Intravitreal Triamcinolone Acetonide Inhibits Choroidal Neovascularization in a Laser-Treated Rat Model

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Objective: To determine if intravitreal triamcinolone acetonide (TAAC) inhibits experimental choroidal neovascular membranes induced by laser trauma in a rat model.

Methods: Nineteen anesthetized male Brown Norway rats received a series of 8 krypton red laser lesions per eye (647 nm, 0.05 seconds, 50 µm, and 150 mW in 17 rats, and 200 mW in 2 rats). One eye received an intravitreal injection of triamcinolone acetonide (20 µL, 0.8 mg) and the other eye received an injection of isotonic sodium chloride solution. Fundus and fluorescein angiography examinations occurred just before euthanasia and tissue processing for histopathology on day(s) 0, 1, 3, 7, 14, 21, 28, and 35.

Results: From the control eyes that underwent photocoagulation at 150 mW, 57 discrete lesions with definitive fibrovascular proliferations were observed at 21, 28, and 35 days, arising from a total of 72 spots placed (79% yield). From the control eyes that underwent photocoagulation at 200 mW, 11 discrete lesions with definitive fibrovascular proliferations were observed at 28 days, arising from a total of 16 spots placed (69% yield). In the TAAC-treated group, no fibrovascular proliferations were observed in the 72 lesions and in the 16 lesions created with 150 mW and 200 mW, respectively.

Conclusion: Intravitreal TAAC is a potent inhibitor of fibrovascular proliferations in a rat model of choroidal neovascular membranes induced by laser trauma.

Clinical Relevance: This study corroborates previous investigations that propose TAAC as a potential treatment for choroidal neovascular membranes in humans.

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AGE-RELATED nacular degeneration (AMD), the leading cause of irreversible visual loss in the United States, has many treatment limitations. Steroid compounds are well-known antiangiogenic agents, which have been suggested as treatment for the choroidal neovascular membranes (CNVMs) that cause visual loss in exudative AMD. Although there are no direct animal models of AMD-related CNVMs, there are several animal models of CNVMs secondary to laser trauma. In the current study, we sought to characterize the effect of intravitreal triamcinolone acetonide (TAAC) on CNVM formation in a previously described and validated rat model of CNVM due to laser trauma.

RESULTS

In the controls treated with isotonic sodium chloride solution, gray-white lesion sites were apparent by day 3. By day 14, the lesions had faded on examination (Figure 1). On fluorescein angiography, all lesions showed early hyperfluorescence with late staining and leakage at the border of the lesion (Figure 2). At day 0, there was disruption of the RPE and Bruch membrane, mild hemorrhage in the outer retina, and vascular dilation in the choroid. On day 1, the presence of macrophages was noted. On day 3, there was clearing of red blood cells and resolution of choroidal vascular dilation. Also on day 3, and more prominently on day 7, there was discrete thickening of the choriocapillaris with early FVP, consisting of collagenous tissue and proliferating vessels with erythrocytes in the luminal spaces. At 21, 28, and 35 days, the lesions showed distinct FVP arising from the disrupted RPE and Bruch membrane and infiltrating the retina. In the rats that were laser treated at 150 mW power, 57 discrete lesions with definitive FVP were observed at 21, 28, and 35 days, arising from a total of 72 spots placed (79% yield). Repre-
MATERIALS AND METHODS

ANIMALS

Nineteen male adult (250-g) Brown Norway rats (Harlan Sprague-Dawley Inc, Indianapolis, Ind) were used to evaluate the effect of intravitreal TAAC vs a control injection on CNVMs. All procedures were performed with strict adherence to guidelines for animal care and experimentation prepared by the Association for Research in Vision and Ophthalmology (Bethesda, Md), and by the Indiana University Animal Care Committee. For all procedures, including examination and photography, animals received intramuscular ketamine hydrochloride at 75 mg/kg and acepromazine maleate at 2.5 mg/kg, along with atropine sulfate at 0.05 mg/kg intramuscularly to minimize bronchial secretions. Maintenance amounts (10%-15%) were administered at 45-minute intervals, when necessary. For all procedures, 1% topical cyclopentolate hydrochloride, 2.5% phenylephrine hydrochloride, and 1% atropine sulfate, were administered for pupillary dilation. All animals underwent ophthalmologic examination, color fundus photography, and fluorescein angiography using 25% sodium fluorescein (0.1 mL/kg) administered intravenously. A Zeiss FK 30 fundus camera (Zeiss Instruments, Jena, Germany) was used.

LASER PHOTOCOAGULATION

After receiving anesthesia and undergoing pupillary dilation, the animals were positioned on a Mayo stand before slitlamp laser delivery. The fundus was visualized using a microscope slide coverslip and 2.5% hydroxypropyl methylcellulose solution as a contact lens. A krypton red (647-nm) laser at 0.05 seconds and 150 mW or 200 mW was used. Laser power was verified with a power meter. In all experiments, a series of 8 lesions was concentrically placed at equal distances around the optic discs of both eyes. The 50-µm spot at 150-mW power was the most reliable at producing acute vapor bubbles, which suggested Bruch membrane rupture, fibrovascular proliferations (FVP) emanated from disrupted Bruch membrane and infiltrated the outer retina. To evaluate the effect of intravitreal TAAC vs the control injection, 17 rats underwent laser photocoagulation using the 50-µm spot size at 150-mW power and 0.05 seconds, and 2 rats underwent laser photocoagulation using the same parameters, except at a power of 200 mW. This latter group of rats was killed at 28 days. In the entire group of 19 rats, a total of 304 spots were placed. Histopathological findings of the experimental groups are presented in the Table.

ADMINISTRATION OF ANTIANGIOGENIC AGENT AND CONTROL SOLUTION

A TAAC suspension (Kenalog, 40 mg/mL; Bristol-Myers Squibb, Conn) was administered intravitreally using a 30-gauge needle inserted 1 mm and angled towards the optic nerve immediately after laser photocoagulation. One eye received an intravitreal injection of TAAC (20 µL, 0.8 mg) through the sclera into the vitreous cavity. A higher dose of TAAC was chosen (approximately 9 times the empiric dose used in prior human pilot studies1,2,3) because the pharmacokinetics of the drug in the rat vitreous cavity were unknown, because insufficient dosing was to be avoided, and because this volume could be reproducibly administered. Proparacaine hydrochloride (0.5%) was used for topical anesthesia (in addition to general anesthesia) during this procedure. Prior to injection, 5% povidone was applied to the ocular surface. The posterior segment was evaluated immediately after injection to confirm placement of the drug into the vitreous cavity. The fellow (control) eye received an injection of isotonic sodium chloride solution at the same volume (20 µL) to comparatively evaluate any changes that might have resulted from the physical insertion of the needle tip or from changes in intraocular pressure subsequent to fluid injection. The entire group of 19 rats was treated in this fashion.

ASSESSMENT

Follow-up examination, fundus photography, and fluorescein angiography occurred just before euthanasia on day(s) 0, 1, 3, 7, 14, 21, 28, and 35. The angiograms were subjected to masked analysis by 2 investigators (T.A.C. and M.H.C.) for the presence of staining or leakage at each lesion. Differences in the evaluations were resolved though joint discussions of the data.

Eyes were enucleated immediately after euthanasia and eyecup preparations were fixed in 4% phosphate-buffered paraformaldehyde solution (overnight at room temperature). For each eye, a single square-shaped tissue block (approximately 1.5 mm per side), containing the optic disc and the 8-lesion sites, was hand sectioned from the eyecup preparation. Tissue sections were dehydrated, embedded in paraffin, serially sectioned (6 µm), and stained for light microscopy with hematoxylin-eosin. Each laser lesion site was individually evaluated and photographed. The histologic findings were correlated with those from fundus photography and fluorescein angiography. Specifically, histologic specimens were methodically assessed in a masked fashion by 1 reader (M.H.C.) for the presence or absence of neovascularization; the level of neovascularization with respect to the choroid, Bruch membrane, or the retina; the response of the retinal pigment epithelium (RPE) to the original injury and subsequent FVP; and the inflammatory response to the original injury and subsequent FVP. Lesions that showed FVP with thickening greater than 10 µm between the choroid and retina overlaying the laser lesion were deemed to show the presence of neovascularization.

Sentative histopathologic findings are shown in Figure 3. In the rats laser treated at 200-mW power, 11 discrete lesions with definitive FVP were observed at 28 days, arising from a total of 16 spots placed (69% yield).

In some eyes from the TAAC-treated group, the opaque white drug partially obscured the posterior segment from days 0 to 7 when the drug began to settle inferiorly and clear. At all later time points, when clinical examination was possible, the clinical and angiographic appearance of these lesions was distinct from the lesions seen in untreated eyes. In particular, the lesions showed absolute RPE and Bruch membrane defects with
prominent white sclera evident in the defect (Figure 1). On fluorescein angiography, the absolute atrophy associated with each lesion caused very intense staining, and mild leakage could not be reliably ruled out (Figure 2). It became apparent that histopathologic analysis more reliably determined the presence or absence of FVP. The early findings (days 0 and 1) were similar to the findings in the control eyes treated with isotonic sodium chloride solution. These included disruption of the RPE and Bruch membrane, mild hemorrhage in the outer retina, and vascular dilation in the choroid on days 0 and 1, followed by some clearing of red blood cells and resolution of choroidal vascular dilation on day 3. On day 7, there appeared to be fewer macrophages infiltrating the lesions relative to the controls injected with isotonic sodium chloride solution, but the macrophages were not quantitatively compared with the control eyes by cell-specific staining. At all later time points, the TAAC-treated eyes showed prominent defects in the RPE and Bruch membrane with a striking absence of FVP at each laser lesion. In the rats laser treated at 150-mW power, no FVP were observed in the 72 lesions at 21, 28, and 35 days, arising from the original 72 lesions placed (0%). Triamcinolone acetonide exerted an inhibitory effect on the development of FVP in every recovered laser lesion (P <.001, Fisher exact test).

**COMMENT**

This study demonstrates that intravitreal TAAC is a potent inhibitor of FVP in a laser-treated rat model. The best-known model for CNVMs, however, is the laser-treated primate model, developed by Ryan. In this model, high-intensity laser burns are used to create ruptures in Bruch membrane/RPE complex to initiate a repair process in the fundus that results in the development of subretinal neovascularization. To avoid the use of primates, several groups have validated the laser-treated rat model using intense diode or krypton laser photocoagulation to acutely rupture Bruch membrane, which leads to rapid reproducible CNVM. This model does not show rapid spontaneous regression of the neovascular process, as in the laser-treated primate model, which is a useful feature when evaluating

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**Histopathologic Findings**

<table>
<thead>
<tr>
<th>Day</th>
<th>No. of Eyes</th>
<th>Findings</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Intravitreal Triamcinolone Acetonide</strong></td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>Disruption of Bruch membrane; RBC infiltration of outer retina; vascular dilation in choroid</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Infiltration by macrophages</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>Clearing of RBCs; resolution of choroidal vascular dilation</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>Prominent defect in RPE and Bruch membrane; retina drawn into defect; no FVPs</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>No FVPs</td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>No FVPs</td>
</tr>
<tr>
<td>28</td>
<td>2 (200 mW)</td>
<td>No FVPs</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>No FVPs</td>
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<tr>
<td></td>
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<td><strong>Isotonic Sodium Chloride Solution</strong></td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>Disruption of Bruch membrane; RBC infiltration of outer retina; vascular dilation in choroid</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Infiltration by macrophages</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>Clearing of RBCs, resolution of choroidal vascular dilation; early FVPs formation arising through the disrupted RPE and Bruch membrane and infiltrating the retina with RBCs seen in lumenal structures and macrophages</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>FVPs</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>FVPs</td>
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<tr>
<td>28</td>
<td>4</td>
<td>FVPs</td>
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<td>28</td>
<td>2 (200 mW)</td>
<td>FVPs</td>
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<tr>
<td>35</td>
<td>3</td>
<td>FVPs</td>
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* All eyes except those noted underwent laser photocoagulation at 150-mW power. RBC indicates red blood cell; RPE, retinal pigment epithelium; and FVP, fibrovascular proliferations.
antiangiogenic treatments. Other investigators have developed rat models using lower-intensity krypton laser photocoagulation, which did not result in acutely rupturing the Bruch membrane. This led to slower development of CNVM, presumably through focal digestion of Bruch membrane by enzymes elaborated by proliferating choroidal endothelial cells.\textsuperscript{26,27}

The stimulus for neovascularization in the laser models obviously differs from that in AMD, because the laser models invoke a traumatic repair process, which may better mimic traumatic CNVMs and not AMD-related CNVMs. This point may be especially relevant for the high-intensity laser photocoagulation models that cause acute rupture of Bruch membrane, as in our study. Nevertheless, the laser trauma in both the high-intensity and low-intensity laser models may initiate a cascade of angiogenic growth factors, which may be relevant to AMD-related CNVM. For example, one group of investigators has demonstrated the expression of basic fibroblast growth factor in their rat model using lower-intensity krypton red photocoagulation.\textsuperscript{28,29} An Australian group has found evidence of expression of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{Fluorescein angiography (late phase, 10 minutes postinjection) of the identical region at 28 days. \textbf{A}, Control eye treated with isotonic sodium chloride solution. There is late leakage at the borders of the lesions. \textbf{B}, Triamcinolone acetonide–treated eye. There is intense late staining of the lesions due to the absolute defect in the Bruch membrane and retinal pigment epithelium. Mild leakage cannot be reliably ruled out owing to the very intense staining.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Histopathologic analysis of the identical region at 28 days (hematoxylin-eosin; retinal thickness adjacent to the lesion measures 100±10 \textmu m). \textbf{A}, Control eye treated with isotonic sodium chloride solution. Note the fibrovascular proliferations (FVP) arising through the disrupted retinal pigment epithelium (RPE) and the Bruch membrane and infiltrating the retina. There are red blood cells seen within lumenal structures. \textbf{B}, Triamcinolone acetonide–treated eye. Note the prominent defects in the RPE and the Bruch membrane with a striking absence of FVP. The retina is drawn into the defect.}
\end{figure}
cell adhesion molecules and vascular endothelial growth factor in their model using intense krypton laser photocoagulation. A Japanese group, who has had the most published experience with the intense krypton laser-treated rat model, has demonstrated expression of basic fibroblast growth factor, vascular endothelial growth factor, and transforming growth factor β. 

Several of these growth factors have been implicated in human CNVM formation. For example, surgically excised and postmortem CNVM tissue, as well RPE cells, have been shown to be immunoreactive for vascular endothelial growth factor, transforming growth factor β, platelet-derived growth factor, and basic fibroblast growth factor.

Our study establishes the potent inhibition of FVP by intravitreal TAAC in the laser-treated rat model, corroborating and amplifying some of the findings from prior studies. Unlike some recent studies using a diode laser in the rat, however, fluorescein angiography seemed to be of limited value in determining the presence or absence of CNVMs. This could be due to the absolute atrophy associated with the TAAC-treated lesions, which caused very intense staining, limiting the ability to rule out superimposed mild leakage in these eyes. Alternatively, the diode laser could induce lesions with different angiographic characteristics compared with the krypton red laser used in this study. Nevertheless, the differences seen on histopathologic analysis were striking.

Steroid compounds have long been known to possess angiostatic properties via alteration of extracellular matrix degradation and possibly through inhibition of leukocytes that release angiogenic growth factors. Oral prednisone or sub-Tenon injections of depot forms of steroids have been advocated for treatment of CNVMs due to the presumed ocular histoplasmosis syndrome, although no controlled studies have been performed. Intravitreal steroid injections potently inhibit experimental subretinal and preretinal neovascularization in primates and pigs, respectively. Intravitreal TAAC produced an apparent beneficial effect in an uncontrolled pilot study of CNVM treatment in AMD, which was followed (in a study by Challa et al) by a favorable effect on the course of the disease during an 18-month period. More recently, a prospective pilot study involving 27 patients randomized to intravitreal TAAC vs observation demonstrated a statistically significant beneficial effect of TAAC on best-corrected visual acuity at 3 months. These authors speculate that intravitreal TAAC has a beneficial effect on AMD-related CNVM development through the inhibition of leukocytes, including macrophages, which release angiogenic factors. Investigators using the Ryan primate CNVM model have also postulated that macrophages involved in the initial response to Bruch membrane injury secrete angiogenic growth factors. In this study, there appeared to be relatively fewer leukocytes observed at the TAAC-treated laser lesions at day 7, although macrophages were not quantitatively assessed and their role in CNVM development in this model remains speculative.

Obviously, the TAAC dosage level used in this study probably accounts for the absolute inhibition of FVP. It should also be noted that this study, by its design, demonstrated inhibition or prevention of FVP, but not regression of preexisting FVP, which would be more relevant to human CNVM. Nevertheless, this study corroborates and amplifies previous investigations that propose TAAC as a promising potential treatment modality for CNVM in humans. Further studies of the mechanism by which TAAC exerts its antiangiogenic effect to inhibit the action of growth factors, as well as the determination of optimal TAAC dose-response levels for both inhibition of FVP formation and regression or stabilization of preexisting FVP are warranted.

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