Functional Efficacy of Glatiramer Acetate Treatment for Laser-Induced Retinal Damage in Rats

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Background and Objectives: To functionally evaluate the efficacy of glatiramer acetate (Cop-1) as a neuroprotective treatment for laser-induced retinal injuries in rats.

Study Design/Materials and Methods: Using standard lasering and flash ERG techniques, we evaluated the effect of photocoagulation and of Cop-1 treatment on retinal function 3, 20, and 60 days after covering one-half of the retina with of 23 rats with argon laser lesions.

Results: Significant neuroprotective effects of Cop-1 treatment on functional recovery were observed 20 and 60 days after retinal photocoagulation. Two months post-lasering, the amplitude of electroretinographic signals in lasered eyes (mean $\pm$ SEM) was $99.5 \pm 10.2\%$ of that of intact eyes in the Cop-1-treated group and $85.8 \pm 5.5\%$ in the untreated lasered control group ($P<0.05$).

Conclusions: Cop-1 immunization in rats is neuroprotective against laser-induced injuries to the outer retina and improves functional recovery of the injured retina. Studies have documented effective neuroprotective treatment after laser damage to myelinated neurons, but this is the first report of neuroprotection of nonmyelinated neurons. Lasers Surg. Med. 40:196–201, 2008.

Key words: electroretinogram; laser; neuroprotection; retinal injury; wound healing

INTRODUCTION

In many types of central nervous system (CNS) injuries, including those caused by ischemia or mechanical trauma, the eventual loss of tissue and functional damage are greater than those caused by the initial injury. This is because the spread of injury often continues for long periods after termination of the primary injurious event, via a process known as secondary degeneration [1–3]. The progressive degeneration begins when noxious compounds, such as glutamic acid, free fatty acid, and reactive oxygen species, are released from cells damaged by the primary injury and spread to neighboring cells. The main compound causing secondary degeneration is glutamate. An increase in extracellular glutamate is assumed to result from the death of neurons, with subsequent release of their intracellular contents, which contain approximately 10 mM glutamate [4]. Extracellular glutamate in excessive amounts has been implicated in the pathogenesis of many neurological and ophthalmic conditions, including stroke, trauma, epilepsy, and glaucoma [5,6]. Glutamate causes damage to the neurons via an excitotoxic pathway, mediated primarily through the *N*-methyl-D-asparate (NMDA) subtype of glutamate receptors [1].

Neuroprotection is an attempt to prevent this secondary degeneration, and hence to minimize the damage and maximize the recovery of a neural system from acute or chronic neural insults [7,8]. This can be accomplished by lessening the noxious effects of the extracellular environmental changes brought about by the directly injured cells, preventing their adverse effects on the healthy neighboring neurons, and assisting the latter to withstand those effects. Different types of competitive and noncompetitive NMDA-receptor antagonists were examined for their ability to reduce excitotoxic damage [9]. The most potent of these was found to be MK-801. The neuroprotective and antiproliferative properties of this noncompetitive NMDA-receptor antagonist have been convincingly demonstrated in the retinas of rats [10], but its neurotoxic and psychotoxic reactions have precluded its use in human studies.

In a number of models of CNS injuries, a well-controlled T cell-mediated response was shown to play a key role in the ability to fight off conditions causing degeneration [11–18]. It was further shown that such T cells, to be protective, should be directed against antigens that reside at the site of the lesion, and not against the harmful self-compounds that might accumulate there [14,15,19,20]. Not all individuals are equally endowed with the capacity to spontaneously recruit a T cell-dependent protective mechanism, but all individuals can benefit if this protective mechanism is boosted in a well-controlled way [13,15,21]. Thus, even strains in which this spontaneous ability is limited can derive the benefit of a protective T cell-dependent response induced by active immunization or by passive transfer of T cells directed against autoantigens or their weak agonists [14,19,21–23].

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Boosting of the immune response can be achieved by immunization with certain peptides derived from relevant autoantigens, such as the synthetic oligomeric copolymer glatiramer acetate (copolymer-1; Cop-1) [24,25], shown to be neuroprotective in rodent models of acute or chronic neurodegenerative conditions when administered, together with an adjuvant, as a single injection [15,26,27]. Because of its low affinity for a wide variety of autoreactive T cells the Cop-1 molecule acts as a weak agonist of numerous autoantigens, and can thus circumvent the tissue-specificity barrier found to be critical for T cell-dependent neuroprotection [14,15]. The neuroprotective action of Cop-1 has been demonstrated in models of neuronal damage caused by glaucoma [15,28], optic nerve trauma [22], glutamate toxicity [29], spinal cord injuries [12,20], CNS trauma [13,16], as well as amyotrophic lateral sclerosis (ALS) and other motor neuron diseases [24,26,30].

Lasers are widely used in ophthalmology, most commonly for treatments involving purposeful retinal destruction. Other types of laser surgeries can result in complications, including retinal damage [31]. Accidental retinal damage has also been reported in ophthalmic practice [32], as well in the course of laboratory, industrial, and military use of lasers [8]. Laser weapons aimed at damaging electro-optical sensors and visually incapacitating soldiers by destroying parts of their retinas have recently been developed [32]. As in other CNS injuries, laser burns to the retina are also characterized by secondary degeneration that causes destruction of tissue adjacent to the primary laser lesion [1,8,33–36]. We recently reported that secondary degeneration following laser retinal burns in rats can be demonstrated functionally by electroretinography (ERG) [37]. In that study, this noninvasive electrophysiological tool was used to monitor and record the progressive loss of function of the outer retina following a primary injury caused by laser burns [37]. The aim of the present study was to functionally evaluate the neuroprotective effect of Cop-1 treatment for retinal laser burns in rats.

METHODS

Experimental Model

Twenty-three 90-day-old pigmented DA male rats (strain DA/Ola/Hsd, purchased from Harlan Olac, Blackthorn, Bicester, England, and raised in the animal house of Tel-Aviv University) were used in this study. All procedures in the rats were carried out according to regulations formulated by the Institutional Animal Care and Use Committee (IACUC) and conformed with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Animal Care and Use Committee of Tel-Aviv University.

Cop-1 and Placebo Treatments

Seven days before the laser photocoagulation session, 11 rats were immunized by injection into both hind-footpads of 200 μg of Cop-1 (Teva Pharmaceutical Industries, Petah Tikva, Israel) emulsified in an equal volume (0.2 ml) of complete Freund's adjuvant (CFA). At the same time, 12 rats received placebo treatment with saline and served as a negative control group. This time schedule was chosen since neuroprotective effect of Cop-1 was demonstrated when it was given 10 or 7 days prior to glutamate intravitreal injection [15,38] or 7 days ahead the injury in an animal model of head injury [39].

Laser Injury

Rats were anesthetized by intraperitoneal injections of 20 mg ketamine and 1 mg xylazine. The right pupil of each rat was dilated with sterile drops of tropicamide 0.5%. A contact lens, designed and crafted in our institute to fit a rat eye for retinal laser irradiation, was attached to the cornea with hydroxypropyl methylcellulose 2.5%. Standard argon laser lesions were produced in the visible retina of the right eye of each rat by the use of a Coherent Novus 2000 argon laser (Laser Innovations, Santa Paula, CA). Laser settings were 514 and 544 nm, 200 μm spot size at 0.1 W for 0.05 seconds. These settings were previously found by our group to result in lesions of uniform size and configuration, involving mainly the outer retinal layers [10]. The histological and functional consequences of these lesions in the rat retina have been described in detail [37]. The left eye of each rat served as a normal control.

Flash Electroretinographic Recordings

Flash ERG was used to assess changes in function of the outer retinal layers of the lasered right eye relative to the normal left eye in all 23 rats (i.e., in both the Cop-1-treated and the saline-treated group). The sequential order of eye recordings in each rat at 3, 20, or 60 days after laser injury was determined randomly. Recording was done by an investigator who was unaware of the treatment received by the rats.

Rats were anesthetized by intramuscular injection of ketamine 85 mg/100 g and xylazine 3 mg/100 g body weight and their pupils were dilated with sterile drops of tropicamide 0.5%. The rats were placed in a Faraday cage on a pad heated with warm water. Before recording was started the rats were dark-adapted for 10 min. A white stroboscopic stimulus was then delivered by a xenon flash stimulator (Nicolet Biomedical, Madison, WI) at an intensity of 2.1 log cd/m^2 and a frequency of 0.1 Hz. Retinal signals were recorded with a corneal contact lens electrode designed for use in rats (Medical Workshops, Groningen, The Netherlands). Subcutaneous needles served as reference and ground electrodes, and were placed at the temporal canthus of the ipsilateral eye and at the base of the ear, respectively. Signals were amplified with a 2–250 Hz band-pass (without a notch filter), averaged online ($n = 10$), and stored for subsequent analysis.

The effect of lasering on the *a*- to *b*-wave amplitude was measured in terms of the ratio, defined as the ratio (expressed as a percentage) of the amplitude of the right (lasered) eye to that of the left (intact) eye at each of the three time points after lasering. A paired Student's *t*-test was used to test for differences between eyes on the same individuals. A Student's two-group *t*-test was used to test for differences between treatment groups at

individual time points. A sequentially rejective method of adjusting for multiple comparisons [40] was used for decision-making as to whether *P*-values were significant at the nominal Type I error probability of 0.05.

RESULTS

Sample traces recorded from both eyes of a treated animal and a control animal (3 and 60 days post-lasering) are shown in Figure 1. The traces are the mean. To assess the functional impairment of the lasered eye, we calculated the ERG response ratio of the injured to the uninjured eye. This was done by averaging response to 10 flashes of light by the recording software and dividing the *a*- to *b*-wave amplitude of the lasered eye by that of the intact (contralateral) eye and multiplying by 100. Figure 2 presents the mean ($\pm$SEM, %) ratios of the responses of the experimental and control groups, recorded 3, 20, and 60 days post-lasering.

In the untreated group, the responses of the lesioned eyes were significantly lower than those of the intact eyes at 3 days ($P = 0.0035$), 20 days ($P = 0.021$), and 60 days ($P = 0.029$) post-lesion. In other words, in untreated animals the lesion caused a functional deficit that lasted at least 60 days, as reported in our previous study [40]. In the treated group, the responses of the lesioned eyes were significantly lower than those of the intact eyes at 3 days ($P = 0.005$), but there were no significant differences between lesioned and intact eyes at 20 days ($P = 0.36$) and 60 days ($P = 0.37$). This indicates that in the treatment group there was a temporary deficit due to the lesion (at 3 days), but treatment enabled recovery at 20 and 60 days.

The ERG response ratios (means $\pm$ SEM) of the lasered eyes relative to the intact eyes in saline-treated control animals were $69.8 \pm 8.5\%$, $83.5 \pm 8.0\%$, and $85.8 \pm 5.5\%$, at 3, 20, and 60 days post-lasering, respectively. The respective ratios for the Cop-1 treated animals were $68.7 \pm 8.7\%$, $99.7 \pm 7.1\%$, and $99.5 \pm 10.2\%$. There was no significant difference in the response ratio for the 2 groups at 3 days ($P = 0.59$), that is, in both groups there were deficits in the injured eye. However, there was evidence that the ratios were different at 20 days ($P = 0.03$) and 60 days ($P = 0.04$), indicating a functional improvement in the treated group but not in the control group.

DISCUSSION

The results of this study show that in rats subjected to retinal laser injury, pre-immunization (7 days prior to lasering) with Cop-1, a small synthetic amino-acid polymer which acts as a weak universal antigen, has a neuroprotective effect on the function of the outer retina. As we reported previously, our laser photocoagulation protocol

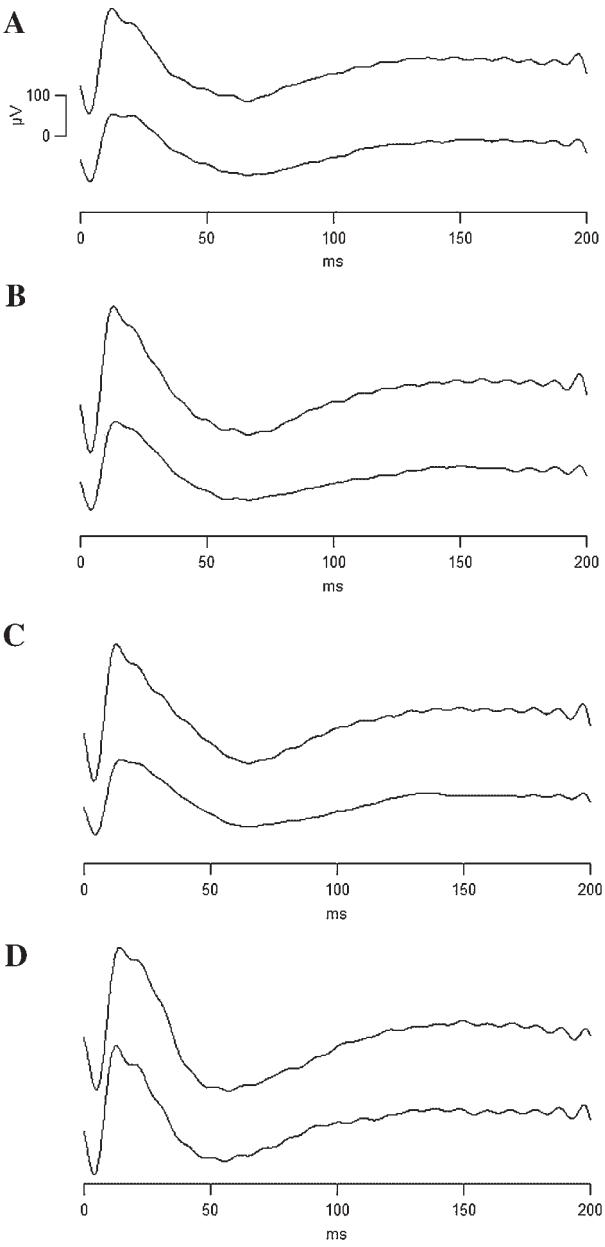

Fig. 1. Sample traces recorded from both eyes of a treated animal and a control animal. The traces are the mean response to 10 flashes of light (0.1 Hz), averaged online by the recording software. Signals recorded 3 and 60 days post-lasering are shown. In each panel, the response of the intact (unlasered) eye is shown at the top and the response of the lasered eye is shown at the bottom. It can be appreciated that the signals in the intact eyes of both animals are similar, and did not change between 3 and 60 days. **A**: Control (untreated) animal 3 days post-lasering. A significant decrease in the response of the lasered eye (bottom trace) may be seen. **B**: Same animal as in panel A, 60 days post-lasering. Minimal recovery in the response of the lasered eye may be seen. **C**: COP-1 treated animal 3 days post-lasering. A significant decrease in the response of the lasered eye (bottom trace) may be seen, similar in magnitude to the decrease seen in panel A. **D**: Same animal as in panel C, 60 days post-lasering. A significant recovery in the response of the lasered eye is seen (compare to the minimal recovery seen in panel B).

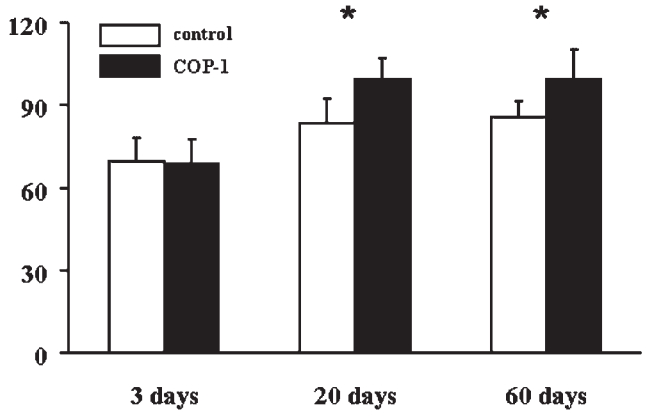

Fig. 2. The ERG (a- to b-wave) response ratios (means ± SEM, %) of the lasered eyes relative to the intact eyes in saline-treated and in Cop-1-treated rats. For each animal we calculated the ratio between the responses of the lasered eye relative to the intact eye. Mean (± SEM, %) ratios of the responses of the experimental and control groups, recorded 3, 20, and 60 days post-lasering are presented. * $P<0.05$.

causes significant functional and morphological damage in the outer retina of the rat [37]. The present study suggested, however, that in rats pre-immunized with Cop-1, the functional deficit is transient. Normal (baseline) function was restored here by 20 days after the injury, and continued for at least 60 days after the laser session. In contrast, in saline-treated rats the decrease in retinal function persisted throughout the follow-up period, and any recovery observed was insignificant. It can be thus concluded that Cop-1 is an effective neuroprotectant in laser-induced retinal lesions.

The increasing use of lasers in medicine, industry, laboratory, entertainment, and the military has resulted in many accidental ocular injuries. The lesions are almost always retinal, because this is the site where visible and near infrared radiation are concentrated by the eye's refractive media. In the retina, as in any injured neural tissue, the lesion spreads because noxious agents are released by the directly injured neurons. This secondary degeneration, as well as the scarring process that follows severe injuries, is responsible for the frequently observed increase in morphological and functional damage beyond that directly caused by the primary lesion. Laser lesions have indeed been shown to spread for hours or days after the primary injury [33,41,42], and retinal photocoagulation spots can reportedly progress to retinochoroidal atrophy even years after laser treatment [34,43].

Several modalities of neuroprotective treatment have been proposed in the attempt to halt and treat the progression of secondary degeneration. The theory of neuroprotective immunization is based on the work of Schwartz and Kipnis [44], who showed that a T cell-mediated immune response is neuroprotective after injury, and that its effect can be boosted [16,45–47] both by passive transfer of T cells specific to myelin basic protein [45] and by active therapeutic vaccination [46,48]. Adoptively transferred, activated T cells accumulate at the site of optic nerve injury in rats [49] and in mice [22]. Active immunization also results in increased homing of T cells to the site of a CNS lesion [13,19,50,51].

Neuroprotective immunization with Cop-1 has been found beneficial in animal models of glaucoma [15,28], optic nerve trauma [22], glutamate toxicity [29], spinal cord injuries [12,20], CNS trauma [13,15], and ALS and other motor neuron diseases [24,26,30]. The present finding that Cop-1 treatment is neuroprotective in laser-induced retinal lesions is of particular interest because it demonstrates, for the first time, that Cop-1 is neuroprotective in unmyelinated neural tissue. All previous studies that demonstrated the neuroprotective action of Cop-1 were conducted in models of damage to myelinated neural cells [12–16,20,22,24,26,28–30].

ERG response measurements are known to vary widely between individuals, probably because of technical difficulties in achieving exactly the same setup for recording electrodes and depth of anesthesia in different experimental animals. We minimized this variability by comparing signal amplitudes in both eyes of the same animal (i.e., by providing an internal control). Comparison of the amplitude ratio between the lasered and the intact eye of the same animal is obviously more meaningful than a comparison of the absolute ERG amplitudes of different animals from different experimental groups.

Because Cop-1 was administered together with an adjuvant in the present study, the present formulation is not suitable for testing of humans. Future animal studies should also address the question of dosage, and extend the investigation over a longer follow-up period. It should be noted, however, that the neuroprotective effect of Cop-1 without adjuvant has been successfully demonstrated in recent studies using other animal models of retinal damage [15,28]. Future studies should therefore evaluate the neuroprotective effect of Cop-1 without adjuvant in the rat model of retinal laser burns. Pending successful results, the next step will be to conduct clinical trials in humans, because Cop-1 without adjuvant is approved for use in patients with multiple sclerosis. Depending on the outcome of clinical studies, it might be possible to develop the neuroprotective vaccination for prevention and treatment of various neurological and ophthalmic diseases, including glaucoma, ischemic optic neuropathy, and different kinds of retinal degeneration and optic neuropathies, in addition to its potential prophylactic use against laser-induced retinal injuries.

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