b-series Ganglioside Deficiency Exhibits No Definite Changes in the Neurogenesis and the Sensitivity to Fas-mediated Apoptosis but Impairs Regeneration of the Lesioned Hypoglossal Nerve*

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The polymorphic carbohydrate structures of gangliosides play regulatory roles. In particular, b-series gangliosides, all of which contain α2,8 sialic acids, have been considered to be critical in various biological events such as adhesion, toxin binding, neurite extension, cell growth, and apoptosis. To clarify the physiological functions of b-series gangliosides in vivo, we have established a gene knockout mouse of GD3 synthase. Although all b-series structures were deleted in the mutant mice, they showed an almost complete nervous tissue morphology with no apparent abnormal behavior. Moreover, no differences in Fas-mediated apoptotic reaction of lymphocytes between wild type and the mutant mice were detected. However, the mutant mice exhibited clearly reduced regeneration of axotomized hypoglossal nerves compared with the wild type, suggesting that b-series gangliosides are important in the repair of damaged nerves rather than in the differentiation of the nervous system.

EXPERIMENTAL PROCEDURES

Generation of GD3 Synthase Gene Knockout Mice—The chromosomal GD3 synthase gene was isolated from the agt11 phage library using GD3 synthase cDNA (pD3T-31) and mapped as described previously (11). To distinguish the true GD3 synthase gene from pseudo-genes, in situ hybridization was performed, and the identity was confirmed based on the correspondence of the gene assignment between humans and mice. The neo+ gene was inserted between the BalI and AccI sites in exon 1 of the gene, and a 9.5-kb gene fragment was used as a targeting vector as shown in Fig. 1A. The diphtheria toxin A gene was attached to eliminate nonhomologous recombinants as described previously (12). Homologous recombination was confirmed by Southern blotting using a probe as shown in Fig. 1, generating 4.5- and 2.8-kb fragments by BamHI and 4.6- and 3.1-kb fragments by HindIII digestion in the wild-type and the recombinant allele, respectively (Fig. 1A).

Ganglioside Analyses—Changes in the ganglioside profile of the GD3 synthase gene knockout mice were examined by extracting glycolipids from the brain and liver as described previously (13). Briefly, glycolipids were extracted with chloroform/methanol (2:1/1:1/2) sequentially, and ganglioside fractions were separated by DEAE-Sephadex (A-50) ion-exchange column chromatography. The extracted gangliosides were separated by thin layer chromatography (TLC2) using HPTLC plates (Merck). Gangliosides were visualized using a resorcinol/HCl spray.

Preparation of Thymocytes and Spleen Cells—The thymus and spleen were dispersed using syringe needles, and the cell number was counted using a hemocytometer. Cells were used for flow cytometry and proliferation and apoptosis assays. Spleen cells were used after elimination of adherent cells with plastic dishes.

Flow Cytometry—Ganglioside expression was analyzed by flow cytometry with monoclonal antibodies (mAbs) for individual structures as described previously (14). Cell subsets were analyzed by fluorescein isothiocyanate-conjugated anti-CD4 mAb and phycoerythrin-conjugated anti-CD8 mAb as described previously (14). Cell proliferation was

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1 Ganglioside nomenclature is based on that of Svennerholm (24).
2 The abbreviations used are: TLC, thin layer chromatography; mAb, monoclonal antibody; HRP, horseradish peroxidase; Ex (number), embryonal day x.
analyzed by stimulating with concanavalin A, anti-CD3 mAb, or interleukin-2 as described previously (14).

Apoptosis Induction Assay—Apoptosis induction was performed by adding anti-Fas mAb Jo2 (1–1,000 ng/ml) to the culture medium of leukin-2, or diphtheria toxin A gene; H, HindIII; B, BamHI; TM, transmembrane; E, EcoRI; LacCer, lactosylceramide; U, units; St, standard.

RESULTS AND DISCUSSION

The established GD3 synthase gene knockout mice (Fig. 1C) exhibited changes in the ganglioside components as expected from biochemical enzyme analyses as shown in Fig. 1B (17). Namely, all b-series gangliosides including GD3, GD2, GD1b, GT1b, and GQ1b were deleted, and a-series gangliosides such as GM1, GD1a, and GM2 were accumulated (Fig. 1Ea). In the enzyme assay with membrane fractions of brain tissues, GD3 synthase activity completely disappeared in the mutant mice, whereas GM3 synthase and GD1a synthase activity were retained (Fig. 1D, a and b). Heterozygotes showed approximately half the level of GD3 synthase activity and the corresponding profiles of gangliosides. It was also confirmed that neither GD3 synthase activity nor b-series gangliosides were detectable in the mutant embryonal brains throughout development (E10–E18) (Fig. 1, De and Eb), indicating that there was no different isoform of GD3 synthase expressed in early development. Brains from newborn homozygotes were also negative for the enzyme activity and its products (data not shown). No changes in the neutral glycolipid components were detected (data not shown). b-series ganglioside-lacking mice were born and grew up without gross abnormal behavior. They showed normal hindlimb reflex, postural changes, spontaneous motor activity, swimming ability, flinch hearing, and no sign of ataxia. Current analyses for the activity of memory and learning such as the Morris water-maze test, passive avoidance test, Y-maze test, etc. also showed no abnormal findings in the mutants (data not shown). In macroscopic and histological analysis of brain tissues, the mutant mice had almost normal size, shape, and weight of brains being indistinguishable from those of wild type. The following sites were carefully examined and exhibited almost normal morphology: cortices, striatum, callosum, hippocampus, ventricles, choroid plexus, cerebellum, and brain stem. The retina also showed no abnormalities in the laminar
architecture and number of neurons, although it was one of the sites where the GD3 synthase gene was most strongly expressed (18).

The majority of b-series gangliosides deleted in the mutant mice were also deleted in GM2/GD2 synthase knockout mice (19) except for GD3. Therefore, the phenotypes of the mutant mice might be similar to those of complex ganglioside-lacking mice. The findings described above indicated that b-series gangliosides including GD3 are not indispensable for the morphogenesis of nerve tissues as previously described (20), and probably the remaining a-series gangliosides (and asialo-series) substitute for the roles of b-series structures.

A number of studies have been performed to investigate the involvement of gangliosides in the regulation of the immune system (14, 21, 22). Although GD1b expression was completely deleted and GM1 expression was moderately increased in mutant thymocytes (Fig. 2A) and splenocytes (data not shown), the thymus and spleen were normally formed, and no changes were detected in size, shape, and cell number in the mutants (Fig. 2C). The ratio of CD8+ thymocytes was reduced (Fig. 2B), and the proliferative response to various stimulations in the mutant were generally increased (concanavalin A, anti-CD3) or not changed (interleukin-2) (data not shown), although the mechanisms remain to be clarified. Recently it was reported that Fas-induced apoptosis of leukocytes was mediated via GD3 (10). To analyze the resistance of b-series ganglioside-lacking mice to Fas-induced apoptosis, thymocytes from the wild-type and the mutant mice were used for the induction of apoptosis. However, no reduction in the sensitivity to the Fas-induced apoptosis could be detected (Fig. 3). In the presence of cycloheximide, 50% of thymocytes from the wild type underwent apoptotic cell death at 12 h of incubation with Jo2 (10 ng/ml). Roughly 55% of those from the mutant also died approximately at the same time point. The sensitivity to apoptosis with various concentrations of Jo2 was also very similar between the two genetic types (Fig. 3A). The intensities of fragmented DNA and the formation of sub-G1 in the DNA histogram during the induced apoptosis were also very similar (Fig. 3, B and C). Thus, deletion of b-series gangliosides did not alter the sensitivity of thymocytes to Fas-induced apoptosis, although DeMaria et al. (10) reported the significant role of GD3 as a mediator of apoptosis.

We have established an experimental system to analyze the regeneration activity of damaged hypoglossal nerves (16) and reported that exogenous gangliosides were very effective for the promotion of regeneration. In the case of GD3 synthase gene

![Fig. 2. Immune tissues in GD3 synthase gene knockout mice.](http://www.jbc.org/)

**A**. ganglioside expression in the mutants. Thymocytes and spleen cells were analyzed for ganglioside expression with mAbs as described under “Experimental Procedures.” GD1b disappeared and GM1 slightly increased in the mutant thymocytes and spleen cells (data not shown). B, the ratio of CD4+/CD8+ cells in the thymus and spleen cells was examined by two-dimensional flow cytometry. C, total numbers of thymus and spleen cells were counted, and the ratio of single positive T cells (CD8+ /CD4+) were compared between +/+ and −/−. The results from five mice each were presented as mean ± S.D. * represents p < 0.005.

![Fig. 3. The sensitivity of thymocytes to Fas-induced apoptosis did not change in the mutant mice.](http://www.jbc.org/)

**A**. apoptosis induced by Jo2 (1–1,000 ng/ml) with cycloheximide. The percentage of viable cells at 12 h of treatment was counted and plotted. Results at 8 and 20 h were essentially similar. **B**, DNA ladder formation in thymocytes treated with Jo2 for 12 h. Cytoplasmic DNA was collected and electrophoresed as described under “Experimental Procedures.” **C**, DNA histogram of thymocytes during apoptosis. Cells were stained with propidium iodide and analyzed by flow cytometry. Note that the population of the sub-G1 fraction was similar between the two genetic types.
knockout mice, regeneration levels were definitely reduced compared with the wild type, i.e. by ~45% (Fig. 4, A and C). The surviving neuron number was also reduced to ~50% (Fig. 4B), suggesting that b-series gangliosides are essentially needed for the protection of neuronal death and repair of damaged hypoglossal nerves. In the axotomized rat, exogenously added b-series gangliosides, i.e. GT1b and GD1b, were most effective in the regeneration (23). The present findings shown here appear to be in good agreement with those of the previous rat experiment (23). Furthermore, GM2/GD2 synthase gene transgenic mice in which b-series gangliosides were reduced also showed similar levels of reduction in the surviving neuron number and the regeneration activity (data not shown). Thus, endogenously generated b-series gangliosides turned out to be critical in the repair of damaged neural tissues in vivo, while their roles in the neurogenesis or apoptosis were not clearly elucidated.

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REFERENCES

1. Wiegandt, H. (ed) (1985) in Glycolipids, pp. 199–260, Elsevier Science Publishing Co., Inc., New York
2. King, C. A., and van Heyningen, W. E. (1975) J. Infect. Dis. 131, 643–648
3. Kleinman, H. K., Martin, G. R., and Fishman, P. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3367–3371
4. Cheresi, D. A., Pieruschka, M. D., Herzig, M. A., and Mujoo, K. (1986) Cancer Res. 46, 688–696
5. Tsuji, S., Arita, M., and Nagai, Y. (1983) J. Biochem. (Tokyo) 94, 393–396
6. Nakano, J., Raj, B. K., Asagami, C., and Lloyd, K. O. (1986) J. Investig. Dermatol. 107, 545–548
7. Birkle, S., Ren, S., Slinominski, A., Zeng, G., Gao, L., and Yu, R. K. (1999) J. Neurochem. 72, 954–961
8. De Maria, R., Lenti, L., Malisan, F., d’Agostino, F., Tomasini, B., Zeuner, A., Rippo, M. R., and Testi, R. (1997) Science 277, 1652–1655
9. Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K. O., Shiku, H., and Furukawa, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10455–10459
10. De Maria, R., Rippo, M. R., Schuchman, E. H., and Testi, R. (1998) J. Exp. Med. 187, 897–902
11. Furukawa, K., Soejima, H., Niikawa, N., Shiku, H., and Furukawa, K. (1996) J. Biol. Chem. 271, 20386–20394
12. Aizawa, S., Suda, Y., Furuta, Y., Yagi, T., Takeda, N., Watanabe, N., Nagayoshi, M., and Ikawa, Y. (1990) EMBO J. 9, 2107–2116
13. Furukawa, K., Clausen, H., Hakonson, S., Sakamoto, J., Look, K., Lundblad, A., Mattes, M. J., and Lloyd, K. O. (1985) Biochemistry 24, 7820–7826
14. Zhao, J., Furukawa, K., Fukumoto, S., Okada, M., Miyazaki, H., Shiku, H., Aizawa, S., Matsuyama, M., and Furukawa, K. (1999) J. Biol. Chem. 274, 13744–13747
15. Ogasawara, J., Suda, T., and Nagata, S. (1995) J. Exp. Med. 181, 485–491
16. Itoh, M., Fukumoto, S., Kuga, Y., Mizuno, A., and Furukawa, K. (1999) Glycobiology 9, 1247–1252
17. Pohlentz, G., Klein, D., Schwarzmann, G., Schmitz, D., and Sandhoff, K. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7044–7048
18. Yamamoto, A., Yamashiro, S., Fukumoto, S., Haraguchi, M., Atsuma, M., Shiku, H., and Furukawa, K. (1996) Glycobiology 6, 1247–1252
19. Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Shih, M., Okada, M., Fukumoto, S., Haraguchi, M., Takeda, N., Fujimura, K., Sakae, M., Kishikawa, M., Shiku, H., Furukawa, K., and Aizawa, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10662–10667
20. Kawai, H., Allende, M. L., Wada, R., Kono, M., Sango, K., Deng, C., Miyakawa, T., Crawley, J. N., Werth, N., Bierfreund, U., Sandhoff, K., and Proia, R. L. (2001) J. Biol. Chem. 276, 6885–6888
21. Nakamura, K., Suzuki, H., Hirabayashi, Y., and Suzuki, A. (1995) J. Biol. Chem. 270, 3876–3881
22. Nohara, K., Kunimoto, M., and Fujimaki, H. (1998) J. Biochem. (Tokyo) 124, 194–199
23. Itoh, M., Fukumoto, S., Iwamoto, T., Mizuno, A., Rokutanda, A., Ishida, H., Kiso, M., and Furukawa, K. (2001) Glycobiology 11, 125–130
24. Svennerholm, L. (1963) J. Neurochem. 10, 455–463
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