Refractory Skin Injury in Complex Knock-out Mice Expressing Only the GM3 Ganglioside

We generated double knock-out mice lacking the GM2/GD2 and the GD3 synthase gene by mating single gene mutants, and we analyzed the abnormal phenotypes of the mutant mice expressing only the GM3 ganglioside. We observed a refractory skin lesion that appeared primarily on the face of the mutant mice at 25 weeks after birth or later. Frequent scratching of the wound sites was observed in mutant mice with the skin injury, suggesting that it is a triggering factor that exacerbates the injury. This was confirmed by isolating mice in special cages for metabolic study in which the skin injury developed more rapidly. Characteristic proliferation of nerve fibers was found in the epidermis and subepidermis at the injured sites of the mutants, probably as a result of continuous skin injury. Peripheral nerve degeneration was observed in young mutant mice, suggesting that reduced sensory function induced over-scratching and the resulting skin lesion. The fact that sensory response to mechanical stimuli decreased while that to hot stimuli increased in the mutant mice supports this interpretation. Thus, only GM3-expressing mice displayed the important role of gangliosides in maintaining skin integrity via regulation of the peripheral nerves.

Acidic glycosphingolipids, gangliosides, are ubiquitously expressed in the various tissues of vertebrates and play important roles in the regulation of highly organized multicellular systems (1). In particular, they are very much enriched in the nervous system and have been considered to control development, proliferation, differentiation, and maintenance of the neural tissues and cells (2, 3). Expression patterns of various ganglioside species in the nervous system vary during development and are strictly regulated in a spatio-temporal manner (4), suggesting that individual structures of gangliosides contain significant implications for individual situations.

A number of studies have been performed to demonstrate the biological function of gangliosides primarily by modifying their structures with enzymes or using inhibitors to block some steps of synthesis (5). Otherwise, the effects of addition of gangliosides to the culture medium or injection into the conditioned animals have been the primary approaches in addressing the significance of gangliosides. However, recent success in the molecular cloning of glycosyltransferase genes brought about an evolutionary change in the experimental approaches for carbohydrate function analysis. Availability of glycosyltransferase genes responsible for the synthesis of gangliosides enabled us to remodel ganglioside compositions of cells and tissues in vivo (6, 7) and in vitro (8).

Because we isolated GM2/GD2 synthase (EC 2.4.1.92) cDNA (9) and GD3 synthase (EC 2.4.99.8) cDNA (10), we established gene knock-out mice lines of the individual glycosyltransferases. GM2/GD2 synthase gene knock-out mice lacking all complex gangliosides showed almost normal morphogenesis of the nervous system and no definite abnormal behaviors (11), although they showed nerve degeneration along with aging (12, 13) as well as immunodeficiency (14) and male infertility (15). GD3 synthase gene knock-out mice lacking all b-series gangliosides also showed no marked abnormal appearance in nerve tissues and exhibited almost intact behaviors (16, 17), although regeneration of the lesioned hypoglossal nerve was largely reduced (17).

In the present study, we established double knock-out mice of the above described GM2/GD2 synthase gene and GD3 synthase gene by mating them to each other, and we analyzed the abnormal phenotypes of the mutant mice expressing only the GM3 ganglioside. Although these mutant mice might have a serious deficit in the nervous system (16), we observed a characteristic skin lesion that appeared mostly on the face of the mutant mice 25 weeks after birth or later. We have investigated the mechanisms for the development of the skin lesion and have elucidated the reduced sensitivity of the sensory nerve based on nerve degeneration as a primary triggering factor for the lesion.

**EXPERIMENTAL PROCEDURES**

*Mice—*The mice were maintained in our laboratory. GM2/GD2 and GD3 synthase gene knock-out mice were mated, i.e. heterozygotes of both mutants were mated, and genotypes of the offspring were screened for the two genes as described previously (11, 17). To generate double knock-out mice efficiently, we also mated female homozygotes of the GD3 synthase gene and male heterozygotes of the GM2/GD2 synthase gene. Double knock-out mice with the two genes were designated as Ho/ho2 mutant, and those with wild type for both genes were presented as Wd/Wd. Body weight was measured every week, and mice were observed every day.

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*This abbreviations used are: Ho/Ho, homozygous mutant for GD3 synthase; Wd/Wd, wild type for GD3 and GM2/GD2 synthase.

**Nomenclature of gangliosides is based on that of Svennerholm (35).**
Typing—Genetic typing of GD3 synthase gene was performed by polymerase chain reaction with mouse tails using primers as follows, 5’-GCTGAGGTACACTGACCCTGGGACATCGA-3’, 5’-TCGTGCTTTACGTGCTTTACGTGCTTTACGTGCT-3’, and 5’-ACTAGGGACAGACCGGCGAAATCCTTGATT-3’. The reaction was started with a 3-min cycle at 95 °C followed by 30 cycles for 1 min at 55 °C, 2 min at 72 °C, and 1 min at 95 °C. Genetic typing of the GM2/GD2 synthase gene was performed as described previously (11).
Extraction of Glycolipids—Glycolipids were extracted as described previously (18). Briefly, lipid fractions were extracted by chloroform/methanol at ratios of 2:1, 1:1, and 1:2, sequentially. Glycolipids were isolated by a Florisil column after acetylation, and then neutral and acidic fractions were separated by DEAE-Sephadex (A-50) column chromatography.

Isolation of Mice in Metabolic Cages to Induce Wounds—Seven each of Wd/Wd-type and Ho/Ho-type 25-week-old male mice were housed in special cages usually used for metabolic study and were fed regular chow. Daily observation was performed for 2 months to check the body condition. FIG. 2. Generation of skin injury in Ho/Ho mice. A, appearance of a 30-week-old Ho/Ho mouse with serious skin injury on its face. Arrows indicate injured fingers. B, time course of the generation of skin injury shown as the ratio of mice with skin injury to the face and neck. In Ho/Ho mice only, this emerged immensely in adulthood (25 weeks after birth). C, example of the scratching movement observed in Ho/Ho mice with the injury. D, frequency of scratching in 30-week-old Ho/Ho and Wd/Wd mice. Note that only wound (+) Ho/Ho showed high frequency.

FIG. 3. Promotion of skin injury in the metabolic study cages. A, representative examples of the injury at 2 weeks (top panel) and 5 weeks (bottom panel) after accommodation were shown. Arrows indicate lesions around the eyes. B, time course of the development of the skin injury in Ho/Ho and Wd/Wd mice housed in the metabolic cages. The ratios of injured/whole mice examined are shown as a function of weeks after isolation. C, frequency of scratching as measured every 5 min. Average numbers of the movement of three sequential counts (mean ± S.D.) are presented.
weight, generation of wounds, and frequency of skin scratching. Scratching measurements were performed every 5 min twice a week.

Pathological Studies—Tissue sections from the skin lesions of the injured sites were prepared. The serial sections were divided into three groups, i.e. the first group was stained with hematoxylin-eosin, and the other two groups were stained with toluidine or used for immunohistochemical studies with anti-PGP9.5 antibody directed toward the N terminus of the protein (19). The areas occupied with nerve fibers in the

**Fig. 4. Histological analysis to elucidate the peripheral nerve hypertrophy.** A, erosion and marked infiltration of inflammatory cells in epidermis of injured Ho/Ho mice skin (hematoxylin-eosin). B, hypertrophic nerve fibers in epidermis of injured Ho/Ho mice (hematoxylin-eosin). Arrows indicate nerve fibers. C, toluidine blue staining of Wd/Wd mice skin. D and E, hypertrophic nerve fibers in Ho/Ho mice skin stained with toluidine blue. Arrows in C, D, and E indicate nerve fibers. Arrowheads in D indicate cells with granules. F, proliferated nerve fibers in epidermis in the injured lesion of Ho/Ho mice stained by an anti-PGP-9.5 antibody; note that a number of nerve fibers were stained. G, electron microscopic analysis of nerve fibers in the epidermis of Ho/Ho. H, area occupied by nerve fibers in the skin sections were measured by NIH image as described under “Experimental Procedures.” Percent areas is shown as mean ± S.D. *, p < 0.05. 30-week-old Ho/Ho and Wd/Wd mice were used.
epidermis were quantitatively measured in consecutive sections, and the results were presented as an average of the values obtained from three sections. Actual measurement of the nerve-occupying area was performed as follows. Images were captured using the Olympus digital camera DP-11 or classical photomicrography on film. Analysis was performed using the public domain NIH Image program (anonymous FTP from zippy.nimh.nih.gov). These analyses were performed by two independent persons with no preliminary information.

Electron Microscopic Study—Skin tissues were fixed by immersion in a fixative containing 1.5% paraformaldehyde and 1.5% glutaraldehyde in phosphate buffer (pH 7.4) and osmicated in 1% OsO₄ solution for 1 h. Fixed tissues were dehydrated in alcohols and embedded in Epon 812. After staining with uranylacetate and lead citrate, ultrathin sections were prepared and observed using the JEOL 100 CX electron microscope.

Sensitivity Test—To analyze sensory function in the mutant mice, we examined sensitivity to pain stimulation using the hot plate method and with the von Frey strings test as described previously (20).

Statistical Analysis—Numeric data are expressed as mean ± S.D. The number of mice used in the experiments are as indicated in each figure. The significance of differences among groups was determined using analysis of variance for comparison.

RESULTS

Generation of Double Knock-out Mice of GM2/GD2 and GD3 Synthase Genes—The heterozygous mutants of GM2/GD2 synthase and those of GD3 synthase were mated, resulting in nine combinations of the genotypes. Eventually, Ho/Ho mutants were obtained at the approximate ratio of 1:16, similar to the Wd/Wd mutants, corresponding to the classic Mendelian rule, indicating that Ho/Ho mutants had no critical disadvantage in development and birth. They appeared somewhat normal and could not be distinguished from their littermates at birth.

Only GM3 Remained in Double Knock-out Mice—Brain, liver, and skin tissues were used for the extraction of glycolipids and exhibited dramatic changes in the compositions of gangliosides, i.e. all complex gangliosides were deleted, and only GM3 was found in brain extracts in the double mutants (Fig. 1B) as expected from the synthetic pathway (Fig. 1A). In the skin extracts GM3 was a major ganglioside, and an additional faint band of GD1a was present in Wd/Wd skin. Otherwise, no marked change was found in the extracts of Ho/Ho mice skin; only GM3 was detectable at similar levels during the course (Fig. 1C).

Skin Injury Observed at 25 Weeks Old—Approximately 35% of the 30-week-old mutant mice (Ho/Ho) showed, more or less, skin injury primarily on their faces as shown in Fig. 2, A and B, and the frequency increased up to 50% as they aged to 40 weeks old. In this situation, the injured mice showed characteristic scratching toward the injuries on their faces and necks (Fig. 2C). The frequency was about 90× per 5 min, whereas non-injured Ho/Ho and Wd/Wd mice scarcely exhibited this scratching movement (Fig. 2D).

Promotion of Skin Injury by Isolation of Mice into Metabolic Cages—It was discovered occasionally that the skin lesions were generated easily when mice were housed in special cages for the studies of metabolism in which they frequently underwent trauma to the face while taking foods through wire mesh (Fig. 3A). Therefore, we tried to house Ho/Ho and Wd/Wd mice in the metabolic cages under an isolated condition. Consequently, only Ho/Ho mice exhibited development of the skin injury earlier than they did in conventional cages (Fig. 3B). Wd/Wd mice showed no injury. Ho/Ho mice undergoing the skin injury showed a much higher frequency of scratching than Wd/Wd mice (Fig. 3C), suggesting that this scratching might induce the skin injury and its exacerbation.

Proliferation of Peripheral Nerves in the Skin Lesion—In the sections of the injured skin tissues, skin erosion and granulocyte infiltration were observed in some portions (Fig. 4A). A more characteristic feature in these lesions was proliferated peripheral nerves as shown in Fig. 4B. These hypertrophic nerves were stained with toluidine blue and were found frequently in the epidermis and subepidermis in Ho/Ho mice skin (Fig. 4, D and E) but rarely in Wd/Wd mice (Fig. 4C). A few cells with granules were found occasionally in Ho/Ho skin (Fig. 4D). To identify the nerve-like structures that appeared characteristically in the Ho/Ho mice skin, immunohistochemistry with anti-PGP9.5 antibody was performed. As shown in Fig. 4F, a large number of nerve fibers were found in the subepidermis and epidermis, and electron micrographs exhibited almost normal-shaped, slightly invaginated, myelinated, and unmyelinated nerve fibers (Fig. 4G).

Increased Area Occupied by Nerve Fibers in Ho/Ho Mouse Skin—As shown in Fig. 4H, measurement of nerve fiber-occupied area revealed that 6.0 ± 5.2% (as an average) of the skin was occupied by nerve fibers in the skin of the mutants with injury. In contrast, non-injured skin showed a minimal number of nerve fibers similar to those in Wd/Wd mice skin (1.3% ± 1.5%).

FIG. 5. Abnormal sensory function in Ho/Ho mutant mice. Sensitivity of sensory nerves was examined using two approaches. A, sensitivity to mechanical pain as measured with the von Frey test. The minimum intensity of mechanical stimuli (g, gram) that could induce mouse reaction was determined. B, sensitivity to hot stimuli as measured with hot plates (53 °C). The examination was performed as described under “Experimental Procedures” at least three times. The results from four mice are each presented as mean ± S.D. *, p < 0.05. Note that sensitivity to the mechanical stimuli was already reduced in 25-week-old Ho/Ho mutants.
Reduced Sensitivity to Mechanical Pain Stimuli in Ho/Ho Mice—To investigate the mechanisms for generation of the skin lesion in Ho/Ho mice, we examined the sensory nerve function with two methods using three mouse groups consisting of adolescent (20 weeks old), adult (35 weeks old), and intermediate (25 weeks old). Ho/Ho mice with no injury exhibited a clear reduction in sensitivity to mechanical pain stimuli at 25 weeks old and showed exacerbation at 35 weeks old (Fig. 5A), suggesting that their sensitivity was reduced markedly at a certain time point after 20 weeks and that this may induce over-scratching after the first triggering event. On the other hand, sensitivity to hot stimuli as measured with hot plates was enhanced rather significantly in Ho/Ho mice at 35 weeks old but not at 20 weeks old (Fig. 5B). This result suggests that the response to heat stimuli increased as a result of the injury and subsequent nerve fiber hyperplasia.

Degenerative Changes in Trigeminal and Sciatic Nerves in Relatively Young Ho/Ho Mutants—In observation of the cranial nerve (trigeminal nerve) and the peripheral nerve (sciatic nerve), strong degenerative changes were found even in the myelinated fibers of 20-week-old Ho/Ho mice. Nerve fibers of Ho/Ho mice showed an irregular array, swollen, beaded, and duplicate myelin appearances compared with those of Wd/Wd mice (Fig. 6, A and B). Degeneration in the sciatic nerves was more prominent (Fig. 6B, bottom panel). Width measurement of nerve fibers revealed that the diameter of fibers including myelin sheath in homozygotes generally was increased in both trigeminal and sciatic nerves compared with wild type (data not shown). The width of axon and myelin sheath also was significantly increased in homozygotes in both nerves as indicated in Fig. 6, C and D. However, the ratio of myelin sheath/axon was almost equivalent between homozygotes and wild type mice in both sciatic nerve and trigeminal nerve, suggesting that nerve fibers enlarged by a whole unit (Fig. 6E).
DISCUSSION

Although the double knock-out mice containing only the GM3 ganglioside were born and grew up almost normally at a glance, they started to die suddenly 12 weeks after birth. The accumulative survival rates were 92.2% after 10 weeks, 77.4% after 20 weeks, and 33.2% after 30 weeks of birth. The cause of death is not clear and may not be due to audiogenic convolution for these mutant mice in contrast with those reported previously (16).\(^3\) Namely, 12- and 18-week-old Ho/Ho mice showed no seizure when treated with various audiogenic stimulations such as the noise of key bundles (16) or sounds generated by a PA audiometer (Nagashima Medical Instruments Co., Nagoya, Japan) (21) at 200, 500, 2,000, 4,000, 8,000, 10,000, and 15,000 Hz (80–108 decibels) (data not shown). In surviving mice, serious skin injury was found in the significant population of Ho/Ho mice, which never has been observed in single mutant mice, e.g., GM2/GD2 synthase or GD3 synthase gene knock-out. Even with the isolation in metabolic cages, the single knock-out mice as well as the Wd/Wd mice did not show such serious injury, suggesting that the deletion of all ganglioside structures except for GM3 induced a novel defect in the skin. Because a definite difference in ganglioside composition in the double mutants from that of GM2/GD2 synthase gene knock-out mice (11) is the lack of GD3, this result indicates a significant role by at least one ganglioside (GD3). The reason why our Ho/Ho mice showed no audiogenic seizure yet exhibited rather prominent skin injury is not known. Genetic backgrounds of the embryonic stem cells used are different between the two groups, i.e., 129SvEv (TC-1) (16) and (CBA × B6)F1 (TT2) (11, 17), and seem to be a major factor for the phenotypic differences.

There were no complex gangliosides other than GM3 and a low level of GD1α on a thin layer chromatography of the extracted glycolipids from Wd/Wd mice skin. This pattern was similar to that of Ho/Ho mice skin, suggesting that the change in ganglioside composition in skin tissue might not be a primary factor to induce the serious skin injury. The amounts of GM3 in Ho/Ho mice showed no marked change in either brain or skin during observation, despite the progress of skin injury. We also focused on their movement to scratch the initially injured sites to observe whether it induced exacerbation of the wound. Particularly in the special cages, it was apparent that the scratching became more frequent as the injury advanced and was tightly associated with the development of refractory lesions.

In human atopic dermatitis, scratching the itchy sites was thought to induce serious dermatitis, and it frequently caused hypertrophy of peripheral nerve and hyperplasia of neural fiber bundles (22–24), leading to a high density of peripheral nerve fibers along with the progress of the disease. This bad cycle might be one of the bases for the refractory atopic skin inflammation (25). A similar situation may occur in the skin of Ho/Ho mice, i.e., the reduced sensation to mechanic pain might promote very frequent and repetitive scratching, and the resulting inflammatory processes might induce peripheral nerve hyperplasia.

Histological analysis of the sciatic nerve and trigeminal nerve revealed that peripheral nerves in Ho/Ho mutant mice underwent serious degeneration more prominently than those in the single mutants as reported previously (12).\(^4\) The reduced sensitivity to the mechanic pain observed in Ho/Ho mice with no injury might reflect these degenerative changes.

Of course, Ho/Ho mutants should have various defects other than those in peripheral nerves as expected from abnormal phenotypes such as immunodeficiency and endocrinological defects in the single mutant mice (14, 15). These factors might also be involved in the generation of the severe skin injury. However, it seems reasonable to consider that a defect in peripheral nerve regulation is one of the major causes of the skin injury. Furthermore, there have been no clear studies on the role of gangliosides in skin, although we reported previously that transgenic mice of the GM2/GD2 synthase gene showed a rather increased reaction to exogenous stimulation and a delay in healing (26). Therefore, this study demonstrates for the first time an important role of gangliosides in the regulation of skin integrity by manipulating ganglioside synthase genes.

For a long time, gangliosides have been thought to play an important role in the nervous system as a sort of neurotrophic factor (27, 28). Actually, neurite extension, synaptogenesis, or regeneration of damaged nerves was enhanced in the presence of gangliosides (29). If this is the case, the hyperplastic nerve fibers found in Ho/Ho mice skin seem to be a paradox. Could the deletion of all gangliosides except for GM3 result in increased neural regeneration? If so, might GM3 itself induce and promote neurogenesis? The hypertrophic neural fibers also might be an abnormal proliferative reaction as a result of deregulation after the deletion of complex gangliosides triggered by repetitive scratching and sustained inflammation. As yet, significant mechanisms for promotion of the proliferation of peripheral nerve fibers remain unknown. The sensitivity of neural tissues to neurotrophins, including expression levels of the neurotrophin receptors, might have changed. Neuropeptides (30, 31) or neurotrophins (32–34) from keratinocytes also may be responsible, as reported for atopic dermatitis. True molecular mechanisms remain to be investigated.

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REFERENCES

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