Minocycline Inhibition of Photoreceptor Degeneration

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Objective: To determine whether systemic minocycline can protect photoreceptors in experimental retinal detachment (RD).

Methods: Retinal detachment was induced in mice by subretinal injection of sodium hyaluronate, 1.4%. In 1 experiment, mice received daily injections of minocycline (group 1) or saline (group 2). In a second experiment, mice were treated with minocycline or saline beginning 24 hours prior, immediately after, or 24 hours after experimental RD. In both experiments, photoreceptor cell survival and apoptosis were assessed by immunohistochemistry with primary antibodies against photoreceptor cell markers, rod rhodopsin, and cone opsin, and by terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling.

Results: Photoreceptor cell apoptosis was detected at day 1 after experimental RD, with apoptotic cells peaking in number at day 3 and dropping by day 7. Treatment with minocycline significantly reduced the number of apoptotic photoreceptor cells associated with RD when given 24 hours before or even 24 hours after RD.

Conclusions: Our data suggest that minocycline may be useful in the treatment of photoreceptor degeneration associated with RD, even when given up to 24 hours after RD.

Clinical Relevance: Use of minocycline in patients with macula-off RD may prevent photoreceptor apoptosis and glial cell proliferation, improving final visual outcomes.

Minocycline, a semi-synthetic tetracycline antibiotic, is reported to be neuroprotective in degenerative disease and ischemic models of the central nervous system (CNS). The mechanisms of its neuroprotective properties have not been fully defined, but evidence suggests that it may arise through 2 separate mechanisms that are distinct from the drug’s antibiotic attributes. The first of these mechanisms is its direct anti-apoptotic effects, possibly acting on the caspase cascades in the cell suicide sequence and blocking cytochrome c release from mitochondria. The second mechanism involves anti-inflammatory effects, including prevention of activation and migration of microglia, the resident macrophages of the CNS, whose activity during neuropathologic states and cytotoxic potential have implicated them as mediators of neuronal loss. Minocycline’s neuroprotective properties, high penetration through the blood-brain barrier, and favorable safety profile have made it a desirable candidate as therapy for acute and chronic neurologic injury.

Retinal neurodegeneration characterized by photoreceptor dysfunction, death of rods and cones, and eventually irreversible loss of vision is one of the leading causes of blindness around the world. Such diseases include age-related macular degeneration, diabetic retinopathy, retinal detachment (RD), and retinitis pigmentosa. Retinal neurons share a common feature with those of the CNS—they regenerate poorly or not at all once damaged. Previously, it has been reported that RD-induced retinal damage is most prominent 3 days after RD and drops down to a low basal level by day 7. Currently, no effective treatment is available to either prevent or cure neurodegeneration of retinal neurons, and thus development of drugs that can protect retinal neurons from degeneration is crucial for preserving vision under these pathologic conditions.

It has been reported that minocycline is effective at protecting the retina from light-induced photoreceptor damage when delivered 1 day before damage is induced, implicating the potential application of the drug to treat retinal injury. Previously, we have established an experimental model of RD in mice that enables identification of cellular and molecular targets for pharmacological manipulation and screening of drug candidates for treating retinal photoreceptor degeneration. Multiple lines of evidence have indicated that apoptosis is a major cause of photoreceptor degeneration and vision loss in experimental RD. The process involves caspase acti-
vation and mitochondrial-dependent cell death pathways.\textsuperscript{12,13} Bax, as a pro-apoptotic member of the Bcl-2 family, which regulates cytochrome c release from the mitochondria, which in turn causes subsequent caspase activation,\textsuperscript{15} is a central player in RD-associated photoreceptor cell death.\textsuperscript{9} Moreover, our recent studies indicate that activation of retinal glial cells contributes critically as a secondary mechanism that induces collateral damage of the retina following RD. Given the 2 independent neuroprotective mechanisms of minocycline, we speculate that the drug may have a potent protective effect for photoreceptor cells after RD.

In the present study, we examined the effect of minocycline in a mouse model of RD. Our results indicate that treatment up to 24 hours after creation of the experimental RD with minocycline significantly attenuates RD-induced photoreceptor cell loss and may be a novel therapy for retinal neurodegeneration in the future.

**METHODS**

**RETINAL DETACHMENT**

All experimental procedures and the use of animals followed the protocol approved by the Schepens Eye Research Institute’s Animal Care and Use Committee, Harvard Medical School, and followed the standards of the Association for Research in Vision and Ophthalmology. Adult (aged 8-12 weeks) C57BL/6J mice were anesthetized by intraperitoneal injection of a mixture of ketamine (12.5 mg/mL) and xylazine (2.5 mg/mL) (Pfizer Pharmaceutical Inc, St Joseph, Missouri). The left pupil of the mouse was dilated with topical application of cyclopentolate, 1%, and phenylephrine hydrochloride, 2.5% (Akorn Inc, Buffalo Grove, Illinois). Sclera puncture was made using a glass micropipette to lower the intraocular pressure, and 2 µL of sodium hyaluronate, 1.4% (Healon GV, Pharmacia & Upjohn, Sweden), was injected slowly into the subretinal space with a glass micropipette.

For the first experiment, mice that underwent the retinal detachment procedure were randomly divided into 2 groups: 1 group received daily intraperitoneal injection of minocycline hydrochloride (50 mg/kg; Sigma, St Louis, Missouri) beginning at 30 minutes after RD, and the other group received injection of the same volume of saline. In the second experiment, mice were divided into 3 treatment groups: the first group received intraperitoneal minocycline or saline 24 hours before RD, the second received intraperitoneal minocycline or saline 24 hours following RD, and the third received intraperitoneal saline 24 hours following RD.

**RETINAL HISTOLOGY AND IMMUNOHISTOCHEMISTRY**

Mice were euthanized at 1, 3, and 7 days after RD in the first experiment and on day 3 in the second. Their eyes were dissected, postfixixed with paraformaldehyde, 4%, for 1 hour, cryoprotected, and sectioned at 14 µm. Retinal sections were then subjected to cresyl violet staining or immunofluorescence labeling. Retinal sections were preblocked with phosphate-buffered saline containing bovine serum albumin, 5% (Sigma),...
and Triton X-100, 0.2%, for 30 minutes. They were then treated with primary antibodies overnight at 4°C, followed by reaction with a secondary antibody: Cy3-conjugated goat anti-mouse (1:200) or AMCA-conjugated donkey anti-chicken antibodies (1:50) (both from Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania). The primary antibodies used include an anti-activated form of caspase-3 (ASP175, 1:100; Cell Signaling Technology Inc, Danvers, Massachusetts), anti-CD11b (1:200; Serotec Inc, Raleigh, North Carolina), anti-rod/rhodopsin (1:2500), and cone opsin (1:2000) as well as glial fibrillary acidic protein (1:500) and vimentin (1:200), 2 hallmark proteins of glial activation. For some experiments, mouse eyes were dissected and fixed with formalin, 10%. Plastic retinal sections (3 µm) were prepared and subjected to hematoxylin- or toluidine blue staining to reveal retinal morphology.

### TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED dUTP-BIOTIN NICK-END LABELING

For terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) stains, retinal sections were permeabilized with a solution containing Triton X-100, 0.1% (Sigma), and sodium citrate, 0.1%, for 2 minutes at 4°C and then incubated with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturers’ guidelines. To identify the type of cells that were undergoing apoptosis, TUNEL and immunofluorescence labeling for photoreceptor and retinal cell markers were carried out on the same slides.

### QUANTIFICATION OF PHOTORECEPTOR CELL SURVIVAL, APOPTOSIS, AND MONOCYTE INFILTRATION

Photomicrographs of retinal sections were taken under a Nikon microscope (TE300) equipped with fluorescence illumination, a color cool digital camera (Spot MVI Inc, Avon, Massachusetts), and a computer. The TUNEL-, caspase-3-, and CD11-positive cells were counted in retinal sections, and areas of the detached retina were measured using National Institutes of Health imaging software (Image J 1.23y). The number of TUNEL-positive or immunolabeled cells per millimeters squared was tabulated. For each mouse, at least 3 separated retinal sections were examined, and the average was recorded. To quantify photoreceptor cell loss, cell layers in the outer nuclear layer (ONL) were counted in cresyl violet–stained retinal sections taken from mice that received treatment with saline (control; n=9) or minocycline (n=9) and were euthanized at various days after retinal detachment. Error bars indicate standard error of the mean. *P<.01, t test.

### RESULTS

#### RD-INDUCED PHOTORECEPTOR CELL DEATH AND MONOCYTE INFILTRATION

Successful RD, defined by detachment of more than one-third of the retina from the underlying retinal pigment epithelium, was confirmed under an ophthalmic microscope. Infiltrated monocytes were identified using immunostaining with antibody against bone marrow–derived monocyte marker CD11b. Consistent with previous reports, pyknotic nuclei, which suggest apoptotic cells, and collapsed inner and outer segment layers were observed at day 1 after RD (Figure 1A and B). In the minocycline group, CD11b<sup>+</sup> cells were found in the subretinal space, most of which attached to the surface of the outer segments of photoreceptor cells (Figure 1C and D). In the saline group, CD11b<sup>+</sup> cells were detected primarily in the inner retina, especially the inner and outer plexiform layers but seldom in the subretinal space (Figure 1C). The number of CD11b<sup>+</sup> monocytes in the subretinal space after RD increased from none in retinas that received saline to 3.5 cells/mm<sup>2</sup> (standard deviation, 1.1 cells/mm<sup>2</sup>) in the minocycline group.

### PHOTORECEPTOR CELL SURVIVAL AFTER RD

Administration of minocycline did not have any effect on normal retinal morphology or ONL thickness (not shown) but did reduce the amount of ONL thinning and attenuate the loss of ONL cell layers associated with RD (Figure 2). Moreover, while the ONL in the retinas of the saline-treated group were disorganized and displayed inner and outer segment deterioration, the ONL and the inner and outer segments in the retinas of the saline-treated group were disorganized and displayed inner and outer segment deterioration, the ONL and the inner and outer segments in the retinas of the saline-treated group were disorganized and displayed inner and outer segment deterioration, the ONL and the inner and outer segments in the retinas of the minocycline group.
minocycline-treated group remained morphologically intact (Figure 1A and B).

**BLOCKAGE OF CASPASE-3 ACTIVATION**

In the saline-treated group, numerous TUNEL-positive cells were seen in the ONL of the detached retinas (Figure 3A-C), whereas only a few were noted in the ONL of minocycline-treated retinal sections (Figure 3D-F). To quantitatively assess photoreceptor cell apoptosis in the retinas of saline- and minocycline-treated mice, we counted TUNEL-positive cells in retinal sections on days 1, 3, and 7 after RD (Figure 4A). Similar to our previous findings, apoptotic cells in the control (saline-treated) group appeared on day 1 following RD, peaked on day 3, and returned to a low level by day 7. At all points that the retinas were examined, the numbers of apoptotic cells counted from the minocycline-treated group were significantly lower than those of the control group. This result was corroborated by studies of caspase-3 activation. With immunohistochemistry that labeled the activated form of caspase-3, more caspase-3–positive cells were noted in the detached retinas of the saline-treated group than that of the minocycline-treated mice (Figure 4B).

**RETINAL GLIAL ACTIVATION AND MONOCYTE INFILTRATION**

Induction of RD-induced glial activation, as reflected by glial fibrillary acidic protein and vimentin immunolabeling, was inhibited by administration of minocycline (Figure 5). In addition, the number of CD11b+ cells in the subretinal space was significantly reduced in the minocycline-treated group compared with the control group (Figure 5).

**DELAYED TREATMENT OF MINOCYCLINE AND PHOTORECEPTOR CELL DEGENERATION**

In the second experiment, with treatment 24 hours prior to, at the time of, and 24 hours after RD, quantification of TUNEL-positive cells was carried out on day 3 after RD induction, a time when RD-associated cell death was most prominent. We noted that even delayed treatment with minocycline significantly decreased the numbers of TUNEL-positive cells compared with saline treatment alone (Figure 6). While there was no significant statistical difference between the 3 minocycline-treated groups, we did see an increase in TUNEL-positive cells in mice with delayed minocycline treatment compared with treatment at the time of RD and even fewer TUNEL-positive cells in mice with minocycline treatment before RD (Figure 6A). These data were confirmed by quantification of anti-caspase-3–labeled cells, which showed similar results for all 3 treatment regimens compared with the control (Figure 6B).

**COMMENT**

Our data suggest that the protective effects of minocycline likely involve both its anti-apoptotic mechanisms and suppression of glial cell activation. In light of its safety record and easy penetration through the blood-retinal barrier, minocycline may hold promise as a neuroprotective agent for patients with RD, age-related macular degeneration, and/or other photoreceptor degeneration. Our results from the second experiment show that even delayed administration of minocycline protects photoreceptor cell degeneration after RD, though apoptosis levels reached a minimum when minocycline was delivered before RD.
Although minocycline has been found to protect CNS neurons in vitro and in vivo from cell death induced by various insults, its protection of retinal neurons has only recently been recognized. For example, it has been shown that application of minocycline attenuates light-induced damage of photoreceptor cells and rod bipolar cells and delays optic nerve injury–induced retinal ganglion cell death. Consistent with these findings, we have shown that minocycline also protects against RD-induced photoreceptor cell damage. Minocycline may act either through an anti-inflammatory mechanism (blocking monocyte infiltration) or as an anti-apoptotic agent (attenuating caspase-3 activation). In addition, other possible mechanisms have been suggested to explain the observed neuroprotective effect of minocycline. These include upregulation of anti-apoptotic proteins, such as Bcl-2 and X-linked inhibitor of apoptosis protein, and inhibition of the release of apoptotic proteins, such as cytochrome c, from the mitochondria. An anti-inflammatory effect of minocycline has also been reported to correlate with its effect on suppression of inducible nitric oxide synthase expression and cytokine release. Further experiments will be needed to define precisely the molecular targets of minocycline against RD-induced photoreceptor cell damage.

Our data also suggest that minocycline treatment is most effective when given 24 hours before RD. This is consistent with the evidence that apoptotic cell death reaches a peak on day 3 after RD, as cells would already have begun entering the apoptotic pathway before minocycline that was delivered 24 hours post-RD could reach therapeutic levels. However, the most promising finding for clinical application of the present study is that even delayed treatment with minocycline can also inhibit photoreceptor degeneration compared with saline. In general, RD can induce neural injury in 2 ways. First, RD causes retinal damage by inducing necrotic and apoptotic cell death of retinal neurons and photoreceptor cells. Next, reactions of multiple cell types, including activations of Müller glia, macrophages/microglia, immune cells, and retinal pigment epithelium, can lead to inflammatory responses, excessive tissue scarring, and wound healing that result in collateral damage of retinal neurons. There is anecdotal evidence that the spreading effect of the primary wound continues at a slow rate for a long time, causing additional loss of vision for many days after the primary injury occurs. Minocycline may therefore be applicable in humans to treat RD or other retinal degenerations that often have a delayed presentation.

Of note for evaluating the clinical relevance of our results is that the experimental RDs were initiated using an injection of sodium hyaluronate, 1.4%, while human RDs are created by liquid vitreous, which contains less than 1% of hyaluronic acid. It is unclear how this difference might affect the diffusion of oxygen or other nutrients across the subretinal space, though future studies of apoptosis time frames in human photoreceptor cells would hopefully answer that question. Likewise, it is unknown whether cells

![Figure 4. Quantification of photoreceptor cell apoptosis in retinal sections of minocycline- or saline-treated mice. Counts of terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL)– (A) and caspase-3– (B) positive cells in the outer nuclear layer of the detached portion of the retinas that received daily injection of saline (n=15) or minocycline (n=17) and were euthanized at different days after retinal detachment. Error bars indicate standard error of the mean. *P < .01, t test.](image)

![Figure 5. Epifluorescence photomicrographs of retinal section labeled with the primary antibody against glial fibrillary acidic protein in saline- (A) or minocycline- (B) treated mice that were euthanized at day 3 after retinal detachment. C, Quantification of CD11b+ cells in retinal sections of mice with retinal detachment that were treated with minocycline (n=4) or saline (n=4). Error bars indicate standard error of the mean.](image)
and monocytes diffuse freely through the subretinal space in the presence of hyaluronate, but it is also possible that they reach the ONL through the retinal vascular system. Furthermore, in clinical RD, the fluid in the subretinal space varies depending on the nature of the RD itself, most likely in some instances allowing for greater nutrient, oxygen, and growth factor permeability compared with sodium hyaluronate. The laboratory results would most likely translate better to clinical RD, where the subretinal fluid was more viscous, such as chronic RD in diabetic patients, compared with acute RD.

While we do not know for certain that it is not the sodium hyaluronate causing apoptosis through contact with the ONL, a previous study has shown that the patterns of photoreceptor apoptosis in animal models using sodium hyaluronate parallel those of human photoreceptors during RD. This implies that apoptosis occurs because of RD and the ischemia resulting from the separation of the retina from the choroidal blood supply rather than the sodium hyaluronate itself. Regardless of the mechanism at work, our results clearly show that minocycline prevents photoreceptor apoptosis. Clinical studies are now needed to evaluate the effectiveness of minocycline in preventing photoreceptor cell loss and vision loss in patients with RD that involves or threatens to involve the macula.

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