Specificity of carbohydrate structures of gangliosides in the activity to regenerate the rat axotomized hypoglossal nerve

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We previously reported that a ganglioside mixture from bovine brain could prevent neuronal death and promote regeneration in rats with hypoglossal nerve resection. In the present study, we have compared the neurotrophic effects of various glycosphingolipids including lactosyl-ceramide. The findings revealed that GT1b had the activity of neuronal death prevention equivalent to a ganglioside mixture or autograft, while other glycolipids exhibited about 60% activity. However, the capability to promote the regeneration varied among glycolipids, that is, GT1b (86%), GD1b (55%), GD1a (35%), GQ1b (34%), GM1 (20%), lactosyl-ceramide (17%) in the number of horseradish peroxidase-positive neurons as an indicator of regeneration. The experiments with oligosaccharides of GT1b or GD1b and ceramide showed that the carbohydrate moiety mainly exerts neurotrophic effects. These findings suggested that fine structures of carbohydrate moiety in gangliosides are critical in the regenerative activity in this hypoglossal nerve regeneration system.

Key words: gangliosides/hypoglossal nerve/nerve regeneration/ceramide/carbohydrate

Introduction

Glycosphingolipids have been thought to play important roles in the regulation of cellular signals by modulating the growth factor/growth factor receptors (Hakomori, 1990). In particular, the neurotrophic effects of acidic glycolipids, gangliosides, have been extensively studied, since they showed various effects on the differentiation and survival of cultured neuronal cells such as neurite extension and protection of apoptosis. They also exhibited marked protective effects on the damaged neuronal tissues. Although it has been difficult to quantitatively evaluate the neurotrophic action of gangliosides in vivo, the hypoglossal nerve regeneration system established is very useful for measuring the surviving neurons or regenerated axons after axotomy (Itoh et al., 1999). We have shown that bovine ganglioside mixture injected at the resection site could cause marked prevention of neuronal death and restore equivalent numbers of HRP-positive neurons at the brain stem to that by autograft.

In many studies, ganglioside GM1 has been used, for example, in experimental rat Parkinsonism (Schneider et al., 1992). This is because GM1 is one of the major gangliosides in vertebrate brain tissues (Suzuki, 1965). However, there have been no systematic studies on the neurotrophic effects of various glycosphingolipids, and no definite evidence to indicate that GM1 is a particularly excellent structure among ganglioside species.

In the present study, we compared the neurotrophic effects of various glycosphingolipids including neutral glycolipids using the rat hypoglossal nerve regeneration system. Then, we analyzed the effects of chemically synthesized gangliosides to eliminate the possibility that the effects were due to contaminants during purification. Furthermore, we compared the neurotrophic effects between the carbohydrate moiety and the lipid moiety to confirm the importance of the carbohydrate structures of gangliosides in their biological functions.

Results

Effects of carbohydrate moiety on the prevention of neuronal death

Since ganglioside mixture from bovine brain showed marked effects on the prevention of neuronal death and regeneration of the rat axotomized hypoglossal nerve (Itoh et al., 1999), we examined those neurotrophic effects using purified single species of gangliosides.

When the numbers of surviving neurons were examined, the majority of glycolipids examined showed fairly good effects as shown in Figure 1. Even LacCer treatment resulted in a neuron survival better than 60%, whereas the non-treated group showed about 15% survival. Significant differences were revealed in the number of surviving neurons between the untreated (5 mm resected group) and the autografted group or glycolipid-injected group (P < 0.005). As shown in Figure 3d, the majority of cell bodies on the right side (with the simple lesion) were lost after 10 weeks. The percent of the number of motor neurons in the right hypoglossal nerve (RHN) over the number in the left hypoglossal nerve (LHN) was 14.4 ± 5.3% (mean ± SD, n = 4). In contrast, the administration of any of the
Materials and methods

The hypoglossal nerve was resected and either treated with the indicated amounts of individual glycolipids or autografted. A solid bar at the left end is the sample of non-treatment (none), dark bars and light bars with names of gangliosides are of treated with 2 µg and 0.2 µg of gangliosides, respectively. A solid bar at the right end is of autografted. The values are means ± S.D. Rat numbers used are as shown in the Figure 1 caption. (B) Summary of P-values calculated as described in Materials and methods to indicate the significances in the individual combinations of treatments. The numbers of rats used were as follows; none (n = 4); LacCer, GM1 (0.2 µg), GD1a (0.2 µg), GD1b (0.2 µg and 2 µg), GD1b (0.2 µg, 2 µg), GT1b (0.2 µg, 2 µg) (n = 5); GQ1b (0.2 µg, 2 µg) (n = 6); G-mix (0.2 µg) (n = 4), (2 µg) (n = 5); autograft (n = 4).

glycolipids to the proximal stump of the nerve in which the lesion had been made resulted in the survival of 60–90% of motor neurons (Figure 1). Among glycolipids, GT1b showed an effect almost equivalent to those of the ganglioside mixture and autograft, and it was significantly better than those of LacCer (P < 0.005), GD1a (P < 0.005), GQ1b (P < 0.005), and GD1b (P < 0.05).

Effects of the carbohydrate moiety on nerve regeneration

The number of HRP-positive neurons was compared after the injection of HRP to analyze the effects on the nerve regeneration. As reported previously (Itoh et al., 1999), in the un-operated rats injected with HRP into the tongue, the number of HRP-positive neurons over that of cresyl violet-stained motor neurons in the RHN was 81.5 ± 4.9% (mean ± SD, n = 30). That in the LHN of operated rats was 80.6 ± 4.5% (mean ± SD, n = 13), and was not significantly different from that of the intact animals (data not shown). The percent of the number of HRP-positive motor neurons in the RHN over that in the LHN was 0% (n = 4) in the 5 mm-resected group, 85.1 ± 13.3% (mean ± SD, n = 4) in the autografted group, and 90.2 ± 5.5% (mean ± SD, n = 5) in the ganglioside-injected group (Figure 2). When compared based on the number of HRP-positive neurons (RHN/LHN), the differences in the effects among the gangliosides were more prominent than those in the number of surviving neurons as shown in Figure 1. GT1b showed the best effect among gangliosides examined (P < 0.005) at both 0.2 µg and 2 µg, and was almost equivalent to that of the ganglioside mixture and autograft. GD1b also showed a better effect than GM1 (P < 0.005) and GD1a (P < 0.005). LacCer showed much less effects than all gangliosides examined except for GM1 in this assay. Figure 3 showed examples of HRP-staining after injection of GT1b (a), GM1 (c), LacCer (b), and none (d).

Effects of chemically synthesized gangliosides

Then, we examined the effects of chemically synthesized gangliosides, to eliminate the possibility that contaminants in the ganglioside preparations did not exert the neurotrophic effects. Both synthetic GT1b and GD1b showed almost equivalent levels of effects in both surviving neuron numbers and HRP-positive neuron numbers (Figure 4).

Comparison between oligosaccharides and ceramides

These findings suggested that the carbohydrate structures in glycolipids were critical in their biological effects particularly for nerve regeneration. Therefore, we examined the effects of two moieties of gangliosides, that is, oligosaccharides and ceramides. Oligosaccharides of GT1b and GD1b exhibited significantly better effects than ceramide in both surviving neuron numbers and HRP-positive neuron numbers (P < 0.005) (Figure 5). Particularly in the number of surviving neurons, oligosaccharides of both GT1b and GD1b showed very good effects similar to those of corresponding native gangliosides (Figure 5A,C). However, the effects of oligosaccharides were slightly less than native gangliosides in the number of HRP-positive neuron (Figure 5B,D).

Ganglioside expression in hypoglossal nerve

To analyze the mechanisms for the neurotrophic effects of gangliosides, expression profiles of gangliosides in the peripheral portion of the rat hypoglossal nerve, and in the hypoglossal
nerve nuclei at the brain stem were examined. In immunohisto-
staining, only GM1 was definitely detected, and GM2 was
faintly stained in the peripheral region of the hypoglossal nerve
(Figure 6A). No clear staining of gangliosides could be
observed in the brain stem due to fragile tissue structure. Then,
ganglioside fractions from the brain stem and peripheral region
were analyzed in TLC. As shown in Figure 6B, major bands
corresponding to GM1 and minor bands of GM2 and GM3
were observed in both the nerve nuclei region and other
regions in the brain stem. A faint band just below GD1a was
not defined. This GM1-dominant profile in the peripheral and
nuclear regions of the hypoglossal nerve had no correlation
with effective ganglioside species in the resection/regeneration
experiments.

Fig. 3. Sections of the brain stem of rats at 10 weeks after the nerve resection.
The hypoglossal nucleus of the intact side is located on the left-hand side. The
hypoglossal nucleus containing the cell bodies of the neurons in which the
resections and treatments were performed are located on the right-hand side.
(a) GT1b-treated animal. Almost all cell bodies are seen, but about 85% of the
HRP-positive cell bodies on the right-hand side nucleus are present compared
with the un-operated side. (b) LacCer-injected animal. The number of
HRP-positive motor neurons on the operated side was markedly reduced (14%).
(c) GM1-injected animal. Note that HRP-positive cell number is almost
equivalent to that in B. (d) Control animal after resection of 5 mm nerve.
Almost all cell bodies on the side with the lesion were lost. Scale bar, 100 µm.

Fig. 4. Comparison of neurotrophic effects of native and chemically
synthesized gangliosides. (A) Numbers of surviving neurons 10 weeks after
operation with GT1b or GD1b injection. (B) Numbers of HRP-positive neurons
in mice treated with synthetic GT1b or GD1b. Synthetic GT1b and GD1b
exhibited equivalent effects to those with individual native compounds both in
the surviving neuron numbers and HRP-positive neurons. Number of rats used
in each group is 5 (n = 5). The values are means ± SD.

Fig. 5. Comparison of neurotrophic effects between carbohydrate moiety and
ceramide portion of gangliosides. The effects of whole GT1b (2 µg),
oligosaccharide of GT1b (2 µg) and ceramide (2 µg) were compared for the
number of surviving neurons (A) and HRP-positive neuron number (B). Ceramide exhibited significantly lower effects (P < 0.005) than the other two
preparations in both experiments. GT1b/oligo showed significantly poorer
effects (P < 0.01) compared with whole GT1b in the regeneration. Whole GD1b
and GD1b/oligo also showed much better effects than ceramide in both
experiments (P < 0.005) (C, D). GD1b/oligo also showed slightly lower effects
than whole GD1b in both the protection of neuronal death and the regeneration,
while they were not significantly different. The numbers of rats used were as
follows; GT1b and GD1b (n = 5), GT1b/oligo and GT1b/oligo (n = 7),
ceramide (n = 6). The values are means ± S.D. *, P < 0.005; **, P < 0.01.

Fig. 6. Expression of gangliosides in the hypoglossal nerve peripheral region
and brain stem nuclei. (A) Immunohistostaining of the peripheral region of the
rat hypoglossal nerve. Antibodies used and detection methods are as described
under Materials and methods. (B) Ganglioside composition in the brain stem.
1, a ganglioside standard from bovine brain. 2, a ganglioside fraction extracted
from the rat hypoglossal nerve nuclei. 3, a ganglioside fraction from the brain
stem after removal of the hypoglossal nerve nuclei region. 4, ganglioside fraction extracted from the peripheral hypoglossal nerve using 10 adult rats.
No definite bands can be seen, suggesting low amounts of gangliosides in this
material. The solvent used was chloroform/methanol/0.2 M CaCl₂, (60:35:8),
and resorcinol spray was performed for the detection of the bands.
Discussion

There have been many studies indicating a neurotrophic activity of gangliosides in in vivo and in vitro systems (Schengrund, 1990). Many of these studies were performed by adding gangliosides to the culture medium of neuronal cells (Ferrari et al., 1983, 1995; Favaron et al., 1988). In in vitro systems, gangliosides were directly administered to animals after generating artificial neurological damage or disorders by mechanical or chemical manipulation (Karpiaik and Mahadik, 1984), injection of toxic reagents (Schneider et al., 1992) or ischemic treatment (Karpiaik et al., 1986). Although ganglioside GM1 was frequently used in these studies, there have been no systematic studies to precisely compare the neurotrophic activity of individual structures of gangliosides and neutral glycolipids. In a study to compare neuritogenic and metabolic effects of individual gangliosides, GM1 produced the greatest morphologic response without changes in metabolism, while GT1b increased both parameters, that is, neurite extension and ornithine decarboxylase activity, suggesting that each ganglioside species has a specific target action in the stimulation of different trophic responses (Matta et al., 1986).

The present findings clearly showed that the carbohydrate structures of gangliosides are critical for the prevention of the lesion-induced death of motor neurons in the hypoglossal nerve nucleus, and in the promotion of the regeneration of the axotomized hypoglossal nerve. In particular, regeneration based on HRP-stained neurons in brain stem nuclei markedly varied depending on the glycolipid species. GT1b and GD1b showed better effects than GM1 or GD1a (representative structures of the a-series) in the regeneration of neurons (the number of HRP-positive neurons). GT1b, in particular, showed the best results among structures examined. GQ1b did not show so excellent effects as expected from a previous report (Tsujj et al., 1983). The relative importance of the carbohydrate structures in these compounds was also supported by the finding that oligosaccharides of GT1b or GD1b showed fairly marked effects, while ceramide did not. However, the presence of the 2-(trimethylsilyl)-ethyl structure in these oligo-saccharides may have some significant roles to give a similar physico-chemical property as ceramide in native glycosphingolipids.

In addition, the finding that the effects on the neuron survival rate and those on the frequency of HRP-positive cells were not necessarily identical among the purified single gangliosides suggested that the prevention of neuronal cell death and the enhancement of neuronal regeneration were achieved by different mechanisms, that is, the former effect was relatively universal to glycolipids and the latter was relatively specific to the restricted carbohydrate structures in gangliosides. The protective effects of polypeptide neurotrophins on the motoneuronal death were examined only by surviving neuron numbers (Sendtner et al., 1990, 1992; Oppenheim et al., 1992; Yan et al., 1992). Therefore, the enhancement of neuronal regeneration might be unique for gangliosides.

The mechanisms by which gangliosides prevented the death of hypoglossal neurons and promoted their regeneration are presently not known. However, gangliosides have been reported to potentiate the in vivo and in vitro effects of NGF on central cholinergic neurons (Cuello et al., 1989; Panni et al., 1998). Mutoh et al. and others reported that the function of a high-affinity NGF receptor (trkA) was markedly enhanced by the ganglioside GM1 (Mutoh et al., 1995; Rabin and Mocchetti, 1995). Recently, we demonstrated that introduction of the GD3 synthase gene into PC12 cells resulted in the continuous activation of NGF receptor TrkA and ERK1/2, leading to accelerated proliferation (Fukumoto et al., 2000). These findings indicated that both exogenous and endogenous gangliosides can modulate the neurotrophin family/neurotrophin receptor interactions regardless of their mechanisms. Moreover, the mRNA expression of neurotrophins and their receptors was differentially regulated after peripheral nerve axotomy (Frisen et al., 1993; Funakoshi et al., 1993). Thus, gangliosides administered at injury sites might alter the expression of these neurotrophic factors and receptors and/or directly modulate the functions of those receptors resulting in the activation of downstream kinases or up-regulation of protein kinase genes such as mitogen-activated protein kinases (Kiryu et al., 1995).

To clarify whether injected gangliosides were transported via axon to the brain stem, we tried immunodetection of GT1b after injection at the resection sites in GM2/GD2 synthase gene knock-out mice. We failed to detect GT1b (data not shown), which suggested that GT1b remained and exerted its role at the resection site, not at the brain stem.

The observations that motor neuron death can be prevented and the regeneration of an axotomized hypoglossal nerve can be promoted by exogenously added gangliosides may provide the basis for a novel therapeutic approach in the treatment of motor neuron injury, and also to the enhancement of the amelioration of other central and peripheral neuronal disorders (Svennerholm, 1994). These findings prompted us to analyze the influence of genetic disruption of complex gangliosides on the regeneration of cleaved hypoglossal nerve (Takamiya et al., 1996). These analyses should reveal the real significance of endogenously-synthesized gangliosides in the nervous system, and provide experimental systems for the analysis of molecular mechanisms of ganglioside actions.

Materials and methods

Gangliosides and other reagents

Gangliosides and lactosylceramide (LacCer) were purchased from Seikagaku Kogyo (Tokyo, Japan) (ganglioside mixture and GM1) and Sigma Chemical Co. (GD1b, GT1b, GQ1b, GD1a, LacCer). Ceramide (C2-ceramide, N-acetyl-D-sphingosine) was also purchased from Sigma. They were tested using thin layer chromatography before use to confirm purity and concentration. Chemical synthesis of gangliosides GT1b and GD1b were as described previously (Ishida et al., 1994). Oligosaccharides of GT1b and GD1b were also synthesized as trimethylsilyl-ethyl compounds as previously described (Ishida et al., 1994). Ganglioside nomenclature was according to that of Svennerholm (1963).

Resection of rat hypoglossal nerve

Experiments of rat hypoglossal nerve regeneration were performed as previously described (Itoh et al., 1999). Briefly, adult Wistar rats were anesthetized by an intraperitoneal injection of sodium pentobarbital, and a 5 mm segment of the right hypoglossal nerve (RHN) was removed (5 mm resected...
group. In the ganglioside-injected group, 2 µg (or 0.2 µg) ganglioside dissolved in phosphate-buffered saline (PBS) was injected into the nerve stump site. In the autografted group, the 5 mm sectioned hypoglossal nerve was placed back between the excised nerve stumps and sutured to the stump. Injection of synthetic gangliosides, neutral glycolipids, oligosaccharides and ceramides were also administered in a similar manner.

**HRP injection and counting of neurons at the hypoglossal nerve nucleus**

Ten weeks after these treatments, 20 µl of 30% horse radish peroxidase (HRP) (Toyobo, Osaka, Japan) in sterile saline was injected into the whole parts of the tongue of the rat as described previously (Streit and Reubi, 1977). After 24 h, the animals were anesthetized deeply and fixed by intracardial perfusion with 0.9% saline containing heparin-Na, then with 10% formalin in 0.1 M phosphate buffer. The lower brain stem was dissected, and 50 µm serial cross-sections were prepared on a freezing microtome. The sections were then incubated with a mixture of 3,3′-diaminobenzidine and hydrogen peroxide at room temperature for 40 min (Svennerholm, 1994), mounted on 3-aminopropyl-triethoxysilane (Aldrich, Milwaukee, WI)-coated glass slides, and counterstained with 1% cresyl violet (Chroma, Kongen, Germany). Only cells containing a clearly visible HRP vesicle in the cytoplasm were quantitated in every fifth section, as described previously (Taniuchi et al., 1986).

All these experimental protocols were approved by the Review Committee of Nagasaki University School of Dentistry, and met the guide-lines of the Japanese Governmental Agency. All efforts were made to minimize animal suffering to reduce the number of animals used, and to utilize alternatives to in vitro techniques, if available.

**Immunohistochemistry**

Expression of gangliosides in peripheral hypoglossal nerves and hypoglossal nerve nuclei in the brain stem was examined using monoclonal antibodies specific for individual ganglioside structures with frozen sections. Briefly, frozen sections were fixed in cold acetone at –20°C for 10 min, then washed in PBS 3 times at room temperature. After blocking with 1% bovine serum albumin in PBS for 1 h at room temperature, anti-ganglioside antibodies were added and incubated for 1 h at room temperature. After washing with PBS, antibody binding was detected with biotin-labeled second antibody and ABC methods (Vectastain). After incubation with DAB for 10 min, samples were washed and counter stained with 2% methyl green for 10 min. The antibodies used were as follows; R24 (GD3, 1:2000 of ascites), 3F8 (GD2, 1 µg/ml), 2590 (GM3 0.5 µg/ml), 10–11 (GM2, 1:200 of ascites), 370 (GD1b, 1:500 of ascites), 549 (GT1b, 1:200 of ascites). For GM1, biotin-conjugated cholera toxin B (List Biological Laboratories, Campbell, CA) and avidin-FITC (EY Laboratories, San Mateo, CA) were used.

**Statistical analysis**

The results obtained were analyzed for significance based on Tukey’s method (Stuart and Ord, 1991).

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**Abbreviations**

mAb, monoclonal antibody; RHN, right hypoglossal nerve; LHN, left hypoglossal nerve; SD, standard deviation; NGF, nerve growth factor; ERK, extracellular signal-regulated protein kinase(s); PBS, phosphate-buffered saline; LacCer, lactosylceramide; HRP, horse radish peroxidase.

**References**


129


