MK-801 Has Neuroprotective and Antiproliferative Effects in Retinal Laser Injury

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**Purpose.** Treatment of the retina by laser photocoagulation often is complicated by an immediate side effect of visual impairment, caused by unavoidable, laser-induced destruction of healthy tissue adjacent to the lesion. A neuroprotective therapy that salvages this healthy tissue might enhance the benefit obtained from the treatment. This study was proposed to determine whether glutamate-receptor blockers can provide adjuvant neuroprotection during laser photocoagulation. The effect of MK-801, an NMDA-receptor antagonist, on laser-induced retinal injury was examined, in a rat model.

**Methods.** Argon laser lesions were created in the retinas of 36 DA rats, and were followed immediately by intraperitoneal injections of MK-801 (2 mg/kg) or saline. The animals were killed after 3, 20, or 60 days and the retinal lesions were evaluated histologically and morphometrically.

**Results.** Photoreceptor-cell loss was significantly less in MK-801-treated rats than in control animals. The proliferative membrane composed of retinal pigment epithelial cells and neovascular blood vessels, which was seen at the base of the lesion in control group retinas, was smaller in the MK-801-treated retinas. In rats treated with a higher dose of MK-801, the lesions showed almost no proliferative reaction.

**Conclusions.** A potent noncompetitive NMDA-receptor blocker, MK-801 exhibits neuroprotective and antiproliferative properties in the retina. Glutamate-receptor blockers should be investigated further as potential adjuvant therapy in retinal photocoagulation treatments.

line on the acuity chart), and 20% of patients suffered from severe visual loss (six or more lines from baseline) as a direct result of the treatment.

A similar, though less severe side effect, was observed when juxtapfoveal macular CNVs were treated with krypton laser photocoagulation. Again, immediately after the treatment, 18% of the eyes exhibited severe visual loss. This side effect is a result of the dynamic expansion of the laser-induced lesion, involving destruction of the neurosensory retinal tissue located in the fovea just next to the treated CNV. Therefore, the immediate disabling side effect of the laser photocoagulation—progressive enlargement of the laser scar accompanied by a significant decrease in visual acuity—is of major concern when selecting and preparing patients for treatment. A neuroprotective therapy capable of preserving the neurosensory retina from the injurious effects of the laser irradiation, though not impairing its efficacy in destroying the CNV, will protect the patient's vision and enhance the beneficial effects of the treatment. Glutamate-receptor blockers are promising candidates for such a neuroprotective role.

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). It is now well established that glutamate plays a crucial role in mediating neuronal damage in CNS injury. After CNS insult the damaged neurons release large amounts of glutamate, which in turn, by activating its N-methyl-D-aspartate (NMDA) receptor, causes damage to the surrounding neurons by changing their intracellular ionic concentrations. This ionic imbalance causes acute swelling of the cell and activation of most of its calcium-dependent enzymes, which in turn leads to the cell's death. The biochemical cascade that originates in the injured neurons amplifies the initial trauma and eventually causes the damage to spread to the adjacent neuronal tissues. It is well documented that NMDA–receptor antagonists (like MK-801) act as neuroprotective agents in a variety of CNS lesions, both ischemic and traumatic. Thus, attempts are now being made to develop an effective clinical therapy using novel NMDA–receptor blockers in the recipients of previously tested agents, including MK-801, demonstrated serious behavioral side effects.

Glutamate, as the main neurotransmitter of the photoreceptor neurons, plays an important role in the normal functioning of the retina and might be involved in the pathogenesis of laser-induced retinal damage and other retinal disorders. This latter possibility is suggested by the vulnerability of retinal neurons to glutamate and NMDA and by the neuroprotective effect of MK-801 in different retinal injuries.

If the pathogenic mechanisms underlying the spread of neuronal death after retinal laser injury are indeed mediated, at least partially, by glutamate, they might be expected to be attenuated as a result of treatment with NMDA antagonists. Such treatment might salvage retinal neural cells from the laser-induced destruction and potentially serve as a candidate for therapy of laser-induced retinal injury, both accidental and iatrogenic.

In the current study, we tested this hypothesis by examining the neuroprotective properties of MK-801, a potent noncompetitive NMDA–receptor blocker. The drug was evaluated in a pigmented rat model of retinal lesion induced by argon-laser irradiation.

**METHODS**

Thirty-six inbred pigmented DA rats (Strain DA/OLa/Hsd, Harlan OLAC, Blackthorn Bicester Oxon, England; raised in Tel-Aviv University animal house), 90 days old, were used. This strain possesses a uniform pigmentation of the posterior segment of the eye, making it particularly useful for use in research on retinal laser injury. The animals were fed ad libitum and maintained on a 12-hour light–dark cycle. The experiments conformed to the Association for Research in Vision and Ophthalmology's statement for the Use of Animals in Ophthalmic and Vision Research.

The animals were anesthetized by intraperitoneal injections of ketamine (40 mg/kg) and xylasine (8 mg/kg). After dilatation of the pupil with sterile drops of topicamide 0.5% (Mydramid, Fischer), a contact lens, specially constructed by the authors to fit a rat eye, was coupled to the cornea with 2.5% hydroxypropyl methylcellulose. Six argon-laser (Emerald Crystal Focus, Mountain View, CA) lesions (514 nm, 200 μm, 0.1 W, 0.05 second) were produced in each eye, two disc diameters from the optic disc. Half of the lesions were inflicted in the superior portion of the retina and the other half in the inferior retinal area. The laser settings were shown in a data from a preliminary study to result in relatively uniform lesions of similar size and configuration, involving mainly the outer retinal layers, retinal pigment epithelium (RPE) and choroid. The experimental group (n = 18) received intraperitoneal injections of MK-801 (Research Biochemical International, Natick, MA) in normal saline, at a dose of 2 mg/kg, immediately after the laser irradiation. At the same time a vehicle-treated control group (n = 18) received the same volume of saline.

The rats were killed 3, 20, or 60 days after laser irradiation (n = 6 in the treated and in the control groups at each time point) with phenobarbital overdose, and their eyes were enucleated and fixed in 2% glutaraldehyde. Using a surgical microscope, the posterior segments of the eyes were dissected into tissue.
samples, each incorporating one retinal laser lesion. The samples were embedded in plastic (Epon) and blocks were serially sectioned (2 μm) with an ultramicrotome and stained with toluidine-blue. Sections from the central part of the lesion, exhibiting the greatest amount of laser-induced retinal destruction, were collected and examined by light microscopy for histopathologic changes.

To evaluate further the neuroprotective effect of MK-801 in the retinal lesions, a masked-fashion, quantitative morphometric assessment was carried out using a computer-assisted image analysis system. The microscopic images (Olympus BH2 microscope with a working magnification of ×66) of the retinal sections were acquired by a CCD video camera (Sony RGB-CCD). The images were digitized by an image-grabber board and analyzed using image analysis software (ScanArray3, Galai Productions, Migdal-HaEmek, Israel). Two morphometric measurements were obtained for each lesion to evaluate the severity of the argon-laser injuries. The first measured the diameter of the lesion by determining the edges of the lesion area, according to the changes in the RPE and in the cytoarchitecture of the different retinal layers (Fig. 1A). The second evaluated the extent of photoreceptor-cell loss. This was done by calculating the ratio between the number of surviving nuclei in the outer nuclear layer (ONL) at the area of the lesion and the number of ONL nuclei in the healthy retina situated at the lesion border (the area of the healthy retina from which the number of ONL nuclei was calculated was equivalent in size to the measured area of the lesion). A total of 432 lesions were subjected to the histopathologic and morphometric evaluations.

Because the results showed both neuroprotective and RPE antiproliferative effects of the MK-801 treatment, the drug was tested further using a higher dose (3 mg/kg intraperitoneally, immediately and 8 hours after the laser irradiation). The lesions in the treated and in the control groups (n = 3 for each time point) were processed as described above.

A two-tailed, unpaired Student's t-test was used to analyze the significance of differences between the results of the MK-801-treated and the control groups at each time point.

RESULTS

Light microscopy revealed histopathologic differences between the saline-treated control and the MK-801-treated groups (Fig. 1). Three days after laser irradiation, the lesions of the control group (Fig. 1A) showed the following local histopathologic changes of the outer retina and of the choroid: At the central area of the lesion there was loss of choriocapillaries and disruption of Bruch's membrane. The RPE showed local proliferation with formation of a fusiform membrane containing macrophagic cells. The outer and inner segments of the photoreceptors were deformed and disrupted, and the ONL showed loss of nuclei and the presence of pyknotic nuclei at the periphery of the lesion. The photoreceptor segments and the ONL tapered off toward the center of the lesion, where they were always completely absent. This central area was filled with cellular debris and pigment-laden macrophages. The outer plexiform layer was disrupted, and the inner nuclear layer was mildly edema-
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IPL = internal plexiform layer; ONL = outer nuclear layer; C = choroid.

tous. The inner plexiform layer, the ganglion cell layer, and the nerve fiber layer were unremarkable.

In MK-801-treated animals (Fig. 1B), the retinal response was milder than that in the controls. Three days after laser irradiation, the RPE at the base of the lesion showed mild proliferation. Only a few macrophages were seen in the area of the disrupted segments and the subretinal membranes were less developed. The ONL showed loss of nuclei and the presence of pyknotic nuclei. Mild cystic changes were seen in the inner nuclear layer and in the inner plexiform layer, whereas the ganglion cell layer and the nerve fiber layer were unremarkable.

By 20 days after irradiation, the proliferative membranes in the lesions of the saline-injected rats had become multilayered and showed occasional neovascularization. The RPE layer had reformed and was lying on the subretinal membrane. The inner nuclear layer was still edematous, and the nerve fiber layer and the inner limiting membrane were folded internally, creating some internal bulging at the inner retinal surface over the area of the lesion. This bulging is assumed to be a result of the edema of the inner retinal layers and the traction of the healthy retinal layers at the edge of the lesion toward its center. In contrast, the proliferating membranes of the RPE in the MK-801-treated animals were smaller and less developed with only occasional neovascular blood vessels. The internal bulging seen in the MK-801-lesions in the untreated group was similar to that seen in the controls.

In control group retinas, 60 days after irradiation (Fig. 2A), the subretinal membrane had diminished in size, had become more fibrotic, and contained numerous small blood vessels. The outer and inner segments of the photoreceptors were disrupted only at the central area of the lesion and had regenerated at its periphery. Occasional pigment-laden macrophages were seen in the central area at the level of the segments and the ONL. The ONL showed fewer pyknotic nuclei. The internal bulging had not changed its shape compared with the 20-day-old lesions. In the MK-801-lesions in the treated group (Fig. 2B), the morphologic appearance 60 days after laser irradiation resembled that of the lesions in their control counterparts. However, in the lesions in the treated group the proliferative membranes were still small and underveloped, and the ONL showed less damage.

To characterize further the effects of treatment with MK-801 on laser-induced retinal lesions, a second stage of the study was performed where the drug was delivered twice (immediately after the laser inflections and again after 8 hours) at a higher concentration (3 mg/kg, intraperitoneally). Three days after laser irradiation of these animals, the histologic appearance of the lesions resembled that of the lesions in their counterparts that had received a single dose of 2 mg/kg. The only difference detected was the complete absence of the RPE proliferative membrane in any of the lesions. By 20 days after irradiation, both the control and the treated animals again resembled their counterparts in the first stage of the experiment. Whereas the proliferative reaction in the RPE in the control group lesions was organized as a thick multilayered fusiform membrane composed of phagocytic cells, fibroblasts and occasional neovascular elements (Fig. 3A), only a thin proliferative membrane was seen in part (40%) of the MK-801-treated rats (Fig. 3B). The same phenomenon was observed 60 days after
FIGURE 3. High-power micrographs of the proliferative membranes seen in lesions in the control group (A) and in lesions in the MK-801-treated group (3 mg/kg) (B), 20 days after exposure. (A) A thick, multilayered fusiform membrane (asterisk) was seen in the central area of the lesion internal to the choroid. It was composed of phagocytic cells, fibroblasts, and occasional neovascular elements (arrows). The retinal pigment epithelium was reformed, after 20 days, and lined the membrane completely. (B) Thin proliferative membranes (asterisk) were seen in some of the MK-801-treated rats. RPE = retinal pigment epithelium; C = choroid.

irradiation when proliferative membranes were absent (or, at most, small and undeveloped) in most of the treated group lesions.

Morphometric measurements of the laser-induced retinal lesions (Figs. 4, 5) also revealed significant differences between treated and control groups (as results for the 2 mg/kg and the 3 mg/kg dosage regimes were similar, only the former are presented here and compared with those of lesions in the control group).

Within both groups, lesions at the superior and inferior halves of the retinas did not differ in their histopathologic appearance or in their morphometric parameters, indicating their equal vulnerability to the destructive effects of the laser irradiation.

The mean diameter of the lesions in the control group decreased during the first 20 days after the injury (Fig. 4), though not significantly. It also did not change significantly thereafter. The ratio between the numbers of surviving ONL nuclei at the area of the lesion and at the adjacent healthy retina became pro-

FIGURE 4. Effect of MK-801 on the mean diameter of the retinal lesion. Diameters of the lesions in the MK-801-treated group (asterisks) were significantly smaller than those of the lesions in the control group.

FIGURE 5. Effect of MK-801 on the mean percentage of surviving cells in the outer nuclear layer. The number of surviving photoreceptor cells (asterisks) was significantly higher in the lesions in the MK-801-treated group compared with those in lesions in the control group.
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gressively higher in the lesions in the control group as time passed (Fig. 5). This probably happened as a result of the traction of healthy retinal layers at the edge of the lesion toward its center, mainly during the first 20 days ($P < 0.0001$). Three days after laser irradiation, the mean diameters of the lesions in the treated and control groups did not differ (Fig. 4). At 20 and 60 days, however, the MK-801–lesions in the treated group became significantly smaller than those in their control counterparts ($P < 0.001$ and $P = 0.01$, respectively). Differences in the ratios of surviving photoreceptor cells between MK-801–treated and lesions in the control group were also significant (Fig. 5): at 3 and 20 days after irradiation the treated and lesions in the control group had similar ratios of surviving ONL nuclei, whereas at 60 days the ratio of surviving photoreceptor cells was higher in the treated group (by 17.2%; $P < 0.01$).

**DISCUSSION**

The results of this study show that MK-801, an NMDA–receptor blocker, exhibits neuroprotective and anti-proliferative properties in retinal laser lesions. The histopathologic changes in argon-laser–induced retinal lesions were followed for 60 days. Throughout the period, evidence of two major histopathologic processes was observed: traction of adjacent surviving photoreceptor cells and other retinal layers into the central area of the lesion, and a proliferative reaction in the RPE associated with subretinal neovascularization and invasion of the retinal lesion site by phagocytes. Treatment with MK-801 substantially attenuated these reactions. Significant numbers of photoreceptor cells were salvaged from laser destruction, as shown by the fact that at 60 days the lesions in the treated group were smaller in diameter than lesions in the control group and the number of surviving ONL nuclei at the area of the lesion was higher. The drug also attenuated the proliferative reaction in the RPE, as indicated by the small size of the subretinal membranes in the MK-801–treated retina. Administration of a higher dose of MK-801 failed to rescue more photoreceptor cells but did enhance its antiproliferative effect on the RPE cells: No membranes could be detected in any of the lesions in the treated group 3 days after irradiation, and the membranes that eventually did form in some of the lesions were small and undeveloped.

It is important to note that the ketamine anesthesia used in this study is known to interact with glutamate receptors and it might have a confounding effect on the extent of the tissue damage. However, because both control and MK-801–treated animals received similar doses of ketamine, the protective effects of MK-801 still stand.

Ocular laser photocoagulation therapy is used as a routine treatment for many retinal disorders. A frequent disabling side effect is an immediate and progressive visual impairment, similar to that described in macular photocoagulation therapy for age-related macular degeneration and in grid photocoagulation treatment for diffuse diabetic macular edema.

This visual detriment is a result of the nonselective destruction of healthy retinal elements by the laser beam and by the spreading of the laser-induced destructive effects to adjacent healthy retinal tissue. An adjuvant therapy that is meant to preserve the neurosensory retina from the injurious effects of the laser irradiation, while not impairing its efficacy in destroying the retinal pathology, will protect the patient’s sight and enhance the beneficial effects of the treatment. Although laser injury to retinal neurons has generally been considered an irreversible phenomenon that cannot be halted or slowed down, new insights into the pathologic mechanisms involved in this process have provided a theoretical basis for evaluating various pharmacologic strategies to induce neuroprotection.

Most of the available information on neuroprotection comes from studies of the CNS after traumatic or ischemic injury. It is now well documented that much of the tissue damage after injury results from delayed inflammation and autodestruction. It seems likely that the retina, which is part of the CNS, is also susceptible to these harmful processes and might respond to drugs shown to have neuroprotective properties in the CNS. Neuroprotection was found to be effective in optic nerve trauma models (Yoles et al). We have previously demonstrated that methylprednisolone exerts a mild though significant neuroprotective effect in the laser-injured retina, as it does in spinal cord trauma.

Among the most intensively studied pharmacologic agents for reducing neurotoxicity during CNS damage are glutamate receptor antagonists. Glutamate plays a dominant role in CNS and retinal neurotransmission. However, exposure of neurons to high concentrations of extracellular glutamate can lead to their death. It is now well established that after CNS injury the damaged neurons release massive amounts of glutamate, which interact with adjacent cells and eventually destroy them. The development of selective NMDA–receptor antagonists has facilitated examination of the role of glutamate in various CNS disorders and demonstrated their neuroprotective properties in these pathologic situations.

The neurocytotoxic effect of glutamate, which is mediated through activation of its NMDA receptors, also was demonstrated in the retina both in vivo and in vitro. Administration of MK-801, a potent noncompetitive NMDA–receptor
blocker, to retinal neurons in culture improved their survival after exposure to glutamate. Asrar et al described similar neuroprotective effects of MK-801 in whole retinas exposed to exogenous glutamate in vitro. In that study, NMDA-receptor antagonists also attenuated neuronal damage in response to hypoxic insult. The results provided evidence that synaptic release of glutamate mediates the death of hypoxic retinal neurons. This finding was further supported by the work of Mosinger et al, who demonstrated the neuroprotective role of MK-801 in an in vivo model of retinal ischemia. The drug reduced the severity of acute ischemia-induced histologic changes in the rat retina. Continuous infusion of dextromethorphan, a powerful, competitive NMDA antagonist, greatly enhanced the immediate and late posts ischemic recovery of the β-wave amplitude of the electroretinogram.

In the results of the histopathologic and functional studies described above, the neuroprotective effect of the NMDA blockers was evident in retinal ganglion cells that were involved in the injury. Our study's findings, on the other hand, demonstrate the neuroprotective effect of MK-801 on photoreceptor cells. This finding is somewhat surprising, because under normal circumstances photoreceptor cells do not express NMDA receptors. They may, however, express NMDA receptors as a result of their exposure to high extracellular levels of glutamate. This phenomenon has been demonstrated previously in the CNS, where NMDA receptors were induced in cerebellar granule cells exposed to high levels of NMDA. MK-801 might also exert its neuroprotective effect by enhancing the release of such growth factors as neurotrophin-3, which by themselves promote the survival of the photoreceptor cells. MK-801 is also known to enhance neuronal survival in a manner independent of its anti-NMDA activity. Nevertheless, the exact mechanism by which MK-801 exerts its neuroprotective effect on photoreceptor-cell survival has yet to be elucidated. Another unanswered question concerns the participation of other types of glutamate receptors, (the kainate-AMPA receptors, for example) in propagating the destructive effect of laser irradiation on the retina, as they do in other retinal insults.

Our study's results also point to a less well recognized property of MK-801, namely its antiproliferative effect on RPE cells. Treatment with MK-801 (3 mg/kg) blocked RPE membrane formation at 3 days after the insult. By 20 and 60 days after laser exposure the membranes had developed but were seen in only 40% of retinas in the treated group and were smaller than in the retinas in the control group. The development of smaller membranes in the treated animals might simply reflect the milder destruction of their internal retinal layers: the smaller the lesion, the weaker the proliferative reaction to it. The antiproliferative property of MK-801 might be expected from the role of glutamate in RPE metabolism, where it enhances both proliferation and phagocytic activity of RPE cells in vitro; both effects are mediated by an NMDA-receptor-coupled mechanism. Blocking of these effects with MK-801 probably resulted in underdevelopment of the subretinal proliferative membranes. It has yet to be determined, however, whether this antiproliferative effect has any clinical implications in different retinal disorders.

Our results suggest that glutamate plays a key role in mediating retinal injury induced by laser irradiation. It contributes to the mechanisms involved in the destruction of the photoreceptor cells, while also enhancing the proliferative response of the RPE to the insult. Antagonism of the glutamate-induced effects at its NMDA receptor significantly improves the outcome of the laser-induced retinal damage. Further studies of glutamate-receptor blockers are warranted to evaluate their therapeutic potential as acceptable therapy for retinal laser injury, both accidental and iatrogenic.

Key Words

glutamate, laser photocoagulation, MK-801, NMDA, retina

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