganglioside can be modified by GM2 synthase to produce GM2 and the a-series gangliosides. Alternatively, by the action of GD3 synthase (CMP-sialic acid: GM3 α-2,8-sialyltransferase; EC 2.4.99.8), GM3 can be converted to the disialoganglioside GD3, diverting the pathway to the synthesis of b-series gangliosides. The ganglioside composition of cells and tissues reflects the relative expression of these two biosynthetic glycosyltransferases (2–4).

Many studies have suggested that gangliosides may be important for neuronal differentiation (for review, see Refs. 5 and 6). In particular, GD3 synthase and its biosynthetic products, the b-series gangliosides, have been implicated as possibly having significant roles in development because of their unique expression pattern during neuronal differentiation (3, 7–9) and their ability to induce neuronal differentiation upon introduction, either by addition or through endogenous expression, into cultured cells (10–13).

To determine directly if b-series gangliosides are essential for neuronal differentiation, we disrupted both copies of the GD3 synthase gene (GD3S) in mouse embryonic stem (ES) cells. ES cells are normal, undifferentiated cells that can form all tissues of a mouse when injected into a blastocyst (14). ES cells also possess the ability to differentiate into many cell types, including neurons, in culture and provide a well-established system that recapitulates the differentiation pathway from undifferentiated cells to fully differentiated neurons (15–19). We report that ES cells with both GD3 synthase alleles disrupted undergo neuronal differentiation in the absence of b-series gangliosides.

EXPERIMENTAL PROCEDURES

Gene Targeting—To disrupt the both alleles of GD3 synthase gene in mouse ES cells, we prepared two targeting vectors with a 13-kb genomic segment isolated from a 129/sv mouse strain library (Stratagene). Within the genomic segment was an exon encoding 486 base pairs (162 amino acids) of GD3 synthase and its 3′-end of the protein coding sequence of GD3 synthase. The entire protein coding sequence of mouse GD3 synthase is 1,023 base pairs (341 amino acids). The neomycin resistance cassette (neo) (20) and hygromycin B resistance cassette (hygro) (21) were inserted into the BamHI site of the exon of GD3 synthase gene to create the GD3S-neo targeting vector and GD3S-hygro targeting vector (Fig. 2). The herpes thymidine kinase gene flanked the GD3S homology region in the targeting vectors. The GD3S-neo targeting vector (50 μg) was linearized and introduced into the J1 ES (22) cells by electroporation (Bio-Rad gene pulser, 400 V and 25 microfarads). Targeted clones were screened by Southern blotting after simultaneous positive-negative selection with G418 (350 μg/ml) and ganciclovir (5 μM). One of the correctly targeted clones was retargeted with the GD3S-hygro vector as above except the ES cells were selected with 75 μg/ml hygromycin B.

Induction of Neuronal Differentiation—Neuronal differentiation of ES cells was performed by using the 4-day/4-day protocol (16) with some modifications (Fig. 3). Undifferentiated J1 ES cells were maintained on mouse embryonic fibroblasts (Genome systems) with Dulbecco’s modified Eagle’s medium (high glucose, Life Technologies, Inc.), 15% fetal calf serum (Hyclone), 100 μM β-mercaptoethanol, and 1,000 units/ml leukemia inhibitory factor (Life Technologies, Inc.). Rapidly growing, undifferentiated ES cells were trypsinized with a balanced salt solution containing 0.25% trypsin and 1 mM EDTA. A portion of the cell suspen-
sion was transferred to a bacterial Petri dish with 10 ml of culture medium without β-mercaptoethanol and leukemia inhibitory factor. Under these conditions, ES cells do not attach to the dishes and readily form floating aggregates termed embryoid bodies (14). Embryoid bodies were cultured for 4 days. Then 50 μM retinoic acid (all-trans form; Sigma) was added to the medium, and the embryoid bodies were cultured for an additional 4 days. After the 8-day induction period, embryoid bodies were transferred to gelatinized tissue culture plates to allow cell attachment and neuronal outgrowth.

GD3 Synthase Assay—The Golgi-rich fraction, obtained from differentiated ES cells according to the method of Sandberg et al. (23), was used for GD3 synthase assay (24). GM3 ganglioside, dissolved in ethanol, was dried in a Speed-Vac and then resuspended in reaction buffer (100 mM sodium cacodylate (pH 6.5), 0.4% Triton X–100, 10 mM MgCl$_2$, 2 mM 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid) by sonication for 1 min. An aliquot of Golgi fraction was incubated with 125 nCi of [3H]CMP-sialic acid (21 μM final concentration) at 37 °C for 1 h in the presence or the absence of exogenous GM3 ganglioside in reaction buffer (20 μl total volume) to control for transfer to endogenous acceptors. The final concentration of exogenous GM3 ganglioside was 0.6 mM. An aliquot of reaction mixture was spotted on flexible TLC plates (Silica Gel IB2-F, J. T. Baker). The plate was developed with water for 15 min to separate the lipid-bound radioactivity, which remains at the origin, from free [3H]CMP-sialic acid. The lipid-bound radioactivity was quantitated on a Fuji BAS-2500 PhosphorImager with MacBAS version 2.52 software.

The GD3S cDNA was prepared by ligating a 950-base pair polymerase chain reaction fragment, encompassing the 5’-end, obtained from a mouse brain cDNA library, to a BamHI-PstI fragment encompassing the 3’-end, obtained from the last exon of the GD3S gene. To remove the sialyl motif S, the GD3S cDNA was truncated at the 3’-end by digestion with BamHI endonuclease. Both cDNAs were subcloned into the expression vector pcDNA3.1/Zeo (Invitrogen) and were transfected into COS-1 cells using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. The cells were harvested 48 h after transfection, and GD3 synthase activity was determined as above except that total cell extracts were used.

Ganglioside Analysis—Neuronally differentiated ES cells were incubated with [3H]galactose (2 μCi/ml, NEN Life Science Products) in Dulbecco’s modified Eagle’s medium with lowered glucose (450 mg/liter) and 10% dialyzed fetal bovine serum for 24 h. Cells were washed with phosphate-buffered saline (PBS), trypsinized, and harvested by centrifugation. Labeled glycosphingolipids were extracted as described by Harel and Futerman (25). Lipid extracts were resuspended with chloroform and methanol (2:1) and separated by TLC using chloroform, methanol, and 0.2% aqueous CaCl$_2$ (60:40:9) as the developing solvent. Labeled glycosphingolipids were detected using a Fuji BAS-2500 PhosphorImager with MacBAS version 2.52 software. Ganglioside standards were developed on the same TLC plates and visualized with orcinol.
ferric-chloride solution (Bial's Reagent, Sigma). The identity of labeled glycolipids was assigned by comigration with standards.

Immunohistochemistry—At day 5, cells were washed with PBS and then fixed with 4% paraformaldehyde at 4 °C for 1 h. Cells were made permeable with 95% ethanol, 5% acetic acid at 20 °C for 30 min. After washing in PBS, the cells were incubated in blocking solution (PBS containing 10% normal horse serum) for 30 min. After washing in PBS, primary antibodies were added, and cells were incubated overnight at 4 °C. Anti-neurofilament-160 kDa (NF-M) antibody was diluted to 1:5,000, anti-growth associated protein-43 (GAP-43) antibody was diluted to 1:200, anti-microtubule-associated protein-2 (MAP-2) antibody was diluted to 1:250. All primary antibodies were purchased from Sigma. Secondary biotinylated antibodies were added and incubated for 1 h at room temperature, followed by avidin and biotinylated horseradish peroxidase complex (ABC Elite kit, Vector Laboratories). The antibody-peroxidase complex was visualized with the substrate 4-chloro-1-naphthol-N, N-dimethyl-p-phenylenediamine monohydrochloride.

RESULTS
To disrupt both alleles of the mouse GD3S gene, we prepared two targeting vectors for use in consecutive rounds of gene targeting; the vectors were identical except that one contained a neomycin resistance cassette, and the other contained a hygromycin B resistance cassette (Fig. 2A). The targeting vectors were designed to disrupt the catalytic domain of the enzyme by introduction of a premature termination codon before sialyl motif S. The sialyl motif S is highly conserved among sialyltransferases and is thought to comprise part of the catalytic domain (26, 27). Removal of the sialyl motif S from the GD3S cDNA rendered the enzyme catalytically inactive as determined by expression in COS-1 cells (data not shown). After the first round of gene targeting with the GD3S-neo targeting vector, 12 out of 24 G418-resistant colonies exhibited the 5.7-kb
bated with $^{14}$C galactose, and the labeled glycolipids were synthesized by the differentiated ES cells, cultures were incubated with wild-type cells (Fig. 2 C). 5.7-kb neo-targeted fragment (Fig. 2 B), and MAP-2 (panels E and F) antigens in differentiated ES cell cultures (day 5) were determined by immunohistochemistry as described under “Experimental Procedures.” Left panels show wild-type ES cells, and right panels show GD3S(-/-) ES cells. EB, embryoid body.

The distribution of NF-M (panels A and B), GAP-43 (panels C and D), and MAP-2 (panels E and F) showed wild-type ES cells on feeder layers, the cells were transferred into bacterial Petri dishes to prevent cell attachment and to allow the formation of embryoid bodies (14). We did not observe any difference in the ability of wild-type and GD3S(-/-) ES cells to form embryoid bodies (Fig. 3B, day -2). After an additional 4-day incubation with retinoic acid, embryoid bodies were transferred to gelatinized tissue culture dishes to allow attachment (day 0). As has been described, a neurite outgrowth from the embryoid body appeared, indicating neuronal differentiation of the cultures (Fig. 3B, day +5) (16).

We measured GD3 synthase enzyme activity from differentiated wild-type and GD3S(-/-) cells. The GD3S(-/-) clones had a 96.4 $\pm$ 2.7% reduction in activity compared with the wild-type cells (Fig. 2C). To assess the gangliosides synthesized by the differentiated ES cells, cultures were incubated with $^{14}$C galactose, and the labeled glycolipids were separated by TLC (Fig. 3, C and D). Both a- (GM3, GM1, GD1a) and b-series (GD3, GD1b and GT1b) gangliosides were synthesized by the wild-type ES cells. By contrast, the GD3S(-/-) cells contained the a-series gangliosides but no detectable b-series gangliosides, consistent with their lack of GD3 synthase activity.

To confirm the neuronal differentiation of wild-type and GD3S(-/-) ES cell cultures, cells were characterized immunohistochemically with antibodies that detect neuron-specific proteins, NF-M (28), GAP-43, and MAP-2 (28). Both cultures were positive for the axon-specific cytoskeletal proteins NF-M and GAP-43 (Fig. 4, A–D). The long neurites emerging from embryoid bodies of both the wild-type and GD3S(-/-) stained strongly for GAP-43 and NF-M. The presence of the dendrite-specific marker, MAP-2, was also determined. In both wild-type and GD3S(-/-) cultures the short, tapered neurites around the embryoid bodies were MAP-2-positive (Fig. 4, E and F). Some neuronal cell bodies around embryoid bodies were also MAP-2-positive in their cytoplasm (arrows).

**DISCUSSION**

The observation that GD3 and other b-series gangliosides are exquisitely regulated during development of the nervous system led to the view that they may be important during neuronal differentiation (3, 8, 9). GD3 is the major ganglioside of the embryonic nervous system and is expressed initially on rapidly proliferating neuroepithelial cells (7, 29, 30). At later stages in development, GD3 ganglioside expression falls drastically while an increase in the synthesis of complex b-series gangliosides occurs. Studies of cultured neurons have shown major changes in ganglioside synthesis coupled to changes in neuronal morphogenesis (31). In particular during axonogenesis, the synthesis of complex gangliosides increases together with the decreased synthesis of GD3 ganglioside. An increase in the ratio of a- to b-series gangliosides occurs during the period of rapid axon growth. Finally, during the neural differentiation of embryonic carcinoma P19 cells, the expression GD3 synthase is specifically induced (32).

Support for the concept that GD3 synthase and b-series gangliosides may be involved in neuronal differentiation comes from experiments showing neuritogenic effects in neuronal cell lines upon ganglioside introduction. The exogenous b-series ganglioside GQ1b, when added at nanomolar concentrations, caused differentiation of neuroblastoma cells (10). Ectopic GD3 synthase cDNA expression in neuroblastoma cells resulted in increased expression of b-series gangliosides together with neurite outgrowth and cellular differentiation (11, 13). Manipulation of GD3 levels in neuroblastoma cells with a transfected O-acetyltransferase gene from influenza C virus also induced morphological changes (12). Although these studies show that GD3 synthase and b-series gangliosides induce differentiation in neuroblastoma cells, they do not address the question of whether b-series gangliosides are required for the differentiation of embryonic precursor cells into neurons.

To investigate this issue we used uncommitted, pluripotent ES cells that, under the appropriate stimulus, can differentiate into functional neurons. We inactivated both alleles of the GD3 synthase gene in mouse ES cells by targeted gene disruption. These double-targeted cells had a profound deficiency of GD3 synthase activity and did not produce b-series gangliosides. Despite this deficiency, ES cells were able to differentiate morphologically into neuronal cells and extend immunohistochemically identified axons and dendrites in a manner indistinguishable from that of wild-type cells. Our results indicate that GD3 synthase and b-series gangliosides are not required for these aspects of neuronal differentiation by uncommitted embryonic precursor cells. However, these gangliosides may have important roles in other nervous system functions such as in cell-cell recognition events (5, 33). Our results suggest that these functions may be evaluated in the context of the whole organism such as in mice with a disrupted GD3 synthase gene without consequences on neuronal differentiation.

Evidence is emerging that large classes of complex gangliosides may be expendable for neuronal differentiation and central nervous system development. Mice deficient in GM2 synthase, lacking a-series and most complex gangliosides, develop a functional nervous system with apparently only subtle abnormalities (34). Inhibition of glycosphingolipid synthesis did not impair growth or morphogenesis of cultured postimplantation embryos (35). The present studies indicate that b-series gangliosides are not essential for neuronal differentiation of
uncommitted precursor cells. In a limited differentiation assay, drug-induced depletion of most cellular gangliosides in neuroblastoma cells did not alter neurite formation (36). The unexpected absence of severe consequences for neuronal differentiation after elimination of gangliosides could indicate that the diversity of ganglioside structures affords some functional redundancy. It is also possible that gangliosides may not be essential for neuronal development and differentiation. The total elimination of ganglioside structures during neuronal development may be needed to settle this issue.

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REFERENCES
1. van Echten, G., and Sandhoff, K. (1993) J. Biol. Chem. 268, 5341–5344
2. Percy, A. K., Gottfries, J., Vilbergsson, G., Mansson, J. E., and Svennerholm, L. (1991) J. Neurochem. 56, 1461–1465
3. Yu, R. K. (1994) Prog. Brain Res. 101, 31–44
4. Yamamoto, A., Haraguchi, M., Yamashiro, S., Fukumoto, S., Furukawa, K., Takamiya, K., Atsuta, M., Shiku, H., and Furukawa, K. (1996) J. Neurochem. 66, 26–34
5. Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716
6. Tettamanti, G., and Riboni, L. (1993) Adv. Lipid Res. 25, 235–267
7. Bouvier, J. D., and Seyfried, T. N. (1989) J. Neurochem. 52, 460–466
8. Rosner, H., al-Aqtum, M., and Rahmann, H. (1992) Neurochem. Int. 20, 339–351
9. Irvine, R. A., and Seyfried, T. N. (1994) Comp. Biochem. Physiol. B Biochem. Mol. Biol. 109, 603–612
10. Tsuji, S., Nakajima, J., Sasaki, T., and Nagai, Y. (1985) J. Biochem. (Tokyo) 97, 969–972
11. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) J. Biol. Chem. 269, 30451–30456
12. Ariga, T., Blaine, G. M., Yoshino, H., Dawson, G., Kanda, T., Zeng, G. C., Kasama, T., Kushiyama, K., and Yu, R. K. (1995) Biochemistry 34, 11500–11507
13. Liu, H., Kojima, N., Kurosawa, N., and Tsuji, S. (1997) Glycobiology 7, 1067–1076
14. Robertson, E. J. (1987) in Teratocarcinomas and Embryonic Stem Cells (Robertson, E. J., ed) pp. 71–112, IRL Press, Oxford
15. Keller, G. M. (1995) Curr. Opin. Cell Biol. 7, 862–869
16. Bain, G., Kitchens, D., Yao, M., Huettner, J. E., and Gottlieb, D. I. (1995) Dev. Biol. 186, 342–357
17. Strubing, C., Ahnert-Hilger, G., Shan, J., Wiedemann, B., Hescheler, J., and Wobus, A. M. (1995) Mech. Dev. 53, 275–287
18. Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995) J. Cell Sci. 106, 3181–3188
19. Okabe, S., Forsberg-Nilsen, K., Spira, A. C., Segal, M., and McKay, R. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 89–92
20. Cao, M. R. (1989) Science 244, 1298–1292
21. van Deursen, J., and Wieringa, B. (1992) Nucleic Acids Res. 20, 3815–3820
22. Lee, K.-F., Li, E., Huber, J., Landis, S. C., Sharpe, A. H., Chao, M. V., and Jaenisch, R. (1992) Cell 69, 737–749
23. Sandberg, P. O., Marzella, L., and Glauermann, H. (1986) Exp. Cell Res. 130, 393–400
24. Kasahara, K., Guo, L., Nagai, Y., and Sanai, Y. (1994) Anal. Biochem. 218, 224–226
25. Harel, R., and Futerman, A. H. (1998) J. Biol. Chem. 268, 14476–14481
26. Tsuji, S. (1996) J. Biochem. (Tokyo) 120, 1–13
27. Datta, A. K., Sinha, A., and Paulson, J. C. (1998) J. Biol. Chem. 273, 9608–9604
28. Peng, I., Binder, L. I., and Black, M. M. (1986) J. Cell Biol. 102, 252–262
29. Goldman, J. E., Hirano, M., Yu, R. K., and Seyfried, T. N. (1984) J. Neuroimmunol. 7, 179–192
30. Rosner, H., al-Aqtum, M., and Henke-Fahle, S. (1985) Brain Res. 350, 85–95
31. Hirshberg, K., Zeling, B., van Echten-Deckert, G., and Futerman, A. H. (1996) J. Biol. Chem. 271, 14876–14882
32. Osanai, T., Watanabe, Y., and Sanai, Y. (1997) Biochem. Biophys. Res. Commun. 241, 327–333
33. Schnaar, R. L. (1991) Glycobiology 1, 477–485
34. Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Chin, M., Okada, M., Fukumoto, S., Haraguchi, M., Takeda, N., Fujimura, K., Sakae, M., Kishikawa, M., Shiku, H., Furukawa, K., and Asahina, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10662–10667
35. Brod, J. V., Platt, F. M., and Seyfried, T. N. (1998) J. Neurochem. 70, 871–882
36. Li, R., and Ladisch, S. (1997) J. Biol. Chem. 272, 1349–1354
37. Svennerholm, L. (1964) J. Lipid Res. 5, 145–155