GM1 Reduces Injury-Induced Metabolic Deficits and Degeneration in the Rat Optic Nerve

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This study demonstrates the earliest reported effects of GM1 treatment on crush-injured axons of the mammalian optic nerve. GM1, administered intraperitoneally immediately after injury, was found to reduce the injury-induced metabolic deficit in nerve activity within 2 hr of injury, as measured by changes in the nicotine-amine adenine dinucleotide redox state. After 4 wk, transmission electron microscopy 1 mm distal to the site of injury revealed a sevenfold increase in axonal survival in GM1-treated compared to untreated injured nerves. These results emphasize the beneficial effect of GM1 on injured optic nerves as well as the correlation between immediate and long-term consequences of the injury. Thus, these results have implications for treating damaged optic nerves. Invest Ophthalmol Vis Sci 33:3586–3591, 1992.

Injury of mammalian optic nerves leads to axonal degeneration followed by a loss of retinal ganglion cells, with failure of axonal regrowth. Initial degeneration of the injured nerve probably results from direct neuronal damage. The associated physiologic and biochemical events that occur in the nerve immediately after injury probably are responsible for the subsequent degeneration, not only of directly injured axons but also of those that escaped the primary damage. These events may contribute significantly to the long-term functional outcome. Moreover, the events that take place at the site of the injury eventually determine the environment encountered by the regrowing axons. This presumably will influence the ability of the injured nerve to regenerate.

The purpose of the present study was to examine the ability of GM1 to attenuate early injury-induced deficits in nerve function and hence the long-term morphologic outcome of injury. Accordingly, we examined the effect of GM1 on early post-traumatic metabolic changes occurring at the injury site as well as on the subsequent axonal morphology. GM1 was chosen based on reports of its effectiveness in vivo at reducing injury-induced behavioral and functional deficits, preventing anterograde and retrograde degeneration, and reducing tissue edema after brain ischemia.

Metabolic activity was monitored before and after a well-controlled crush injury was inflicted on the adult rat optic nerve. Changes in metabolic activity were recorded with the nicotine-amine adenine dinucleotide (NADH) monitoring technique, which we developed for its use in the optic nerve (Yoles E, Mayevsky A, and Schwartz M, unpublished results). Transmission electron microscopy was used to assess morphology 4 wk after injury. The results provide evidence that GM1 reduces the early injury-induced deficit in the nerve’s metabolic activity and attenuates subsequent axonal degeneration.

Materials and Methods

Metabolic Studies

Animal preparation: Animals were used according to the ARVO Resolution on the Use of Animals in Research. Male Sprague-Dawley rats weighing 300–400 g were anesthetized with sodium pentobarbitone (35 mg/kg intraperitoneally). A cannula was introduced into the trachea for artificial ventilation, when required. With the animal’s head held in place by a head holder, a lateral canthotomy was performed under a binocular operating microscope and the conjunctiva was incised lateral to the cornea. After the retractor bulbi muscles were separated, the optic nerve was identified and 3–3.5 mm was exposed near the eyeball by blunt dissection. The dura was left intact and care was taken not to injure the nerve. The first part of a light guide holder was inserted under the optic nerve and the nerve was gently eased into the light guide canal. The second part then was fixed in place so that the light guide was located on the surface.
of the optic nerve 1 mm from the site of injury (Fig. 1A).

Surface fluometry-reflectometry: Monitoring of the intramitochondrial NADH redox state is based on the fact that NADH, unlike the oxidized form NAD+, fluoresces when illuminated by light at 366 nm, resulting in the emission of blue light with peak emission at 450 nm. The source of the 366 nm excitation light was a 100 W air-cooled mercury lamp equipped with a strong 366 nm filter (Corning 5860 [7-37] plus 9782 [4-96]). A flexible Y-shaped bundle of optic fibers (light guide) was used to transmit the light to and from the optic nerve, making in vivo measurements technically feasible. Excitation light (366 nm) was transmitted through the bundle of excitation fibers to the nerve. The light emitted from the nerve, after being transmitted through a second bundle of fibers, was split in a ratio of 90:10 for measuring the fluorescent light (90%) at 450 nm and the reflected light (10%) at 366 nm by two photomultipliers connected to a one-channel direct current fluorometer-reflectometer. To minimize variations among animals, standard signal calibration procedures were applied at the start of the recordings, as described in detail previously. Changes in the fluorescence and reflectance signals during the experiment were calculated relative to the calibrated signals. This type of calibration, although not absolute, nevertheless has been found to yield reliable and reproducible results from various animals and among different laboratories.

Changes in the reflected light were correlated with changes in tissue absorption caused by hemodynamic effects and movements of the optic nerve secondary to alterations in arterial blood pressure and nerve volume. The fluorescence measurements were found to be adequately corrected for NADH redox state measurements by subtracting the reflected light (366 nm) from the fluorescent light (1:1 ratio) to obtain the corrected fluorescence signal (Yoles E, Mayevsky A, and Schwartz M, unpublished results).

Light guide holder: Figure 1B illustrates the two-part light guide holder. The first part of the holder consisted of a hook-shaped spatula that had a canal for the optic nerve. The second part of the holder was used to hold the light guide constantly above the nerve, so the recordings would not be affected by the animal's movements. The two parts were connected tightly by a screw. This device ensured that the optical recordings were only from the optic nerve and not from the surrounding tissues. The hole in the second part of the holder, serving as the light guide cannula, was located directly above the canal in which the nerve lay.

Metabolic measurements: Animals, still anesthetized, were allowed to recover for 30 min from the surgical procedures and then were exposed to anoxic and hyperoxic conditions. An anoxic state was achieved by having the rat breathe in an atmosphere of 100% nitrogen for 2 min, after which it was returned to air. Whenever animals did not return spontaneously to normal breathing, we ventilated them by blowing twice via the trachea. A hyperoxic state was induced by having the animal breathe 100% O2 for 6–10 min. To evaluate the metabolic activity of the optic nerve, the relative changes in reflected and fluorescent light intensities in response to anoxia and to hyperoxia were measured before and after crush injury.

Fig. 1. Schematic representation of the experimental set-up for monitoring the metabolic activity of the rat optic nerve. (a) One-channel direct current fluorometer/reflectometer connected to the rat optic nerve (ON). A flexible fiber optic bundle (light guide) was inserted into the implanted experimental device and used for the in vivo monitoring of the nerve's metabolic activity, OC, optic chiasm. OD, optic disk. (b) Side view of the two parts of the experimental device used for surface optical measurements from the intact rat optic nerve. 1, screw that keeps the parts tightly connected. 2, light guide holder. The hole in the middle serves as the light guide cannula and is located directly above the canal in which the nerve lies. 3, hook-shaped spatula with a canal for the optic nerve. 4, optic nerve.
the eye and the light guide holder at a pressure corresponding to 120 g for 30 sec, as previously described.\textsuperscript{21} In control groups (13 animals), phosphate-buffered saline (PBS) was injected immediately after injury. In the experimental groups (six animals), GM1 (30 mg/kg; Fidia, Abano Terme, Italy) was injected. All injections were intraperitoneal, because injections via this route are followed by detection of GM1 in the brain.\textsuperscript{22} Metabolic activity before injury was measured in all nerves.

**Morphologic Studies**

**Animal preparation:** Rats were anesthetized by intraperitoneal injections of ketamine (50 mg/kg) and xylazine (0.5 ml/kg of 2% solution). These drugs were chosen because anesthesia was required for only a short period. The nerve was exposed as described above. A well-controlled crush injury then was inflicted between 1 and 2 mm from the optic nerve origin at the sclera, with the aid of a previously calibrated cross-action forceps.\textsuperscript{21} The force generated by the forceps corresponded to 120 g for 30 sec, resulting in a moderate crush lesion, as recently described in detail.\textsuperscript{21}

**Experimental protocol for morphologic studies:** In the control groups (six animals), PBS was injected immediately after injury. In the experimental groups (seven animals), GM1 (30 mg/kg; Fidia) was injected immediately after injury and then for 7 days. All injections were administered intraperitoneally.

**Morphological analysis:** Four weeks after injury, transmission electron microscopy was used to compare the number of axons still viable 1 mm distal to the injury site, in GM1-treated and untreated injured nerves.

With the animals under anesthesia, the nerves were exposed and immersed in situ in fresh Karnovsky fixative (1% paraformaldehyde, 2% glutaraldehyde in Na-cacodylate buffer 0.1 mol/l, pH 7.4, containing 0.25 mmol/l CaCl\textsubscript{2}). About 15 min later, the nerve, from the optic disc to the chiasm, was removed and immersed in the same fixative overnight at 4°C. After 24 hr, the nerves were thoroughly washed with Na-cacodylate buffer, immersed in 7% agar, cooled, and cut into 1 mm fragments in a way that the orientation of the fragments was maintained. The fragments were further osmicated (in 1% OsO\textsubscript{4} in the same buffer), stained en bloc with aqueous 2% uranyl acetate for 30 min, dehydrated in serially graded ethanol solutions, followed by propylene-oxide, embedded in Polybed 812 (Polysciences Inc., Warrington, PA), and cured at 60°C for 2 days. Thin sections were taken from the fragment located 1 mm distally from the injury site, placed on no. 300 copper grids, and analyzed grid square by grid square. Photographs were taken from each grid square at a magnification of ×3600 in a Philips 410 electron microscope. Viable axons were counted in each micrograph. The number of viable axons in each nerve section then was calculated according to the ratio between the area covered by one grid square and the area covered by one micrograph. Axons were considered viable if their cytoplasm was lucent, their microtubules and neurofilaments were well-defined, and their myelin sheath was preserved.

**Results**

After axonal injury, a reduction (about 50%) in NADH fluorescence in response to anoxia was observed (Fig. 2), reflecting an injury-induced decrease in the metabolic activity of the nerve (Yoles E, Mayevsky A, and Schwartz M, unpublished results). In the intact nerve, the mean increase in NADH fluorescence in response to anoxia was 4.38 ± 0.65% (n = 13), whereas after injury (1 hr) the NADH response to anoxia was reduced by 50.6%—ie, the mean increase in NADH fluorescence after anoxia was 2.25 ± 0.45% (n = 13). The difference is statistically significant according to a paired t-test (DF = 12, paired t = 6.48, P < 10\textsuperscript{-4}). At this time—ie, 1 hr after injury,
no effect of GM1 administration was detectable. Injured untreated nerves (four nerves) and injured GM1-treated nerves (six nerves) were followed for 4 hr after injury. With increasing time after injury and GM1 administration, a significant improvement in the GM1-treated animals was observed. As shown in Figure 2, by about 2 hr after injury, the initial reduction in NADH fluorescence was partially compensated for by the post-injury administration of GM1. Compensation by GM1 for the injury-induced reduction of metabolic activity also was manifested by the total range of activity from the anoxic to the hyperoxic state, which, after injury and without treatment, was reduced from 10.6 to 8.7%, whereas after injury and GM1 treatment, it was reduced only to 9.7%.

Analysis of variance showed a positive interaction between the treatment effect and the time elapsed after treatment ($F = 7.79; P < 0.0008$). Therefore, the time effect in each group also was analyzed. In the GM1-treated group, the response to anoxia was found to increase with time ($F = 12.62; P < 0.0002$). In untreated injured nerves ($n = 4$), there was no significant change with time beyond that observed within 1 hr after injury ($F = 1.51; P > 0.2764$).

This early partial compensation for the injury-induced deficit resulting from treatment with GM1 prompted us to examine later morphologic manifestations. Four weeks after injury, the excised nerves from GM1-treated and untreated injured animals were processed for electron microscopy. Representative micrographs from experimental and control nerves 1 mm from the site of injury (Fig. 2) demonstrate the higher rates of axonal survival in the GM1-treated compared to untreated injured nerves.

As shown in Table 1, the mean number of viable axons counted in thin sections of the GM1-treated injured nerves was sevenfold higher than in untreated injured nerves. The difference between treated and untreated injured animals is statistically significant according to Mann-Whitney's statistical test ($P < 0.025$). Nonmyelinated axons were not observed in the GM1-treated injured nerves, indicating that GM1

Fig. 3. Electron micrographs of GM1-treated injured nerves and PBS-treated injured nerves. Ultrathin sections were taken from nerves excised 4 wk after injury, 1 mm distal to the site of injury. (a) GM1-treated injured nerves. (b) PBS-treated injured nerves. Viable surviving axons are marked by arrows. Asterisks mark degenerating axons. Ap, astrocytic processes. ph, phagocytes.
treatment did not induce axonal growth, but apparently slowed down the degeneration of myelinated axons.

Discussion

The results of this study show that intraperitoneal administration of GM1 immediately after axonal injury in the rat optic nerve reduces the metabolic deficit that results from the injury. The improved metabolic activity (manifested by the altered NADH redox state in response to anoxia) already was evident 2 hr after GM1 administration. This early effect is in line with previous findings that GM1 application reduces the proportion of responses induced by anoxia and hypoxia 2 hr after injury. The present study may have significant implications for treating patients with optic nerve injury and blindness.

Table 1. Comparison between the numbers of viable axons distal to the injury site 1 mo after injury in GM1-treated and untreated nerves

<table>
<thead>
<tr>
<th>Group</th>
<th>Injured untreated nerves (control)</th>
<th>Injured GM1-treated nerves</th>
</tr>
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<tbody>
<tr>
<td>Number of axons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mean ± standard error)</td>
<td>216 ± 65</td>
<td>1654 ± 1149</td>
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<td>n</td>
<td>6</td>
<td>7</td>
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Nerves were processed for electron microscopy 1 mo after injury. Injured animals were injected with phosphate buffered saline (untreated controls) or with GM1 for 7 consecutive days (30 mg/kg, intraperitoneally). Myelinated axons were counted by the sampling method using electron micrographs taken 1 mm distal to the site of injury. The corresponding number of viable axons in uninjured animals was 60,432. The difference between treated and untreated animals after 4 wk was significant, according to Mann-Whitney’s test (P < 0.025).

The results of this study show that intraperitoneal administration of GM1 immediately after axonal injury in the rat optic nerve reduces the metabolic deficit that results from the injury. The improved metabolic activity (manifested by the altered NADH redox state in response to anoxia) already was evident 2 hr after GM1 administration. This early effect is in line with previous findings that GM1 application reduces the proportion of responses induced by anoxia and hypoxia 2 hr after injury. The present study may have significant implications for treating patients with optic nerve injury and blindness.

Key words: axonal degeneration, GM1, metabolic activity, optic nerve injury

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References


