Neuroprotective Effect of Intravitreal Triamcinolone Acetonide against Photoreceptor Apoptosis in a Rabbit Model of Subretinal Hemorrhage

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PURPOSE. To study photoreceptor apoptosis and iron migration as mechanisms of retinotoxicity in a rabbit model of subretinal hemorrhage (SRH) and to assess intravitreal triamcinolone acetonide (IVTA) for anti-apoptotic and neuroprotective effects.

METHODS. In adult rabbits, eyes were studied histologically after subretinal injection of autologous blood. For comparisons of control eyes with eyes injected with 2 mg IVTA, morphometric analysis was performed with light microscopy, whereas apoptosis was quantified with terminal dUTP nick end labeling (TUNEL) and fluorescence microscopy. Localization of retinal iron was assessed with Perls' stain.

RESULTS. Photoreceptor degeneration was initiated 48 hours after exposure to subretinal blood and progressed over 7 days. Increased TUNEL positivity demonstrating apoptotic cell death was associated with SRH and photoreceptor loss. VIP–Perls staining demonstrated iron in the photoreceptor layer and retinal pigment epithelium that correlated with photoreceptor degeneration. Treatment with IVTA enhanced photoreceptor cell survival by 11% at 48 hours and by 45% at 72 hours (P = 0.01) and reduced photoreceptor apoptosis ratios by 25% at 48 hours (P = 0.006).

CONCLUSIONS. Photoreceptor toxicity caused by SRH occurs at least in part by apoptosis and is associated with iron migration to the photoreceptor layer. Treatment with IVTA reduced photoreceptor loss and apoptosis, indicating a neuroprotective action. Therapies to target SRH may augment anti–VEGF treatments in exudative age-related macular degeneration and other diseases of choroidal neovascularization. (Invest Ophthalmol Vis Sci. 2008;49:4071–4077) DOI:10.1167/iovs.08-1892

The toxicity of subretinal hemorrhage to the neurosensory retina has been demonstrated in animal models1–3 and in clinical studies.4–5 Possible mechanisms of blood-induced retinotoxicity include mechanical effects such as fibrotic shearing of photoreceptors, hypoxia and metabolic disruption imposed by the clot as a diffusion barrier, and direct retinotoxicity induced by blood components such as iron.1,6,7 The role of apoptosis has not previously been investigated in neuronal toxicity related to subretinal hemorrhage; altering the pathways of programmed cell death may offer prospects for therapeutic intervention and neuroprotection.

A variety of attempts have been used to address submacular hemorrhage as commonly encountered in exudative age-related macular degeneration (AMD) and other diseases of choroidal neovascularization (CNV). Pneumatic displacement or surgical evacuation with the use of recombinant tissue plasminogen activator (tPA) were at one time widely explored; however, a controlled clinical trial failed to show a significant visual benefit of a surgical approach to the removal of submacular blood in AMD.4 Histopathology in human specimens demonstrated that the removal of submacular hemorrhage did not curtail the activity of the causative CNV,5 and at that time no effective antiangiogenic therapies were available. The advent of anti–VEGF agents has greatly advanced the treatment of exudative AMD6–10 and may afford an opportunity to revisit submacular hemorrhage and therapies to ameliorate blood-induced retinotoxicity. AMD patients with significant submacular hemorrhage were excluded from the major clinical trials of anti–VEGF drugs8–10; in current practice, clinicians consider these patients candidates for anti–VEGF therapy. The question is raised whether, in addition to inhibiting choroidal neovascularization and macular edema through anti–VEGF therapies, adjunctive therapies to address the direct toxicity of submacular blood might be used to maximize visual outcomes of in this subset of AMD patients.

In the present study, a rabbit model of autologous subretinal hemorrhage was used to determine the morphologic features and time course of retinal damage, to characterize the apoptotic mechanisms of photoreceptor cell death, and to assess the role of iron toxicity and macrophage activity in blood-mediated retinotoxicity. Intravitreal triamcinolone acetonide (IVTA) was tested for neuroprotective effects on retinal photoreceptors in cell survival and inhibition of apoptosis. The results indicated that apoptosis is a contributing mechanism of photoreceptor cell death caused by SRH and is associated with iron migration from the clot to the photoreceptor layer. IVTA inhibits photoreceptor apoptosis and cell death after exposure to SRH at 48 and 72 hours.

METHODS

All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of California San Francisco Committee on Animal Research.
Animal Surgery
New Zealand White female rabbits (weight range, 2.5–3.5 kg) from the Western Oregon Rabbit Company (Philomath, OR) were anesthetized by inhalation of 2% to 4% isoflurane. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride, and 0.5% proparacaine drops was administered to each eye. A surgical microscope (model 510187; Zeiss, Jena, Germany) was used with a silicone flat lens (Dutch Ophthalmics, Kingston, NH) to visualize the retina. After disinfection with 5% povidone iodine, 100 μL autologous blood obtained through the ear vein was immediately injected into the subretinal space in a nasal juxtapapillary location along the myelinated streak. An angled 1-inch, 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ) was introduced transsclerally 3 to 4 mm posterior to the limbus, and blood was injected subretinally to form a bullous retinal elevation. In other eyes, blood injection was made under the retinal pigment epithelium (RPE). This resulted in a low, diffuse hemorrhage, confirmed histologically to be in the sub-RPE space, allowing comparison with the effects of blood in the subneurosensory retinal space. In selected fellow eyes, 2 mg trimcinolone acetone (IVTA; Bristol-Myers Squibb, Princeton, NJ) was injected intravitreally immediately after the bilateral subretinal blood injection. Subconjunctival antibiotic (cefoxil, 150 μL) was given after the procedure.

Histology
At intervals of 24, 48, 72, 96, or 168 hours after surgery, rabbits were humanely killed under deep anesthesia by intravenous overdose of pentobarbital sodium. Eyes were enucleated and immersed in a solution of 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer solution for 24 hours at 4°C, then washed with PBS. Under a microscope, the site of the autologous blood was identified, and full-thickness wedge dissections were made through the sclera, preserving the hemorrhagic bleb and surrounding retina. Tissue samples were embedded in paraffin, then sectioned at 5 μm and mounted on glass slides. Samples were stained with hematoxylin and eosin or with a DNA fragmentation detection kit (FragEl, catalog no. QIA39; Calbiochem, La Jolla, CA).

TUNEL Experiments
The terminal dUTP-mediated nick-end labeling (TUNEL) technique was used to detect apoptotic degeneration. Staining was performed according to the manufacturer’s protocol. Briefly, sections were deparafﬁnized in xylene and rehydrated in serial dilutions of ethanol. Specimens were then permeabilized in 0.1% Triton X-100 and 0.5% sodium citrate buffer solution for 24 hours at 4°C, then washed with PBS. At room temperature for 30 minutes in 5% potassium ferrocyanide and 5% hydrochloric acid resulted in the Perls Prussian blue reaction product. Sections were counterstained with methyl blue/azure II or with periodic acid–Schiff–hematoxylin.

Morphometric Analysis
Digital images (Spot Flex camera; Diagnostic Instruments, Sterling Heights, MI) were obtained from light microscopy (Eclipse 80i; Nikon, Tokyo, Japan) or fluorescence microscopy (Axioskop; Zeiss), and collected images were analyzed by two independent readers in a masked fashion. Photoreceptor nuclei or TUNEL-positive cells were counted, and cell densities were calculated by superimposing a standard area template (200 × 50 μm) on regions of the outer nuclear layer (ONL) in the retinal images. For each eye, multiple template areas from SRH and control retinal regions were counted in a masked fashion. For controls, multiple adjacent regions of retina not involving SRH were used for intra-eye comparisons of effects on photoreceptor counts. Digital images isolated only to the ONL were gathered from different eyes and different experimental conditions, and cell counts were performed by two masked independent readers; then averages were obtained for each eye. The extent of cell damage was quantified on light microscopic studies (hematoxylin and eosin [H&E] stain) by calculating the following cell survival ratio: ONL density overlying SRH divided by ONL density in retinal regions away from the SRH within the same eye (controls). Apoptotic cell ratios were determined by dividing the density of TUNEL-positive ONL cells overlying SRH by the total density of ONL cells overlying SRH on corresponding H&E stains of the adjacent serial sections.

Iron Histochemistry
Paraffin sections (5 μm) were stained with the very intense purple (VIP)-enhanced Perls’ Prussian blue technique. Incubation at room temperature for 30 minutes in 5% potassium ferrocyanide and 5% hydrochloric acid resulted in the Perls Prussian blue reaction product. Next, incubation for 30 minutes at room temperature using the VIP purple reagent (Vector Laboratories, Burlingame, CA) resulted in a purple reaction product. Sections were counterstained with methylene blue/azure II or with periodic acid–Schiff–hematoxylin.

Statistical Analysis
We estimated time trends and the effects of blood toxicity and steroid injection using linear mixed-effects models because measurements made on the same eye or within the same rabbit cannot be assumed to be statistically independent (note, however, that the measurements at each time point are independent because no repeated measures were made on the same rabbit). Standard errors for estimated survival ratios were computed using bootstrap resampling of ratios within eyes (using 25,000 replicates). Comparing eyes that received IVTA with control eyes, we tested the hypothesis that there were no differences in the survival ratios at 48 and 72 hours using bootstrap resampling of ratios within eyes (using 25,000 replicates). A two-sided P value was approximated by twice computing the relative frequency of a difference between the steroid and nonsteroid groups that exceeded zero (under bootstrap resampling). Statistical computations were performed in R version 2.6.0 (available at http://www.r-project.org).
Morphometric analysis was performed to compare photoreceptor cell densities. Across all analyses there was high agreement between the two masked readers, with an estimated intraclass correlation coefficient of 0.96 (random effects ANOVA model). A trend test analyzing cell counts in all eyes with subretinal hemorrhage at all time points was performed using a linear mixed-effects model fit by restricted maximum likelihood. We found an average decline of approximately 0.51 in the cell count per hour, a statistically significant trend effect with time of exposure to SRH \((P = 0.0001)\).

**TUNEL Studies**

Outer retinal degeneration observed histologically was correlated with photoreceptor apoptosis by TUNEL studies (Fig. 1B). Overlying the SRH, the ONL shows a relative increase in cells positive for TUNEL staining compared with the adjacent retina. The fluorescent signal was localized to the photoreceptor nuclei overlying SRH. The ONL apoptosis ratio was 0.16 ± 0.01 at 48 hours of exposure to SRH and 0.24 ± 0.05 at 72 hours (mean ± SE), mirroring the ONL degeneration seen on histology.

**Iron Staining**

Photoreceptor toxicity was correlated with the presence of iron in the retina. VIP-Perls' staining of a hemorrhage transition zone (Fig. 1C) indicated iron accumulation within the overlying photoreceptor outer segment layer and the underlying RPE. A gradient of retinal iron, with Perls' staining, was most prominent at the site of SRH and decreased with distance from the blood. At higher magnification (Fig. 3), the iron staining was specific for the photoreceptor outer segments. Within the photoreceptor outer segment layer, large cells morphologically consistent with macrophages were associated with areas of photoreceptor degeneration (Fig. 4); these large cells, with large nuclei positive for Perls' staining, were seen at 48 to 72 hours after SRH injection, most likely indicating the ingestion of migrated iron by macrophages.
Intravitreal Triamcinolone Acetonide

Triamcinolone acetonide injected into the vitreous immediately after subretinal injection of autologous blood reduced hemorrhage-mediated retinotoxicity to the ONL. A dose of 2 mg triamcinolone acetonide was selected as below the threshold for toxicity observed in previous studies in rabbit eyes. Control eyes receiving only 2 mg IVTA without SRH revealed no evidence of ONL degeneration at 48 hours (Figs. 6E, 6F); histologic signs of photoreceptor damage were absent in the ONL when the neurosensory retina was in direct contact with SRH.

In eyes with SRH, photoreceptor cell loss was reduced in IVTA-treated eyes. Photoreceptor layer disorganization and nuclear abnormalities were less severe, by histologic examination, at 48 hours in eyes receiving IVTA, though outer segment damage was comparable in both groups (Figs. 6A, 6C). The absolute ONL cell number in each group was counted using light microscopy (H&E stain). Cell survival ratios (Fig. 7A) at 48 hours after SRH injection were 0.96 ± 0.03 in eyes receiving IVTA compared with 0.85 ± 0.01 (mean ± SE) in eyes without IVTA, whereas at 72 hours the cell survival ratios were 0.96 ± 0.06 in those receiving IVTA and 0.51 ± 0.10 in those without IVTA. Data from eyes with subretinal hemorrhage with or without IVTA were analyzed with a linear mixed-effects model (clustering by eye and rabbit). The likelihood ratio test for steroid and its interaction with subretinal hemorrhage indicated a statistically significant protective effect of steroid injection on cell counts (P = 0.01).

In twenty-three eyes studied with TUNEL staining, IVTA was associated with a reduction in apoptotic nuclei in the ONL at 48 hours of exposure to SRH (Figs. 6B, 6D). Morphometry demonstrated that apoptosis ratios were significantly lower in IVTA-treated eyes than in non-IVTA-treated eyes (0.12 ± 0.01 vs. 0.16 ± 0.01; P = 0.006) at 48 hours after injection of SRH (Fig. 7B). At 72 hours, a trend toward reduced apoptosis ratios with IVTA did not reach statistical significance (0.16 ± 0.02 vs. 0.24 ± 0.05; P = 0.15).

DISCUSSION

In these experiments, treatment of rabbit eyes with IVTA was associated with a reduction of photoreceptor apoptosis after exposure to autologous subretinal blood, indicating a neuroprotective effect of steroid therapy at standard concentrations. In rabbit eyes, IVTA is nontoxic to the retina at lower concentrations, but at doses of 4 mg and higher, photoreceptor toxicity ensues. Therefore, in these experiments, 2 mg triamcinolone was used in adult rabbit eyes, roughly equivalent to doses commonly used in patients. Hemorrhage-induced photoreceptor death was reduced in IVTA-treated eyes, with an 11% increase in cell survival at 48 hours and a 45% increase at 72 hours (P = 0.01). This appeared to have resulted from specific cellular mechanisms because photoreceptor apoptosis ratios were reduced in IVTA-treated eyes by 25% at 48 hours (P = 0.006).

Apoptotic photoreceptor death has been implicated in a variety of retinal disorders, including retinal degeneration, age-related macular degeneration, and retinal detachment. Although a number of mechanisms have been postulated for retinal damage in SRH, including iron toxicity, ischemia, and fibrotic shearing, the present experiments are, to our knowledge, the first to implicate apoptosis in photoreceptor death attributed to subretinal blood. Our finding of increased photoreceptor outer segment iron after hemorrhage supports the hypothesis that iron-mediated oxidative...
stress to the easily oxidized outer segment lipids is a possible contributor to photoreceptor degeneration. Antioxidants or iron chelators may warrant testing in this or similar animal models.

Neuroprotective approaches have been studied in a number of animal models of retinal damage. Corticosteroids are effective inhibitors of apoptosis in a variety of cell types including neurons. In the retina, glucocorticoid receptors are present in vertebrate retinal Müller cells and photoreceptors. Corticosteroid therapy has been studied for neuroprotection in animal models, where it has been found to prevent outer retinal damage and photoreceptor apoptosis and to preserve electoretinographic responses in light-induced retinal degeneration. Cytokine upregulation, neurotrophic factor synthesis, antioxidant induction, and transcription factor regulation are among the many proposed mechanisms whereby corticosteroids afford neuroprotection on neurons and glial cells.

The clinical use of intravitreal corticosteroid therapy has become widespread in the management of diverse retinal disorders. The therapeutic mechanisms of ocular corticosteroid therapies include anti-inflammatory, antiangiogenic, and antifibrotic properties as well as effects on vascular permeability and retinal leakage. An additional potential therapeutic effect of corticosteroids, neuroprotection, has been studied most widely in neurologic disorders. High-dose methylprednisolone therapy is a standard in the treatment of acute spinal cord injury and bacterial meningitis, and corticosteroids have been investigated as neuroprotective agents in animal models of hypoxic-ischemic brain injury and corticosteroids have been investigated as neuroprotective agents in animal models of hypoxic-ischemic brain injury. Combination therapy using intravitreal corticosteroids with anti-VEGF agents, with or without photodynamic therapy, has been studied in exudative AMD. With such approaches, the effect of corticosteroids is thought to be against inflammation, vascular leakage, and angiogenic factors, but a neuroprotective steroid effect might now also be considered.

Anti-VEGF therapies are the standard of care for patients with exudative AMD, yet the visual outcomes in AMD accompanied by submacular hemorrhage are unavailable because central macular SRH was an exclusion criterion to participation in the major clinical trials of these agents. Several studies in humans have shown that the presence of submacular hemorrhage in exudative AMD confers a worsened visual progno-
sis. Data from the present study, together with other studies in animal models and in humans, indicate that SRH presents a specific toxicity to photoreceptors. Although anti-VEGF drugs effectively inhibit CNV activity and leakage, they are not expected to directly resolve preexisting SRH or to mitigate its toxicity to the neural retina. This leads to a presumption that visual outcomes with anti-VEGF therapy in exudative AMD may be worsened if significant SRH is present.

Therefore, treatments to address SRH may enhance anti-VEGF therapy in this subset of AMD patients. Removal of SRH has been attempted with several techniques. Vitrectomy has been combined with intravitreal or subretinal recombinant tPA to directly drain SRH or pneumatically displace liquefied blood from the macular region. In a nonsurgical approach, pneumatic displacement of submacular blood has been performed with injections of intravitreal tPA together with an expansile gas bubble. Although uncontrolled studies suggested at least short-term benefits, the only large, prospective, controlled clinical trial has demonstrated no benefit from surgical evacuation of SRH compared with observations in AMD patients with hemorrhagic CNV. Previous investigations of SRH removal for the most part predate the advent of ocular anti-VEGF therapies, and the combination of SRH displacement and anti-VEGF therapies is now being initiated.

The present results indicate that retinal toxicity associated with short-term exposure to subretinal blood is a potential therapeutic target in AMD and other retinal diseases. Although further clinical study is necessary, histologic results in this animal model provide some basis for the clinical practice of combining anti-VEGF therapy with subretinal hemorrhage evacuation or displacement in patients with hemorrhagic AMD and suggest a beneficial mechanism for strategies that incorporate intravitreal corticosteroid or neuroprotective agents in combination treatments.

References

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