Impaired hypoglossal nerve regeneration in mutant mice lacking complex gangliosides: Down-regulation of neurotrophic factors and receptors as possible mechanisms

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Gangliosides, sialic acid-containing glycosphingolipids, have been considered to play roles as neurotrophic factors. Exogenous gangliosides added to the culture medium of neuronal cells or injected in artificially injured sites of nerve tissues actually showed neurotrophic factor-like effects such as neurite extension and alleviation of nerve tissue deterioration. In this study, neuroregeneration in the mutant mice lacking complex gangliosides was examined. To determine whether the nervous system maintains regenerative activity in the long-term absence of complex gangliosides, we analyzed hypoglossal nerve regeneration after axotomy in the mutant mice of GM2/GD2 synthase. These mice exhibited marked impairment of regenerative activity both in the number of surviving neurons and in the number of peroxidase-positive neurons. Moreover, reduced levels of gene expression of neurotrophic factors and their receptors including CNTF, p75 NTR, TrkB, and others in hypoglossal neurons were observed in real-time reverse transcription-polymerase chain reaction combined with laser capture microdissection, suggesting that these molecules are, at least partly, involved in the regeneration of lesioned nerves and that their expression levels are precisely controlled in the presence of intact expression of complex gangliosides.

Keywords: gangliosides/hypoglossal nerve/knockout/regeneration/neurotrophic factor

Introduction

Sialic acid-containing glycosphingolipids, gangliosides, are ubiquitously expressed in vertebrates and are enriched in the brain and spinal cord (Wiegandt 1985). Therefore, it has been considered that they play roles in the development of nervous systems (Suzuki 1965; Ledeen and Yu 1982). Various experiments in vitro and in vivo have suggested that they have neurotrophic factor-like activity (Schengrund 1990) although the mechanisms of their effects have not been clearly determined.

Recent progress in the isolation and characterization of glycosyltransferase genes has enabled direct examination of the biological functions of glycoconjugates in both cultured cells and experimental animals (Marth 1994; Furukawa et al. 2001). To clarify the roles of gangliosides in the nervous system, we have isolated glycosyltransferase cDNAs (Nagata et al. 1992; Haraguchi et al. 1994) and established mutant mice in which the GM2/GD2 synthase gene was disrupted, and which consequently lacked all complex gangliosides as expected from their synthetic pathway (see supplementary Figure 1) (Takamiya et al. 1996). However, analyses of the knockout (KO) mice revealed that ganglioside deficiency did not necessarily induce abnormal morphology and organogenesis of nervous systems, inconsistent with the expected functions of ganglioside (Takamiya et al. 1996). It has instead been demonstrated that complex gangliosides are of critical importance to the maintenance of the integrity of peripheral and central nerve tissues (Sheikh et al. 1999; Chiavegatto et al. 2000; Inoue et al. 2002; Sugiuira et al. 2005; Susuki et al. 2007).

Furthermore, the importance of gangliosides in the nerve repair was reported in the mutant mice lacking b-series gangliosides (Okada et al. 2002). Roles of gangliosides in axonal regeneration have also been reported (Lehmann et al. 2007). Although a number of experimental systems for the analysis of nerve regeneration have been available (Kariaki 1984; Kariaki and Mahadik 1984; Schneider et al. 1992), we generated a hypoglossal nerve clavage system in rats (Itoh et al. 1999, 2001) and recently one in mice as well (Okada et al. 2002). This system is relatively easy to produce and yields clear results enabling the evaluation of regenerative activity. In this, the addition of gangliosides, especially of GD1b and GT1b, very efficiently enhanced the regeneration of lesioned hypoglossal nerves (Itoh et al. 2001). Consistent with this, lack of b-series gangliosides induced by targeting of the GD3 synthase gene resulted in marked impairment of the regeneration of cleaved hypoglossal nerves (Okada et al. 2002).

To investigate whether the nervous system maintains regenerative activity in the long-term absence of complex gangliosides, we examined repair after the cleavage of the hypoglossal nerves in the mutant mice of GM2/GD2 synthase. In addition to degenerative changes in the nervous system, markedly impaired regenerative activity was found in the mutant mice lacking complex gangliosides. Moreover, reduced levels of gene expression of neurotrophic factors and their receptors in the hypoglossal neurons were also found, suggesting that these molecules are, at least partly, involved in the regeneration of lesioned nerves and that their expression is precisely controlled in the presence of intact expression of complex gangliosides.
Results

Impaired regeneration of lesioned hypoglossal nerves in mice lacking complex gangliosides

We examined the roles of endogenous gangliosides in the regeneration of axotomized nerve fibers. We used here a mouse line with knockout of the GM2/GD2 synthase gene (Takamiya et al. 1996). No complex gangliosides and only GM3 and GD3 remained in the KO mouse brain tissues (see supplementary Figure 1). Low levels of sialylated glycolipids belonging to lacto/neolacto series such as LM1 were present though their levels were very low (Takamiya et al. 1996).

In order to examine the time course of changes in surviving neurons and horseradish peroxidase (HRP)-positive neurons after hypoglossal nerve resection in the wild-type mice and the KO mice, animals were allowed to survive for 1–10 (or 20 when needed) weeks after surgical treatment. HRP-positive neurons, which were detected by adding the HRP substrate 3,3′-diaminobenzidine, indicated restoration of transport of HRP injected in the tongue.

In the wild-type mice, there was no neuronal atrophy or cell death after the lesion induction (Figure 1). On the other hand, there was definite neuronal atrophy and apparent cell death in the KO mice. Compared to the wild type, the mutant mice had significantly lower ratios of surviving neurons. At 6 weeks after procedure, there were numerous surviving neurons (almost 100%) in the right hypoglossal nuclei (RHN) in the wild-type mice. On the other hand, approximately 60% of neurons in the heterozygotic KO mice survived, and approximately 35% in the homozygotes, compared to numbers of neurons in the intact light hypoglossal nuclei (LHN) (noncleaved side) (Figure 1).

In this study, we mainly examined HRP-positive neurons, since this staining indicates restoration of connection of the cleaved hypoglossal nerves between the tongue and the proximal region. The wild type exhibited restoration up to 90% of the level of the untreated side. In contrast, the mutant mice exhibited less restoration (below 30%) and even heterozygotes exhibited less than 50% HRP-positive neurons (Figure 1B).

Typical patterns of HRP staining are shown in Figure 3. In the Tg mice, in which GM2/GD2 synthase was overexpressed, there was significant reduction both in the ratio of surviving neurons and in HRP-positive neurons (Figure 2). The intensity of injury found in Tg was similar to that in the heterozygotic KO mice. Actual findings for HRP-positive cells for a Tg mouse line of GM2/GD2 synthase are shown in Figure 3B.

At 10 weeks after the surgical procedure, the percentage number of surviving motor neurons in the RHN compared with the LHN was approximately 96% (n = 5) in the wild type, 38% (n = 6) in the knockout (homozygotic), and 60% (n = 5) in the heterozygotic KO mice (Figure 1A). There were significant differences between the wild type and other mutant lines in this parameter (P < 0.01) (Figures 1 and 3). Significant difference was also found between the homozygotes and heterozygotes (P < 0.01). In the case of GD3 synthase KO mice, the proportion of surviving motor neurons was approximately 50% (n = 5) in the knockout (homozygotic) and 72% (n = 5) in the heterozygotic mice (Okada et al. 2002).

Percentage number of HRP-positive neurons in the RHN compared to the LHN at 10 weeks after the treatment was 94% (n = 5) in the wild-type, 29% (n = 6) in the homozygotic, and 47% (n = 5) in the heterozygotic KO mice. There were significant differences between the wild type and the other groups in this parameter (P < 0.01) (Figure 3). In the case of GD3 synthase KO mice, the proportion of HRP-positive neurons was 43% (n = 5) in the homozygotes and 53% (n = 5) in the heterozygotes (Okada et al. 2002), indicating that complex ganglioside-lacking mice were much worse in the regenerative activity than the mutants lacking b-series gangliosides.

Restoration of nerve regeneration by injecting GT1b at the cleavage site

In order to examine the effects of exogenous gangliosides on the regeneration of cleaved hypoglossal nerves in the KO mice, GT1b was injected at the cleavage site at different doses (0.002–2.0 μg/mouse). As shown in Figure 4C, the...
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Fig. 2. Time course of change in numbers of surviving neurons and HRP-positive neurons after resection of the hypoglossal nerves in the Tg mice. (A) Numbers of the surviving neurons in the hypoglossal nuclei were counted at the time points indicated and presented as percent ratios to those on the untreated side in mean values ± SD. Wt, wild type and Tg, transgenic mice. (B) Numbers of HRP-positive neurons in the nuclei were also counted at the time points and presented as in A. Numbers of mice examined were (wild-type n = 6 and Tg n = 6).

addition of GT1b resulted in the marked improvement of regeneration both in the number of surviving neurons and in the number of HRP-positive neurons (result for 0.2 µg injection are shown). In order to determine whether injected GT1b was transferred to the nuclei at the brain stem, immunohistological analysis was performed using an anti-GT1b mAb. GT1b-positive neurons were definitely detected only on the side of injection (Figure 4D). These results are summarized in Figure 4E and F, which show that injection of GT1b yielded marked enhancement of the repair of cleaved nerves and restored regeneration to levels nearly equivalent to those in the wild-type mice.

Fig. 3. Suppressed regeneration of the hypoglossal nerve after the cleavage. (A–D) Pictures of the hypoglossal nerve nuclei at 10 weeks after the procedure. Sections of the hypoglossal nerve nuclei were stained with the substrate of HRP. (A) Wild-type mouse (Wt), (B) GM2/GD2 synthase transgenic mouse (Tg), (C) heterozygote (Ht), and (D) homozygote (Ho). Representative samples are shown. Bar indicates 100 µm. (E and F) Summary of the numbers of surviving neurons and HRP-positive neurons in the hypoglossal nuclei. Individual groups of mice were examined as shown in Figure 1 and results at 10 weeks after the procedure are presented. (E) Percent ratios of surviving neurons (RHN/LHN) are shown. (F) Percent ratios of HRP-positive neurons (RHN/LHN) are shown. *P < 0.01.
cells were demonstrated by the substrate 3,3′-diaminobenzidine and H2O2.

Changes in levels of gene expression in the mutant mice as determined by laser capture microdissection (LCM) and real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine changes in levels of expression of mRNAs for representative neurotrophic factors and their receptors, neurons in the hypoglossal nuclei were collected with LCM. The yields of RNA from approximately 500 neurons were approximately 2–10 ng. These RNAs were used for reverse transcription and the products were used for quantitative real time RT-PCR analysis (see supplementary Figure 2).

Real time RT-PCR revealed that mRNA levels of a ciliary neurotrophic factor (CNTF), p75NTR, and TrkB were significantly down-regulated in the KO mice (Figure 5). mRNAs of the brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) also exhibited tendencies toward down-regulation in the KO mice although they were not statistically significant. In the case of the IL-6 signal transducer gp130 gene, mRNA expression was instead increased, though not to a significantly extent, either. Other neurotrophic factors and receptors including the nerve growth factor (NGF), trkA, leukemia inhibitory factor (LIF), NT-3, NT-4/5, and others were not evalu-

able due to excessively low levels of gene expression (data not shown). IL-6 mRNA was also undetectable in all mouse samples (data not shown).

Discussion

Although there have been a number of reports on the neurotrophic factor-like activity of gangliosides based on in vitro and in vivo experiments (Schengrund 1990), the mechanisms of the neurotrophic effects of gangliosides have not been clearly demonstrated. There have been reports on the enhancement of signaling via NGF and its receptor TrkA with GM1 (Mutoh et al. 1995) and the enhancement of the signaling pathway of BDNF and TrkB with GM1 (Pitto et al. 1998). These findings were obtained by using cultured cell lines and exogenously added gangliosides, making interpretation of them difficult. Our own findings (Fukumoto et al. 2000; Nishio et al. 2004) make it seem more likely that gangliosides regulate the structures and functions of cis-existing molecules such as receptors on the cell membrane than that they function as neurotrophic factors.

Whatever the mechanisms of their effects are, the findings that gangliosides enhanced neurite extension, protected against neuronal cell death induced by the withdrawal of neurotrophic factors, and alleviated nervous tissue damage after ischemia and/or traumatic insult, have strongly suggested their importance in the maintenance of the nervous tissue integrity. It has correspondingly been thought that lack of gangliosides might induce neuronal degeneration and reduce regeneration after artificial injury.

Reduced regeneration in the complex ganglioside-lacking mutant mice was expected based on a previous report on GD3 synthase KO mice (Okada et al. 2002), in which only b-series gangliosides were deficient. As expected, the impairment of neuronal survival and regeneration rate in the mice lacking complex gangliosides was much worse than in the mutants lacking b-series gangliosides. These findings suggest that not only b-series gangliosides are effective in enhancing regeneration, as shown in the rat system (Itoh et al. 2001), but also that the remaining glycolipids including a-series and asialo-series glycolipids in the GD3 synthase-disrupted mice might compensate for the defects due to the lack of b-series gangliosides. Lack of all complex gangliosides thus resulted in much more serious impairment of neuronal maintenance and regeneration. These findings also suggest that GD3, a major remaining acidic glycolipid in GM2/GD2 synthase KO mice, might not be strongly effective in compensating for the lack of a-series and asialo-series gangliosides (supplementary Figure 1).

On the other hand, the finding that GM2/GD2 synthase Tg mice also exhibited abnormality in hypoglossal nerve regeneration to almost the same extent as the heterozygous KO mice did suggested that precise regulation of ganglioside composition is required for the optimal maintenance of the regeneration activity.

Mechanisms of impairment of integrity and reduction of regeneration have never been determined in any ganglioside synthase-KO mice. Based on the findings obtained from glycosylation-remodeling cells with the manipulation of glycosyltransferase cDNAs (Fukumoto et al. 2000; Nishio et al. 2004), it can be expected that fundamental membrane structures are

Fig. 4. Restoration of nerve regeneration impaired in the KO mice with GT1b.

To examine the effects of GT1b on nerve regeneration in GM2/GD2 synthase knockout mice, GT1b was injected at the site of operation and HRP-positive soma were used for quantitative real time RT-PCR analysis (RT-PCR) and real-time reverse transcription-polymerase chain reaction (LCM and RT-PCR).

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Fig. 5. Results of real time RT-PCR. Relative levels of expression of individual mRNAs were measured according to the manufacturer’s protocol. Relative mRNA levels as determined by real time RT-PCR are presented using GAPDH as a control. Open squares represent wild-type samples and closed squares knockout samples. (A) CNTF, (B) p75NTR, (C) TrkB, (D) BDNF, (E) GDNF, (F) TrkC, (G) GFRα1, (H) CNTFR, and (I) LIFR. Results are presented as mean ± SD, and sample numbers used are n = 3 (Wt) and n = 4 (H0).

abnormal in the mutant mice. In particular, the microdomain structures in the cell membrane might undergo serious injury, leading to insufficient transduction of cell signaling required to maintain intact cell conditions. The findings of this study indicated that levels of expression of mRNAs for multiple neurotrophic factors and their receptors were significantly suppressed in the hypoglossal nerve nuclei as determined by LCM/RT-PCR (Figure 5). These molecules have been reported to be involved in the activation or differentiation of neuronal cells in various developmental and pathological situations. Furthermore, many of them undergo up-regulation during the nerve regeneration that occurs after surgical lesions. For example, after axotomy of sciatric nerves, marked increase in levels of NGF and BDNF was observed (Meyer et al. 1992). Levels of ciliary neurotrophic factor decreased once after axotomy, but returned to normal during regeneration (Smith et al. 1993). In cases of hypoglossal nerve axotomy, GDNF receptor and c-ret were up-regulated in the motor neurons of hypoglossal nerves of an adult rat (Tsujino et al. 1999). Essential roles of BDNF and NT-4/5 were demonstrated by using allografts from BDNF or NT-4/5 KO mice (English et al. 2005). It has also been reported that the expression of GDNF receptor is of crucial importance to the determination of the fate of injured motor neurons (Honma et al. 2002). Moreover, effects of exogenous administration of these neurotrophic factors on the regeneration of lesioned nerves have been widely investigated with fairly good results (Chiu et al. 1994; Tusznyski et al. 1996; Wang et al. 1997; Barras et al. 2002; Zhang et al. 2004). Effects of gene transfer of neurotrophic factors into the lesioned sites on the regeneration have also been reported (Blesch and Tusznyski 2001; Barati et al. 2006). In addition, transgenic mice expressing endogenous chimeric neurotrophins in the lesioned sciatric nerve exhibited accelerated nerve regeneration (Funakoshi et al. 1998). It thus seems reasonable that the suppression of mRNA levels of these genes should be responsible, at least in part, for the impaired regeneration of lesioned hypoglossal nerves in our mutant mice. Since both anterograde and retrograde axonal transport of GDNF and GDNF receptors have been reported (Russell et al. 2000), the determination of the levels of gene expression of critical neurotrophic factors and their receptors in the hypoglossal nerve nuclei in the brain stem is important. The mechanisms of down-regulation of these neurotrophins and their receptors in the absence of complex gangliosides remain to be investigated.

It might seem possible that the neurodegeneration in ganglioside-lacking mutant mice we observed is due to toxic effects of accumulated glycolipids in the individual KO mice. However, this possibility is ruled out by the findings that the double KO mice of GM2/GD2 synthase and GM3 synthase (Yamashita et al. 2005) exhibited much worse phenotypes than did those doubly knocked out for GM2/GD2 synthase and GD3 synthase (Inoue et al. 2002) and those knocked out for GM2/GD2 synthase (Tsujino et al. 1999), indicating that the accumulated GM3 or GM3 and GD3 instead compensate for the defects due to gene disruption and do not injure nervous tissues, respectively.

In this study, we used LCM to obtain a relatively pure population of hypoglossal neurons in the nuclei. Although the recovery of RNA was very low, we could easily dissect areas where neurons were concentrated, enabling accurate analysis of mRNA expression levels in relatively confined cell populations. Use of a rapid procedure for slice staining, laser cutting, and RNA extraction might be critical to obtaining highly reproducible results.

In this study, we focused on the gene expression of known neurotrophic factors and their receptors. It is of interest to
examine whether CNTF injection or p75NTR and TrkB cDNA transfection as well as cDNA expression vectors for various ganglioside synthase genes into sites of injury can restore regeneration. However, other factors may be involved in the regeneration of lesioned nerves. Comparison of gene expression profiles in the hypoglossal nerve nuclei between the wild-type and the mutant mice, and also before and after nerve cleavage is important in identifying molecules of critical importance in nerve regeneration, which should usually play pivotal roles in the presence of full expression of gangliosides.

Materials and methods

KO mice and transgenic mice
Mutant mice lacking complex gangliosides based on the knock-out of the GM2/GD2 synthase gene with the insertion of the PGK neo gene into the exon 4 (Takamiya et al. 1996) were used. The targeting procedures, genetic typing with PCR and Southern blot analysis were as described (Takamiya et al. 1996). The genetic backgrounds of these mutant mice were as described previously (Inoue et al. 2002). Usually, heterozygous male and female mice were mated, and PCR screening was performed for genotyping. Litter mates were used as controls. Wild-type and homozygous mutant mice 8–11 weeks of age were used in this study. The transgenic mice of the GM2/GD2 synthase were as reported previously (Fukumoto et al. 1997).

Unless otherwise indicated, 4–6 mice per group were used for individual analyses. All experiments with mice were approved by the Animal Experimental Committee of Nagoya University School of Medicine and were consistent with the guidelines of the Japanese government (MEXT, Ministry of Education, Culture, Sports, Science and Technology).

Hypoglossal nerve regeneration assay
The regeneration of axotomized hypoglossal nerves was examined as described by Itoh et al. (1999). Briefly, mice were anesthetized by sodium pentobarbital, and the right hypoglossal nerve was cleaved. Skin at the submandibular site was cut and connective tissues were dissected to demarcate hypoglossal nerves (both sides), and the nerve on the right side was cleaved at the mid point of the exposed portion. At 1–10 (or 20 when needed) weeks after the treatment, 20 µL of 30% HRP (Toyobo, Osaka) in saline was injected into the tongue. Then, mice were perfused intracardially with heparin-Na/saline, and fixed with 10% formalin in a 0.1 M phosphate buffer. The lower brain stem containing the hypoglossal nerve nuclei was dissected, and 50 µm serial cross-sections were prepared and then stained by adding 3,3’-diaminobenzidine and H2O2. To confirm the identity of the hypoglossal nerve, real-time RT-PCR was performed for the β3-tubulin gene (a neuron specific marker), with a sufficiently high level of expression observed (data not shown).

Injection and immuno-histostaining of GT1b
Effects of GT1b injection into the site of resection of the hypoglossal nerves were examined as described previously (Itoh et al. 2001). Detection of GT1b at the hypoglossal nerve nuclei was also performed as described previously (Itoh et al. 2001). Monoclonal antibody 549 was used as an anti-GT1b probe (Okada et al. 2002).

Laser capture microdissection (LCM)
LCM was performed using the PALM™ system (Zeiss, Germany). Frozen tissue sections containing hypoglossal nerve nuclei were embedded into OCT compounds and stocked at −80°C. Slice sections were prepared at 7 µm thickness using a CryotomeTM (CM3050S, Leica, Nussloch, Germany) with mounting on P.A.L.M. Slide™ (Zeiss). The slides were treated by RNase zap™ (Ambion, Austin, TX), and rinsed with DEPC water, dried at 37°C, and then UV-irradiated. Samples mounted on the slide glasses were stored at −80°C.

The cryosections were fixed and stained by treatment with 75% ethanol (30 s), distilled water (DW) (30 s), hematoxylin (5 min), 75% ethanol (30 s), 95% ethanol (30 s), 100% ethanol (30 s), and xylene (10–20 min). The sections were dissected using LCM. Hypoglossal nerve nuclei were identified under microscopy, and nuclei containing approximately 50–100 neurons were encircled with laser scissors and then laser-scraped. Every aliquot of slices containing about 500 neurons was subjected to RNA extraction using the Nanoprep kit™ (Stratagene, La Jolla, CA), and extracted RNA was measured for the RNA concentration and quality with the RiboGreen RNA Quantitation kit™ (Molecular Probes, Eugene, OR). The RNAs were converted to cDNA using the Sensiscript kit™ (Qiagen, Hilden, Germany) and stored at −80°C until used for real time RT-PCR.

Real time RT-PCR
RT-PCR was performed using 5 µL of 25 µL RT reaction products, with F-400 in the SYBR green qPCR kit™ (Finnzyme, Espoo, Finland) and Thermal Cycler PTC-200 (MJ Research and subsequently BioRad, Hercules, CA). The RCR conditions were as follows, preheating at 95°C for 10 min and 40 cycles of 95°C (10 s), 60°C (20 s), and 72°C (20 s). Plate read was set at 78°C or 75°C (2 s) depending on individual primer pairs. For melting curve analysis, dissociation temperature was measured every 1 s (0.2°C) starting from 72°C up to 95°C, and finally 7 min incubation was performed. For the internal control in the quantitative analysis, murine glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) was used. Every sample was measured in duplicate, and the gene expression levels were calculated by the 2−ΔΔCt method as described previously (Livak and Schmittgen 2001).

Primers
Primers used for real time RT-PCR were designed according to Primer 3 Input™ (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as shown in supplementary Table 1.

Statistics
Values obtained in the experiments were examined for significance with Student’s t-test. When P values were less than 0.05, they were considered significant.

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.
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Conflict of interest statement.
None declared.

Abbreviations
BDNF, brain-derived neurotrophic factor;CNTF, ciliary neurotrophic factor;GAPDH, glyceraldehyde-3-phosphate dehydrogenase;GDNF, glial cell line-derived neurotrophic factor;HRP, horseradish peroxidase;KO, knockout;LCM, laser capture microdissection;LHN, left hypoglossal nuclei;LIF(R), leukemia inhibitory factor (receptor);NGF, nerve growth factor;RHN, right hypoglossal nuclei;RT-PCR, reverse transcription-polymerase chain reaction;Tg, transgenic.

References
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