Anti-semaphorin 3A Antibodies Rescue Retinal Ganglion Cells from Cell Death following Optic Nerve Axotomy*

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Damage to the optic nerve in mammals induces retrograde degeneration and apoptosis of the retinal ganglion cell (RGC) bodies. The mechanisms that mediate the response of the neuronal cells to the axonal injury are still unknown. We have previously shown that semaphorins, axon guidance molecules with repulsive cues, are capable of mediating apoptosis in cultured neuronal cells (Shirvan, A., Ziv, I., Fleminger, G., Shina, R., He, Z., Brudo, I., Melamed, E., and Brazilai, A. (1999) J. Neurochem. 73, 861–871). In this study, we examined the involvement of semaphorins in an in vivo experimental animal model of complete axotomy of the rat optic nerve. We demonstrate that a marked induction of type III semaphorin proteins takes place in ipsilateral retinas at early stages following axotomy, well before any morphological signs of RGC apoptosis can be detected. Time course analysis revealed that a peak of expression occurred after 2-3 days and then declined. A small conserved peptide derived from semaphorin 3A that was previously shown to induce neuronal death in culture was capable of inducing RGC loss upon its intravitreal injection into the rat eye. Moreover, we demonstrate a marked inhibition of RGC loss when axotomized eyes were co-treated by intravitreal injection of function-blocking antibodies against the semaphorin 3A-derived peptide. Marked neuronal protection from degeneration was also observed when the antibodies were applied 24 h post-injury. We therefore suggest that semaphorins are key proteins that modulate the cell fate of axotomized RGC. Neutralization of the semaphorin repulsive function may serve as a promising new approach for treatment of traumatic injury in the adult mammalian central nervous system or of ophthalmologic diseases such as glaucoma and ischemic optic neuropathy that induce apoptotic RGC death.

Axon guidance molecules are a large family of secreted and membrane-bound proteins that participate in axonal pathway formation during development of the nervous system in a receptor-mediated process (reviewed in Refs. 1–4). In particular, several members of the semaphorin family of proteins may play an important role in regulating axonal networks throughout neuronal development via their chemorepulsive activity on neuronal growth cones (5–7). The basic structure of sema includes a semaphorin domain of 500 bp as well as immunoglobulin-like and thrombospondin domains. Semaphorins are currently grouped into nine subclasses, which contain both axon guidance molecules, and cyclic nucleotides can regulate their activity from repulsion to attraction (15, 16). Involvement of semaphorin family members in other physiological phenomena was suggested, such as regulation of organ formation outside the nervous system (17), regulation of the immune response (18, 19), and tumor-related cell survival mechanisms (20, 21). We have recently shown a functional involvement of semaphorins in neuronal apoptosis (22). In a model system of cultured chick sympathetic neurons undergoing dopamine-induced apoptosis, induction of both collapsin-1, now termed chick semaphorin 3A (Sema3A)1; a secreted and diffusible type III semaphorin with repulsive activity), and CRMP-62 was detected. This up-regulation was evident at the early stages following exposure to the apoptotic trigger, even before the emergence of the typical morphological signs of apoptosis. The up-regulation of Sema3A expression was coupled to the onset of the commitment time point of the neurons to the death process. Moreover, marked and prolonged protection of neurons from dopamine-induced apoptosis was achieved by co-treatment of cells with function-blocking anti-Sema3A antibodies. Antibodies against neuropilin-1, the putative semaphorin III/Sema3A receptor (23, 24), also inhibited neuronal apoptosis. In addition, induction of apoptosis was evident by treatment of neurons with a recombinant human semaphorin III protein (22). These results demonstrated a correlation between the semaphorin death-inducing activity and the repulsive activity on axonal

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1 The abbreviations used are: Sema3A, semaphorin 3A; RGC, retinal ganglion cell(s); 4-Di-10-Asp, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide; PBS, phosphate-buffered saline; KLH, keyhole limpet hemocyanin.

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nerve fibers were completely transected at a distance of 2 mm from the globe. The procedure was performed without damage to the nerve cell bodies in all animals, and the other eye served as a control.

The molecular mechanisms responsible for transforming the repellent guidance cue from the damaged axon into a death signal that may affect the cell body are unknown. Therefore, it was important to further substantiate and extend our in vitro findings to an experimental in vivo model of neuronal apoptosis. One of the models used to study the fate of injured neurons in the adult central nervous system is the visual system, in which retinal apoptosis occurs following axonal injury to the optic nerve. In rats, transection of the optic nerve close to the cell bodies is a trigger for retrograde degeneration and delayed death of retinal ganglion cells (RGC) (27). The mechanism that initiates the loss of retinal cell bodies as a function of axonal injury is still unknown, and several hypotheses have been suggested (reviewed in Ref. 28). However, many lines of evidence indicate that apoptotic processes are responsible for the loss of RGC in the retina following axotomy of the optic nerve and for the loss of other axotomized or traumatized central nervous system neurons (27, 29–31). This secondary death of the RGC also serves as an example of the correlation between neuronal cell survival and axonal integrity. Such a correlation is supported by studies indicating that when an axon is injured, the cell body goes through a series of changes that may culminate in the death of the axotomized neurons (32).

Following optic nerve axotomy in adult rats, initial reduction of the original population of RGC is observed 5–7 days after injury. A rapid process of cell death then takes place, and >80% of the RGC die within 2 weeks after the injury (27, 33–35). Because a relatively long interval precedes this massive death process, it can be speculated that a time window exists in which protective intervention with modulators that attenuate or prevent cell death can be employed. Indeed, partial and temporal rescue of RGC from axotomy-induced apoptosis was obtained by several approaches. These included early treatment with microglial inhibitors (36, 37), glial or brain-derived neurotrophic factors (38, 39), caspase inhibitors (40, 41), implantation of phagocytic factors (38, 39), caspase inhibitors (40, 41), and overexpression of Bcl-2 (43, 44). Therefore, we chose the visual system as an in vivo model in which neuronal apoptosis can be induced and, more importantly, that can also offer an opportunity to intervene and alter the fate of the injured RGC.

We now report that high levels of semaphorin expression are transiently induced in the rat retina at an early post-axotomy stage and before any RGC death is observed. Furthermore, we demonstrate that rescue of RGC from axotomy-induced degeneration can be achieved by treatment of the injured retina with intraocular administration of anti-Sema3A function-blocking antibodies.

MATERIALS AND METHODS

Optic Nerve Transection in the Rat

Adult male Sprague-Dawley rats (8–10 weeks old, 300 g) were deeply anesthetized (50 mg/kg xylazine and 35 mg/kg ketamine), and their left optic nerves were exposed by lateral canthotomy. The conjunctivae were incised lateral to the cornea, and the retractor bulbus muscles were separated. Through a small opening in the meninges (~200 μm), the nerve fibers were completely transected at a distance of 2–3 mm from the globe. The procedure was performed without damage to the nerve vasculature and optic nerve blood supply and with minimal damage to the meninges by the use of a specially designed glass dissector with a 200-μm tip and a smooth blunt edge (45). The injury was unilateral in all animals, and the other eye served as a control.

growth cues. We therefore proposed that repulsive diffusible cues, acting on appropriate receptor-bearing cells, could modulate neuronal cell fate (22). Further support for our studies was provided by the indication of involvement of semaphorins in apoptosis of sensory neurons (25) and neuronal progenitor cells (26).

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Antiserum to chick Sema3A was obtained by immunization against Pep1A, a 363-380 synthetic peptide corresponding to amino acids 363-380 from chick Sema3A (46). The sequence of this peptide is identical in chick Sema3A, human semaphorin III, and rat and mouse semaphorin D. These antibodies were therefore expected to react with the highly conserved Sema3A/semaphorin III/semaphorin D proteins present in those species. Synthetic peptides were obtained and purified as described above. Peptide conjugates with keyhole limpet hemocyanin (KLH; Sigma) were prepared in rabbits by immunization against Pep1A, a chicken Sema3A-derived peptide (Pep1A, corresponding to amino acids 363–380 from chick Sema3A, and the control peptide Pep0A, corresponding to amino acids 249–256 according to Ref. 46) were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA) and purified to homogeneity on a RP-18 column (Vydac, Hesperia, CA) using a Gilson Model 303 HPLC system. Prior to injection, each peptide was dissolved in PBS, and a total amount of 0.33 mg/kg in a volume of 2 ml was injected into the vitreous body of one eye, and the opposite eye served as a control. Insertion was at the corneal limbal border behind the lens, over the optic nerve head area and close to the retinal surface.

Antibody Preparation

Polyclonal Anti-Sema3A Antibodies—Polyclonal anti-Sema3A antibodies were prepared in rabbits by immunization against Pep1A, a peptide corresponding to amino acids 363–380 from chick Sema3A (46). The sequence of this peptide is identical in chick Sema3A, human semaphorin III, and rat and mouse semaphorin D. These antibodies were therefore expected to react with the highly conserved Sema3A/semaphorin III/semaphorin D proteins present in those species. Synthetic peptides were obtained and purified as described above. Peptide conjugates with keyhole limpet hemocyanin (KLH; Sigma) were prepared by incubation of the peptide (3 mg) with 1 ml of KLH containing 10 mg of protein and 1.25% glutaraldehyde. After 3–4 min at room temperature, the reaction was stopped by adding 0.1 ml of 1 M glycine in water. Two rabbits were immunized against the peptide. Rabbits were immunized by subcutaneous injections of 0.1 mg of KLH-conjugated peptide. Booster shots were injected 2, 5, and 8 weeks after immunization. The titer of the antibodies in the serum was 1:10\(^4\) as determined by enzyme-linked immunosorbent assay and compared with the preimmune serum. Both the injected peptide and preimmune sera were fractionated using 40% saturated ammonium sulfate and dialyzed against PBS. These antibodies were previously characterized and shown to react with the chick Sema3A protein and to act as function-blocking antibodies that block the repellent activity of human sema-
phorin III (29). These antibodies were used in this study for inhibiting the effect of axotomy.

**Monoclonal Anti-sephorin Antibodies**—Balb/c mice were immunized subcutaneously with 50 µg of KLH-conjugated Pep1A in complete Freund’s adjuvant and boosted four times subcutaneously with 50 µg of KLH-conjugated Pep1A in incomplete Freund’s adjuvant at 3-week intervals. Three days before fusion, mice were boosted by intravenously injection of 50 µg of KLH-conjugated Pep1A in PBS. One mouse that had a relatively high titer (as evidenced by enzyme-linked immunosorbent assay with bovine serum albumin-conjugated Pep1A) and that was also positive in Western blot analysis, immunohistochemistry, and immunoprecipitation was chosen for fusion. Hybridomas were prepared by fusion of spleen cells with mouse myeloma N50S cells (donated by Dr. C. Milstein). Following the cloning procedure, positive clones were chosen for the preparation of ascitic fluids, which was carried out by intraperitoneal injection of 2–5 × 10⁶ hybridoma cells into pristane-primed male mice. The fluid was harvested starting at 7–10 days and then twice every 2nd day. Ascitic fluids were centrifuged at 800 × g and kept frozen at −80 °C. The antibodies were characterized by their ability to react with a single protein with the right molecular mass (110 kDa) in chick embryo retinas at embryonic day 18, by their ability to immunoprecipitate this protein, and by their ability to label the ganglion cell layer of embryonic day 18 retinal slices. These antibodies were used in this study for both immunohistochemistry and Western blot analysis.

**Protective Antibody Treatment**

For protective antibody treatment, the axotomized animals were grouped into four groups of four to six rats each. Two groups were injected with the partially purified polyclonal anti-Sema3A antibody (at a single dose of 50 µg in a volume of 2 µl). One of the groups was injected immediately after optic nerve transection (time 0), and the second group was injected with the antibody 24 h post-injury. The other two groups served as controls: one group was injected with preimmune serum (30 µg in a volume of 2 µl), taken from the same rabbit used for immunization, and the second with PBS. The anti-Sema3A antibody was injected into the vitreous body of the axotomized eye. Injection was performed using a glass pipette that was inserted into the eye globe. Insertion was at the corneal limbic border behind the lens, over the optic nerve head area and close to the retinal surface. Injection of the anti-Sema3A antibody did not cause any signs of inflammation or irritation or any other changes that may indicate that this treatment is toxic. Clinical examination of the eyes was performed using a slit lamp, and the eyes were monitored for any signs of hyperemia, edema, discharge, fibrin, and other inflammatory parameters.

**Western Blot Analysis**

Western blot analysis was performed as described by Harlow and Lane (65) using 8% polyacrylamide gels. Each lane was loaded with an equal amount of protein extracts (175 µg), which, following electrophoresis, were transferred to an Immobilon (polyvinylidene disulfide) membrane for 1.5 h. Blots were stained with Ponceau to verify equal loading and transfer of proteins. Membranes were then probed with monoclonal anti-sephorin antibodies (1:1000 dilution). The intensity of the signal was determined using the ECL-Plus detection system (Amersham Biosciences, Buckinghamshire, UK).

**Immunohistochemistry**

**Tissue Preparation**—Dissected retinas from optic nerve-axotomized rat eyes and from non-injured control were from optic nerve-axotomized rat eyes, and controls were fixed in 3.5% paraformaldehyde in PBS for 1–2 h and then infiltrated for cryoprotection with 4% sucrose for 2 h at 4 °C, followed by 20% sucrose + 5% glycerol in PBS at the same temperature, overnight. Fixed tissue was quickly frozen in liquid nitrogen. Cross-sections (10 µm) were placed on subbed slides (1% gelatin containing 0.3% chromium potassium sulfate) and stored at −20 °C. Sections were washed with PBS and incubated with PBS and 1% bovine serum albumin for 30 min at room temperature. The sections were incubated overnight with the primary monoclonal antibody against Sema3A at 4 °C. The slides were then washed three times with PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h at room temperature. After washing three times with PBS, the sections were mounted with galvanol. Observations were made and photography was carried out with a Zeiss Axioplan microscope with an appropriate filter. As a control, the sections were reacted either with preimmune rabbit serum or with the secondary antibody only.

**RESULTS**

**Experimental Model of Complete Axotomy of the Rat Optic Nerve**—In the adult rat, injury to the optic nerve leads to delayed apoptotic death of the RGC. To establish the experimental model, rats were subjected to intrameningeal complete axotomy of the optic nerve in one eye, whereas the second eye served as a control. To demonstrate the effect of axotomy on RGC in this experimental system, two approaches were used. One method estimated the number of live RGC that were still detected in retinal cross-sections following 12 days of axotomy. The RGC from one eye that was subjected to optic nerve axotomy were retrograde-labeled by application (at day 8) of the neurotracer dye 4-Di-10-Asp and were taken from intact (A) and axotomized (B) eyes. The difference in RGC densities. The two photomicrographs were taken of the peripheral retinal area under identical conditions, and the difference in contrast between A and B stems from the higher fluorescence of the live RGC in A. Bar = 55 µm.

**Hoechst Staining**

Cross-sections were stained with a cell-permeable form of bisbenzimide (Hoechst 33342) at a concentration of 4 µg/ml for 30 min at 37 °C. Cells were visualized by UV light microscopy, and the relative number of apoptotic nuclei with condensed or fragmented chromatin was then evaluated and compared with non-apoptotic nuclei, showing a pale and diffuse staining. Photomicrographs using a UV filter were taken for documentation.
33342, after which the RGC were examined for the presence of chromatin changes. The relative number of cells in the axotomized eye with condensed or fragmented chromatin, indicating apoptosis, was evaluated and compared with that in control retinas of non-axotomized eyes, which showed only a pale and diffuse staining. Fig. 2 shows that, as a result of axotomy, there was a massive loss of the ganglion cells in the injured eye compared with the non-injured eye. Twelve days after the damage, the estimated density of ganglion cells/mm² in this layer was dramatically decreased. Moreover, a significant number of apoptotic cells could be identified using the Hoechst nuclear staining method (Fig. 2), which identifies pyknotic nuclei and chromatin fragmentation as hallmarks of apoptosis. In comparison, no apoptotic nuclei could be detected in the non-axotomized eye. Although the number of nuclei showing apoptotic morphology in these retinas is small, it is consistent with previous estimates that cells undergoing death in vivo are eliminated from the tissue within 3 h or less (47).

**Induction of Semaphorin Expression in the Retina following Axotomy of the Optic Nerve**—To examine whether secreted semaphorins participate in the degeneration process of RGC, we followed the retinal expression pattern of semaphorins in the above model system of optic nerve axotomy. Following axotomy in one eye, the retinas from both eyes were removed at different time intervals and used for immunohistochemical analysis with the anti-Sema3A antibody. As controls, we used retinas from the non-axotomized eyes of the same rats as well as retinas from eyes of different rats that were not subjected to axotomy. Four to six rats were analyzed at each time point.

Temporal analysis of semaphorin expression as a function of advancement of the death process was performed. Retinas at 1–4, 10, 12, and 16 days following axotomy were analyzed, and data from several time points are presented in Fig. 3. Axotomy initiated a gradual increase in semaphorin expression, starting 24 h post-injury and peaking at 48–72 h. At 96 h, staining declined, indicating decreased expression. No additional wave of semaphorin expression could be detected at days 10, 12, and 16, at which time the RGC death process was fully manifested (data not shown).

The up-regulation of semaphorins reflects induction of their expression in the RGC layer and the inner nuclear layer, where the amacrine and bipolar cells reside. Some label was also detected in the outer nuclear layer, where the horizontal cells are found. It is possible that the induction of semaphorin expression is transmitted through a transsynaptic pathway, which may explain why the labeling of semaphorins could be detected in cell structures of neural origin.

Western blot analysis of retinas prepared at different time points revealed two specific bands that reacted with the anti-semaphorin antibodies and showed elevated levels following axotomy (Fig. 4). One protein has a molecular mass of 110 kDa and corresponds to the full-length semaphorin protein (46). A lower molecular mass protein of 36 kDa appeared to be up-regulated and may represent cleavage of a degradation product of the full-length protein. Furthermore, at 72 h post-axotomy, another protein band of 38 kDa could be detected, which appeared only at this time point; and its relation to the other two bands has yet to be investigated. Quantitative analysis of the results showed that, whereas the higher band peaked at 48 h
post-injury, the lower band reached its peak at 72 h. The level of the 110-kDa protein was elevated 2.3-fold 24 h after the injury and 4-fold after 48 h and fell to 1.8-fold after 96 h. The 36-kDa protein was elevated 4.2-fold 72 h after axotomy and fell to 2.5-fold after 96 h. The kinetics of up-regulation of both bands (in particular, lag time of appearance of the 36-kDa protein) and the fact that both bands showed a similar level of up-regulation support the assumption that the two bands are related and that the small band indeed represents a cleavage product of the large band.

These results indicate that the up-regulation of semaphorins in the retina peaks shortly after induction of damage, at a time point that precedes the manifestation of the actual death process of the RGC. High expression of semaphorins in neurons following nerve injury could have a potentially inhibitory effect on the outgrowth of nerve sprouts, thereby preventing regeneration processes.

The Sema3A-derived Peptide Is Capable of Inducing RGC Loss—Our previous studies indicate that exposure of neuronal cells to the full-length Sema3A protein can induce apoptotic death in cultured neurons, and a similar process was observed when neurons were exposed to a small conserved peptide derived from Sema3A (Pep1A) (48). The apoptotic process, accompanied by shrinkage of the axonal network was observed in both cases, suggesting that Pep1A can mimic the death-inducing activity of the full-length protein (48). Based on the evidence of involvement of semaphorins in RGC loss following axotomy, it was of interest to test whether direct application of the semaphorin-derived peptide to healthy animals has an effect on survival of RGC. As a control, a different Sema3A-derived peptide (Pep3A) was applied to retinas of healthy rats. Pep3A was previously shown to be nontoxic in neuronal cultures (48) and therefore was tested as a control peptide. Injection of Pep1A into healthy eyes resulted in 57% neuronal loss compared with only 9% RGC death in Pep3A-treated rats, indicating that the death-inducing activity is specific to Pep1A (Fig. 5). These results suggest that Pep1A has toxic effects when applied both in vitro (48) and in vivo and may imply that the exposure of RGC to secreted Sema3A can propagate the death process within neighboring RGC.

Rescue of RGC from Axotomy-induced Degeneration by Anti-Sema3A Antibodies—To examine whether the function-block-
Anti-Sema3A Antibodies Inhibit RGC Death

Fig. 4. Up-regulation of semaphorin extracted from the rat retina following axotomy. A, retinas from axotomized eyes were dissected at different intervals (0 (control (C)), 24, 48, 72, and 96 h) after lesion, and total retinal proteins were extracted, separated on 12.5% polyacrylamide gel, and blotted onto polyvinylidene disulfide membrane. The membrane was stained with Ponceau, after which the blot was reacted with the monoclonal anti-semaphorin antibody (1:1000 dilution). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (1:25,000 dilution). The blots were developed using the ECL system. B, quantitative analysis of bands was performed using TINA software, and data are normalized to the corresponding tubulin. The results are represented as percent of control untreated animals. Three retinas isolated from three different animals were used per time point.

Fig. 5. Intraretinal injection of Sema3A-derived peptide Pep1A leads to RGC loss. Both peptides Pep1A and Pep3A were injected at a concentration of 0.33 mg/kg; and 8 days later, survival of the RGC was monitored following the application of the neurotracer dye as described under “Materials and Methods.” A, whole-mounted retinas showing 4-Di-10-Asp-labeled RGC cells that were exposed to PBS, Pep1A, and Pep3A. Excessive RGC loss was evident when Pep1A (but not Pep3A) was applied. Bar = 25 μm. B, quantitative analysis of Pep1A- and Pep3A-treated RGC. Results are presented as means ± S.E. of live cells/mm². Statistical analyses were performed with the Mann-Whitney non-parametric test. *** p < 0.01.

Sema3A antibodies at a single dose of 30 μg (0.1 mg/kg) was administered. In the fourth group, identical quantities of anti-Sema3A antibodies were injected 24 h post-axotomy. Eight days later, the RGC were retrograde-labeled with the neurotracer dye 4-Di-10-Asp (which is specifically stains live neurons). The retinas were removed 4 days following the staining (12 days post-injury), and the number of stained neurons was counted. In addition, retinas from untreated animals served as a reference for evaluating the protection level achieved by the different treatments. The retinas were analyzed by fluorescence microscopy, and the results are presented in Fig. 6 (A–C). A massive loss of RGC was observed in retinas from axotomized eyes treated with PBS, and these retinas appeared to be very depleted. Similar retinal RGC depletion was obtained when the injured eyes were injected with preimmune serum (data not shown). However, remarkably higher RGC densities comparable to those in axotomized retinas were displayed in retinas treated with anti-Sema3A antibodies (Fig. 6A). Furthermore, the surviving large neuronal cells appeared to have long processes of axons, indicating that the axonal network remained intact (Fig. 6B). For quantitative analysis of the results, we evaluated the distribution of the surviving retinal neurons. The number of live (stained) RGC in each experimental group was further estimated by counting the RGC in the central and peripheral retinal areas (for specific location of the central and peripheral areas, see “Materials and Methods”). The results are shown in Fig. 6C and Table I. In untreated animals, ~65% (central area) and 50% (peripheral area) of the RGC population had degenerated 8 days following optic nerve axotomy (comparable to the results obtained by Isenman et al. (35)). However, treatment of the axotomized retinas with anti-Sema3A antibodies either at the time of axotomy (designated time 0) or 24 h
later resulted in full protection, and retinas were indistinguishable from those of the control animals (Fig. 6C and Table 1). Treatment of retinas at time 0 with preimmune serum had no protective effect (Fig. 6A). The RGC from the central retinal areas compared with the peripheral areas were more sensitive to the apoptotic trigger, showing lower survival rates, and were also less responsive to the antibody treatment. Overall, these results indicate that treatment of axotomized eyes with the anti-Sema3A antibody leads to a marked increase in the number of surviving RGC in the central as well as peripheral areas of the retina, which, with no other treatment, would otherwise suffer a massive loss of RGC. These findings further substantiate the concept that axon guidance molecules with repulsive cues play a role in degeneration of RGC in response to axotomy. The ability for neuroprotection at time points of up to 24 h following the injury indicates the existence of a therapeutic time window during which RGC death is still reversible and can be modulated.

**DISCUSSION**

Our study shows that a peak of induction of semaphorin expression in the RGC and in the inner plexiform layer at the early stages following complete axotomy of the optic nerve. This up-regulation was detected in the RGC and in the inner plexiform layer of the axotomized eyes and preceded the actual death process of the ganglion cells, which is the inevitable outcome of such optic nerve injury in mammalians. Moreover, we have shown that, by neutralizing semaphorin repulsive activity using a single intravitreous injection of function-blocking anti-Sema3A antibodies, we could prolong the survival of RGC and obtain dramatic protection from axotomy-induced degeneration. These results are consistent with our previous *in vitro* experiments on cultured neuronal cells exposed to an apoptosis-inducing challenge (22). In these studies, we have shown that a peak of induction of Sema3A expression occurred just prior to the commitment of sympathetic neurons to apoptosis, and prevention of cell death was achieved using the same anti-Sema3A antibodies (22). Although the *in vitro* experimental system utilized peripheral neuronal cells harvested from post-mitotic chick embryos, and the animal model described in this study focused on central nervous system ganglion cells from the rat retina, taken together, these data indicate the existence of a common pathway. The correlation between the pivotal roles played by semaphorins as mediators of neuronal fate in both the *in vitro* and *in vivo* models suggests that diffusible chemorepulsants may participate in a crucial step of the apoptotic cascade. Indeed, a diffusible apoptosis-promoting activity that is heat-labile was isolated from R28 retinal cell-conditioned medium (56). The heat lability of the compound may indicate that it is likely to be a secreted protein. This apoptosis-inducing activity has not yet been character-
ized, but it is supportive of our findings suggesting that semaphorins are candidates for serving as diffusible apoptosis-promoting elements.

More importantly, we have shown that apoptosis can be blocked during its early stages by antibodies that inhibit the axonal collapse activity. Indeed, recent lines of evidence support the view that repulsive cues are not restricted to the early stages of neurodevelopment, but may also play a role in the response of axons to their environment during adulthood. For instance, adult sensory neurons were shown to retain the ability to respond to semaphorin III (57). In addition, expression of semaphorin III was recently shown in selective adult brain tissue (58). It is also likely that semaphorins are involved in the pathogenesis of human neuronal degenerative diseases. A further example of the role of semaphorins in pathological conditions was reported by Fujita et al. (59). They found transient up-regulation of neuropilin-1, neuropilin-2, and semaphorin 3A in rat brain after occlusion of the cerebral artery distal to striate branches. They suggest that neuropilin-1, neuropilin-2, and semaphorin 3A are involved in microglial/neuron interactions to phagocytose the dying neurons. Increased levels of CRMP-62, a protein involved in the intracellular response to Sema3A, are associated with the pathological process of neurofibrillary tangle formation in Alzheimer’s disease brains (60). Another study described an altered expression pattern of semaphorin IV in Alzheimer’s disease brains (61). Although semaphorin IV expression does not co-localize with CRMP-2 expression in Alzheimer’s disease brains (61), these findings may still link its accumulation with some of the pathological changes accompanying nerve degeneration. The emerging concept therefore suggests that, as a result of injury, neurons may react by up-regulation of proteins with a repulsive signal.

Furthermore, we have shown that, by applying the function-blocking anti-Sema3A antibodies to rats following optic nerve injury, we were able to protect the RGC. Several possible explanations can be suggested for the inhibitory mechanism displayed by neutralizing repulsive signals. The first is based on the apoptosis-inducing capability of semaphorins. When adult neurons are exposed to unscheduled high levels of semaphorins, death signals are conveyed to the cell bodies, leading to neuronal apoptosis (22, 25). Evidently, neutralizing the activity of these proteins can prevent their toxic effects. A second possible explanation is that the increase in repulsive signals can contribute to unfavorable conditions leading to the failure of regenerative processes, thus culminating in neuronal cell death. Indeed, decreased Sema3A mRNA expression was recently reported as a temporary event accompanying peripheral nerve crush (62). This down-regulation was interpreted as an important mechanism enabling regenerative attempts that occur after nerve injury (62). In accordance with this concept, a transient up-regulation of semaphorin III mRNA was indicated at the lesion site following axotomy of olfactory axons and in neural scar tissue after injury to the adult central nervous system (62, 63). It was even speculated that, by inhibiting the activity of repulsive cues, promotion of regeneration processes could take place (62). A third possible explanation is that anti-Sema3A antibodies interfere with some other regulatory step of axotomy-induced apoptosis that is non-relevant to inhibition of repulsive signals. Obviously, more studies are needed to gain a better understanding of the molecular events that underlie the rescue of RGC from axotomy-induced death. It is still unresolved whether neuropilin-1, the downstream receptor of semaphorin III (23, 24), is involved in conveying the repulsive cue into a death signal in axotomized RGC. Although neuropilin-1 is known to be down-regulated in adult rat RGC (64), it appears from our study that the RGC retain their responsiveness to chemorepulsive signals following optic nerve injury. Further support for a possible involvement of neuropilin-1 in this process comes from our studies in cultured neuronal cells, in which function-blocking anti-neuropilin-1 antibodies were able to inhibit the death process (22).

The induction of semaphorins in the rat retina occurred during the early stages of the time window that separates the retrograde death of the RGC from the onset of the injury (i.e. axotomy). We have taken advantage of this time lag and the temporal up-regulation of semaphorin expression to treat the damaged eye with a neuroprotective antibody. The delayed administration of the antibody did not impair its neuroprotective ability, suggesting that a high percentage of the neurons are still in the reversible stage and that it is therefore still possible to rescue RGC even 24 h after the injury and to achieve a high survival rate. It may be speculated that, in the case of optic nerve injury in humans, physicians will have a time window of at least 24 h to evaluate the clinical situation prior to treating the patients with neuroprotective treatment or anti-apoptotic drugs. There are many eye conditions in which neuroprotective treatment can rescue RGC until the primary cause of injury to the optic nerve and retina can be abolished. Among them are acute increase in intraocular pressure, sudden occlusion of the central retinal artery, ischemic optic neuropathy, retinal vein occlusion, and others (Refs. 66–68; for review, see Ref. 69). It is tempting to speculate that this neuroprotective approach may be applied not only to the visual system, but also to injuries to the nervous system function. Obviously, further studies need to be employed to test this theory. Our successful results obtained in this study raise the possibility that blockage of axon guidance molecules with chemorepulsive activity can serve (either alone or in combination with other neuroprotective agents) as an important novel strategy for modulation of neurodegeneration and of traumatic injury in the central nervous system.

It is of interest that the kinetics of semaphorin induction following optic nerve axotomy are similar to those of c-Jun after optic nerve crush (30). Both are rapidly up-regulated during the early days post-injury; they reach a maximum of labeling intensity after 3 days and then decline. Therefore, the list of immediate-early genes whose expression is up-regulated in response to apoptotic triggers may also be extended to include the semaphorins, which can be used as early predictors of retinal degeneration.

In summary, we have described a model in which RGC survival in the retina following optic nerve injury was enhanced by inhibiting repulsive cues. Our results suggest that diffusible chemorepulsive signals may have a direct effect on the survival of injured neurons and that a therapeutic benefit can be obtained by inhibiting their function. In our experiments, we did not include functional studies of the surviving neurons, and further studies of their ability to receive and integrate synaptic inputs are still needed. In addition, future studies should also examine whether longer-term survival can be obtained using this strategy.

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