Oxidative Stress and Neuroprotection

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PURPOSE. To establish a method for morphometric analysis of retrogradely labeled retinal ganglion cells (RGCs) of the mouse retina, to be used for the study of molecular aspects of RGC survival and neuroprotection in this model; to evaluate the effect of overexpression of CuZn-superoxide dismutase (CuZnSOD) on RGC survival after severe crush injury to the optic nerve, and to assess the effect of the α2-adrenoreceptor agonist brimonidine, recently shown to be neuroprotective, on RGC survival.

METHODS. A severe crush injury was inflicted unilaterally in the orbital portion of the optic nerves of wild-type and transgenic (Tg–SOD) mice expressing three to four times more human CuZnSOD than the wild type. In each mouse all RGCs were labeled 72 hours before crush injury by stereotactic injection of the neurotracer dye FluoroGold (Fluorochrome, Denver, CO) into the superior colliculus. Survival of RGCs was then assessed morphometrically, with and without systemic injection of brimonidine.

RESULTS. Two weeks after crush injury, the number of surviving RGCs was significantly lower in the Tg–SOD mice (596.6 ± 71.9 cells/mm²) than in the wild-type control mice (863.5 ± 68 cells/mm²). There was no difference between the numbers of surviving RGCs in the uninjured retinas of the two strains (3708 ± 231.3 cells/mm² and 3904 ± 120 cells/mm², respectively). Systemic injections of brimonidine significantly reduced cell death in the Tg–SOD mice, but not in the wild type.


Optic nerve injury triggers a process of degeneration in the damaged fibers as well as in fibers that escaped the primary lesion (secondary degeneration). In both cases, the outcome is the death of retinal ganglion cells (RGCs). Using a model of partial crush injury of the rat optic nerve, we have previously shown that the secondary degeneration of spared fibers, which results from the extracellular toxicity produced by the degenerating axons, may be prevented or at least delayed by treatment with neuroprotective drugs. Such drugs may neutralize the effects of the mediators of toxicity and/or enhance the ability of the vulnerable cells to cope with the stressful conditions. Among the potent compounds shown to be neuroprotective in the partially lesioned optic nerve are α2-adrenoreceptor agonists. The mechanism of neuroprotection by these drugs is not yet fully understood.

To identify and characterize the molecules participating in the process of RGC death, and to discover how the α2-adrenoceptor agonists act against such degeneration, it was necessary to devise an animal model that allows molecular manipulation. Establishment of the mouse model would provide a way to study the effects of the injury in genetically manipulated mice. In the present study, we used transgenic mice overexpressing superoxide dismutase (CuZnSOD), a key enzyme in the metabolism of free oxygen radicals.

Free oxygen radicals are highly reactive molecules that contain one or more unpaired electrons. Mounting evidence points to the involvement of these molecules in a broad range of neuropathologic disorders, as well as in apoptosis, presumably by increasing the peroxidation of fatty acids or nucleic acids and eliminating protein cross-linking. SOD catalyzes the conversion of superoxide radicals (O2·−) to hydrogen peroxide (H2O2). Catalase and glutathione peroxidase remove H2O2 from the intracellular environment by reducing it to H2O and O2. Under normal conditions, most of the H2O2 molecules generated by CuZnSOD are further metabolized to water by catalase and glutathione peroxidase.
CuxZnSOD overexpression in transgenic mice causes physiological abnormalities similar to those seen in patients with Down’s syndrome.11–14 These abnormalities are attributed mainly to excessive accumulation of H2O2, facilitating its reaction with transition metals (Fenton’s reaction).15 By using the transgenic mice in the present study, we were able to examine the contribution of oxidative stress to the posttraumatic death of RGCs and to examine the effect of activating the α2-adrenoreceptor pathway with brimonidine in preventing or delaying such death.

METHODS

Animals

CB6F1 transgenic mice harboring the human SOD-1 gene were produced by microinjecting fertilized eggs with a linear 14.5-kb fragment of human genomic DNA containing the entire CuZnSOD gene, including its regulatory sequences. Expression of the transgene in these mice is similar to its expression in humans, with 0.9- and 0.7-kb transcripts in a ratio of 1 to 4, and the human enzyme is synthesized in an active form. CuZnSOD activity in the brains of these transgenic mice is three to four times higher than in the wild type. Male transgenic mice and age-matched wild-type (CB6F1) mice aged 13 to 16 weeks were used. All mice were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized by intraperitoneal administration of ketamine (80 mg/kg) and xylazine (16 mg/kg). Before tissue extraction the mice were killed by an overdose of pentobarbitone sodium.

Labeling of RGCs

Mice were anesthetized and placed in a stereotactic device. The skull was exposed and kept dry and clean. The bregma was identified and marked. The designated point of injection was at a depth of 2 mm from the brain surface, 2.92 mm behind the bregma in the anteroposterior axis and 0.5 mm lateral to the midline. A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurontracer dye FluoroGold (4% solution in saline; Fluorochrome, Denver, CO) was then applied (1 μl/kg) and xylazine (16 mg/kg). Before tissue extraction the mice were killed by an overdose of pentobarbitone sodium.

Crush Injury and Brimonidine Injection

Seventy-two hours after RGC labeling, the mice were anesthetized and subjected to severe crush injury in the intraorbital portion of the optic nerve, 1 to 2 mm from the eyeball. With the aid of a binocular operating microscope the conjunctiva was incised, and the optic nerve was exposed. Using cross-action forceps and taking special care not to interfere with the blood supply, the nerve was crushed for 1 second. Immediately thereafter, the α2-adrenoreceptor agonist brimonidine (100 μg/kg) was injected intraperitoneally. A control group of crush-injured mice received intraperitoneal injections of saline.

Assessment of RGC Survival

Two weeks after the crush injury, the mice were given a lethal dose of pentobarbitone (170 mg/kg). The eyes were enucleated, and the retinas were detached and prepared as flattened wholemounts in 4% paraformaldehyde solution. At approximately the same distance (0.3 mm) from the optic disc, six to eight fields of identical size (0.07 mm2) were randomly chosen, and labeled cells were counted under a fluorescence microscope (magnification, ×800) by observers blinded to the identity of the mice. The location of the fields was specified to avoid variations in RGC density as a function of distance from the optic disc. The average number of RGCs per field was calculated in each retina.

Optic Nerve Excision

For evaluation of macrophage invasion of the injured optic nerve, mice were killed 1 day or 1 week after crush injury. A segment of the injured nerve between the optic chiasma and the eyeball, including the entire area of injury, was removed. The nerve was immediately frozen at −70°C.

Immunocytochemical Staining for Macrophages and Astrocytic Markers

Longitudinal cryosections of the excised optic nerves (10 μm thick) were picked up onto gelatin-coated glass slides. Sections were fixed in absolute ethanol for 10 minutes at room temperature, washed twice in double-distilled water, and incubated for 3 minutes in phosphate-buffered saline (PBS) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween-20). Sections were then incubated for 1 hour at room temperature with rat monoclonal antibodies to MAC-1 (PharMingen, San Diego, CA) diluted 1:50 in PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were washed three times with PBS and Tween-20 (0.05%) and incubated for 1 hour at room temperature with FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBS containing 3% fetal calf serum and 2% bovine serum albumin. After they were washed again with PBS containing Tween-20, the sections were viewed under a fluorescence microscope (Carl Zeiss; Oberkochen, Germany).

Statistical Analysis

The results were evaluated using Student’s t-test, or analysis of variance.

RESULTS

Kinetics of RGC Death after Severe Crush Injury of the Mouse Optic Nerve

As a baseline, the total number of RGCs was determined by retrogradely labeling the RGCs with a fluorescent dye applied in the superior colliculus and then counting the cell 72 hours after dye application. In the retinas of wild-type mice subjected to severe unilateral optic nerve injury, fields of 0.07 mm2 were found to contain approximately 296.5 ± 13.3 (mean ± SEM) RGCs on the uninjured side (n = 7) and 80.2 ± 3.4 on the injured side (n = 5) 2 weeks after the injury. The same method of assessment was used to determine the kinetics of RGC death after crush injury of the optic nerve. Wild-type CB6F1 mice were subjected to unilateral crush injury of the optic nerve, their retinas were excised at the indicated times after injury, and labeled RGCs from both retinas were counted. The labeled RGCs on the uninjured side were counted to rule out the possibility of a decline in their number due to decay of the dye.
in the eye on the uninjured side, therefore represents the real loss of RGCs as a result of the injury (Fig. 1B). RGC survival on the injured side after crush injury was found to be 47% ± 0.05% after 1 week and 27% ± 0.01% after 2 weeks. Representative micrographs showing a retrogradely labeled retina excised 2 weeks after injury and the corresponding uninjured retina are shown in Figure 2.

**Effect of CuZnSOD Overexpression on RGC Survival**

To assess the effect of excessive SOD activity on the postinjury survival of RGCs, we compared RGC survival 2 weeks after injury in wild-type and Tg−SOD mice. As shown in Table 1, there was no difference between the average number of RGCs in the normal retinas of the two strains. Two weeks after optic nerve crush injury, however, the number of surviving RGCs in the Tg−SOD mice was significantly lower than in the wild-type mice (t = 2.49, df = 30; P = 0.02). Thus, overexpression of CuZnSOD significantly enhanced RGC death.

To determine whether the higher death rate in the transgenic mice is accompanied by excessive degeneration occurring in the nerve itself, we examined the extent of inflammation in the injured nerves of both groups of mice. As early as 1 day after crush injury, reproducibly more macrophages and/or activated microglia (identified immunocytochemically by anti-MAC-1 antibodies) were seen around the lesion in the Tg−SOD mice (n = 5) than in the wild-type mice, although macrophages were also seen in the wild-type mice (n = 4; Fig. 3).

**Effect of Brimonidine on Posttraumatic RGC Survival**

It has been shown that a single systemic injection of the α2-adrenoceptor agonist brimonidine can reduce the degeneration of spared fibers (secondary degeneration) after partial injury of the optic nerve in the adult rat. In the present study, using the mouse model, we examined whether brimonidine, in addition to rescuing undamaged fibers immersed in a degenerative environment, can also protect the cell bodies of injured axons. After a single intraperitoneal injection of brimonidine, RGC survival 2 weeks after injury was 68% higher in the Tg−SOD mice than in untreated injured Tg−SOD control mice (analysis of variance [ANOVA], P < 0.002). In injured wild-type mice treated with brimonidine, RGC survival was only 15%
higher than in the untreated injured wild-type control mice; this difference was not significant (Fig. 4). It therefore seems that brimonidine effectively counteracted the contribution of CuZnSOD overexpression to the postinjury death of RGCs, because there were no differences in RGC survival between Tg–SOD and wild-type mice after brimonidine treatment.

**DISCUSSION**

Neurodegenerative diseases and acute central nervous system traumatic injuries in adults result in irreversible functional losses, because neurogenesis does not take place, and the damaged neurons cannot regenerate. Understanding of the processes and mechanisms of neuronal death and neuronal survival is an essential prerequisite for the development of neuroprotective measures. The mouse model of optic neuropathy used in this study was established with the object of exploiting the availability of transgenic and knockout mice to investigate mechanisms of neuronal death. In our model, the inflicted injury is severe enough to damage all the nerve fibers at once, and consequently the process of degeneration is uniform in time and pathway.

Death of RGCs in this study occurred gradually. By 7 days after the injury, the RGC population was reduced to 47% of normal, similar to the loss in adult rats after complete optic nerve transection. Yet, 2 weeks after the injury 27% of the normal, similar to the loss in adult rats after complete optic nerve transection.27,28 In a different model of neuronal stress, increased peroxidase activity has been proposed as a possible cause of neurodegeneration in familial amyotrophic lateral sclerosis,30–34 Down's syndrome,35 and aging.36,37 The accelerated toxicity in the these disorders may be explained by the fact that an increase in SOD1 activity does not significantly affect the rate of apoptotic death of RGCs during development and maturation. The rate of RGC death after optic nerve crush, however, was higher in the Tg–SOD mice than in the wild-type animals. There are conflicting reports about whether an increase in SOD1 expression exacerbates neuronal damage or protects against it. Overexpression of SOD1 reduces the damage resulting from cerebral reperfusion in adult animals,25,26 but worsens the outcome in immature animals.27,28 In a different model of neuronal stress, in which excitotoxicity was mediated by kainic acid, neurons from Tg–SOD mice were more susceptible than neurons from wild-type mice.29

Increased peroxidase activity has been proposed as a possible cause of neurodegeneration in familial amyotrophic lateral sclerosis,30–34 Down's syndrome,35 and aging.36,37 The accelerated toxicity in the these disorders may be explained by the fact that an increase in SOD1 activity is accompanied by an

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<th>Tg–SOD</th>
<th>Wild-Type</th>
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<tr>
<td>RGCs/mm² in retinas with uninjured optic nerve</td>
<td>3708 ± 231 (n = 6)</td>
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<tr>
<td>RGCs/mm² 2 weeks after optic nerve injury</td>
<td>596.6 ± 71.9 (n = 20)</td>
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Values are mean number of RGCs ± SD.
accumulation of H$_2$O$_2$ and facilitation of its reaction with transition metals (Fenton’s reaction), leading to increased hydroxyl radical production and thus increasing oxidative stress. It is interesting that at the very early posttraumatic moment is not clear. A number of pathways are possible. The spared neurons in the partial injury of the rat optic nerve which brimonidine exerts its neuroprotective effect on the wild-type mice than in transgenic mice. We have shown that injury-induced death of RGCs of severely injured axons in the wild type. This enhanced local inflammatory reaction function (see comments). *In the Tg–SOD group, the difference between the brimonidine-treated mice and the saline-treated controls is significant (P < 0.002). **The difference between saline-treated Tg–SOD mice and saline-treated wild-type mice is significant (P < 0.02).

In this study, excessive death of RGCs resulting from overexpression of SOD1 in transgenic mice was reversed by treating the mice with the α2-adrenoreceptor agonist brimonidine. Brimonidine had only a slight, nonsignificant effect on RGC death after optic nerve injury in the wild-type mice. These findings suggest that brimonidine exerts its effect, at least in part, on death involving oxidative stress, and therefore that oxidative stress may play a less prominent part in the injury-induced death of RGCs of severely injured axons in wild-type mice than in transgenic mice. We have shown that brimonidine can attenuate the spread of neuronal damage caused by partial injury of the rat optic nerve. The way in which brimonidine exerts its neuroprotective effect on the spared neurons in the partial injury of the rat optic nerve model is not clear. A number of pathways are possible. The α2-adrenoreceptors are coupled to multiple second-messenger pathways and can also upregulate basic fibroblast growth factor (bFGF), a neuronal survival factor and anti-apoptotic factors such as bcl-2 and bcl-xl. Which of these pathways, if any, were operative in the present study is not known.

In summary, our mouse model of severe optic nerve axonal injury may be useful for investigating the effects of various genes on the degeneration and death of RGCs. Using this model, we showed that interference with the equilibrium of free oxygen radicals may have a neurotoxic effect, which may be partially blocked through activation of the α2-adrenoreceptor pathway by selective agonists such as brimonidine.

**References**


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