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Effects on XIAP Retinal Detachment–Induced Photoreceptor Apoptosis

Laura A. Zadro-Lamoureux^{1,2}, David N. Zacks³, Adam N. Baker¹, Qiong-Duan Zheng³, William W. Hauswirth⁴, and Catherine Tsilfidis^{1,2}

¹Vision Science Program, Ottawa Health Research Institute, Ottawa, Ontario, Canada

²Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada

³Department of Ophthalmology and Visual Sciences, Kellogg Eye Center, University of Michigan Medical School, Ann Arbor, Michigan

⁴Department of Ophthalmology, University of Florida College of Medicine, Gainesville, Florida

Abstract

Purpose—To evaluate the ability of X-linked inhibitor of apoptosis (XIAP) gene therapy to provide neuroprotection to cells of the outer nuclear layer (ONL) of the retina after retinal detachment.

Methods—Subretinal injections of a recombinant adenoassociated virus (rAAV) encoding either XIAP or green fluorescent protein (GFP; injection control) were performed in the left eye of Brown Norway rats. Two weeks later, retinal detachments were created at the site of viral injection by delivering sodium hyaluronate into the subretinal space. Retinal tissue was harvested at 24 hours after retinal detachment and was analyzed for caspase 3 and 9 activity. Histologic analysis was conducted on samples taken at 3 days and 2 months after detachment to confirm the presence of XIAP or GFP expression and to assess levels of apoptosis and changes in retinal thickness.

Results—Caspase assays performed 24 hours after detachment confirmed an expected increase in caspase 3 and 9 activity in the detached regions of GFP-treated retinas, whereas XIAP-treated detached retinas behaved comparably to attached controls. TUNEL analysis of 3-day tissue samples showed fewer apoptotic cells in XIAP-treated detachments than in GFP-treated detachments. At 2 months after the detachment, histology and immunohistochemistry confirmed the preservation of the ONL at sites of XIAP overexpression, whereas the GFP-treated detached retinas had significantly deteriorated.

Conclusions—The results suggest that XIAP confers structural neuroprotection of photoreceptors for at least 2 months after retinal detachment.

Retinal detachment involves a separation of the neural retina from the underlying retinal pigment epithelium (RPE). It is a common form of retinal injury and a significant cause of visual loss,^{1,2} especially if it involves the macula. The incidence of retinal detachment is approximately 1 in 10,000 people per year, with a lifetime prevalence of 1 in 300.³ Risk increases dramatically when factors such as trauma, myopia, cataract surgery, previous

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Corresponding author: Catherine Tsilfidis, University of Ottawa Eye Institute, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6; ctsilfidis@ohri.ca.

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retinal detachment, or family history are considered.⁴ Retinal detachment may also be associated with ocular disorders such as age-related macular degeneration (AMD), diabetic retinopathy, retinopathy of prematurity, retinoschisis, and central serous retinopathy among others. Rhegmatogenous detachment is the most common type; it involves a retinal tear that allows vitreal fluid to leak into the subretinal space and to detach the retina from the underlying RPE. In tractional detachment, fibrovascular tissue caused by injury, inflammation, or neovascularization pulls the neurosensory retina away from the RPE. Exudative retinal detachment results from subretinal fluid accumulation in the absence of a retinal tear.

Recovery of vision after retinal separation from the RPE depends on the nature, severity, and duration of the detachment. The primary retinal cell types affected by detachment are the photoreceptors. These cells undergo characteristic changes such as shortening of the outer segments, retraction of rod terminals from the outer plexiform layer,⁵ and opsin redistribution.⁶ In addition, synapses of second-order neurons are remodeled and retinal glial cells proliferate.⁷ Some of these changes are significant obstacles to recovery but may be reversible after reattachment of the retina.⁸ However, the primary cause of visual loss is most likely photoreceptor death by apoptosis.^{1,9} In animal studies, apoptosis is initiated within 24 hours after retinal detachment and peaks at 3 days.^{2,8} Apoptosis most often involves the activation of cysteine proteases, called caspases, which are involved in the proteolytic digestion of the cell and its contents. Retinal detachment causes cell death through the activation of caspases 3, 7, and 9.²

X-linked inhibitor of apoptosis (XIAP) is a key member of the inhibitors of apoptosis (IAP) gene family. Members of this family all share at least one baculoviral IAP repeat (BIR) domain, so named because it was first discovered in baculoviruses. XIAP has three BIR domains, which, in combination with the linker regions between them, are involved in binding to and suppressing the activity of caspases 3, 7, and 9.¹⁰ XIAP also contains a carboxyl-terminal RING zinc finger domain that has E3 ubiquitin ligase activity. This domain determines the fate of XIAP or the cell, or both, depending on the severity of the cellular insult. Under conditions of severe cellular stress, XIAP will undergo auto-ubiquitination and degrade, allowing the apoptotic cascade to culminate in the death of the cell. Under lower apoptotic stress, XIAP will promote the ubiquitination and degradation of the caspases, leading to cell survival through the suppression of apoptosis.¹⁰

In disease models, XIAP has been shown to confer protection in forebrain ischemia,¹¹ in methyl-phenyl-tetrahydropyridine-induced Parkinson disease,^{12,13} and in cisplatin-induced ototoxicity.^{14–16} In gene therapy studies in the eye, XIAP protects ganglion cells after axotomy of the optic nerve,^{17,18} increased intraocular pressure,¹⁹ and retinal ischemia.²⁰ It also protects photoreceptors from chemotoxic insult^{21,22} and in genetic models of retinitis pigmentosa.²³ The present study examines the protective effects of XIAP delivered through an adenoassociated virus (AAV) vector on retinal detachment-induced photoreceptor apoptosis. XIAP is an ideal therapeutic agent because it suppresses caspases 3, 7, and 9, whose activation has previously been shown to be responsible for apoptotic cell death in animal studies of retinal detachment.²

Methods

Animals

Adult male Brown Norway rats, at least 6 weeks of age, were purchased from Harlan (Indianapolis, IN) and Charles River Laboratories (Wilmington, MA). Animals were maintained under standard laboratory conditions, and all procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the

guidelines of the University of Ottawa Animal Care and Veterinary Service. Rats were divided into two groups, with one group receiving XIAP gene therapy and the other receiving the green fluorescent protein (GFP) control.

Construction of the Recombinant Adeno-Associated Virus Vectors

A cDNA construct encoding the full-length, open-reading frame of human XIAP with an N-terminal hemagglutinin (HA) tag was inserted into a pTR vector under the control of the chicken β -actin promoter. A GFP construct was similarly generated for use as a surgical and viral control. XIAP viral transgene expression was enhanced by inserting a woodchuck hepatitis virus posttranscriptional regulatory element in the 3' untranslated region of the construct. Serotype 5 recombinant adenoassociated virus (rAAV) was generated^{24,25} and purified²³ as previously described. Viral titers were 2.33×10^{12} physical particles/mL for rAAV-GFP and 1.87×10^{13} physical particles/mL for rAAV-XIAP. Ratios of physical to infectious particles were less than 100.

Subretinal Injections

An injection of rAAV carrying either XIAP or GFP was delivered to the subretinal space of the left eye of each rat. The right eye served as an untreated control. Animals were anesthetized by 2% isoflurane gas inhalation. Eyes were dilated using 1% tropicamide (Mydracyl; Alcon Canada, Mississauga, ON, Canada) and 2.5% phenylephrine hydrochloride (Mydrin; Alcon). Proparacaine hydrochloride drops (0.5%, Alcaine; Alcon) were administered as a local anesthetic. Pain management was achieved by buprenorphine injection (0.04 mg/kg). To maintain lubrication throughout the procedure, 0.3% hypromellose (Genteal gel; Novartis Pharmaceuticals, Mississauga, ON, Canada) was applied to the eye. Subretinal injections were performed by creating a sclerotomy approximately 2 mm posterior to the limbus with a 20-gauge V-lance knife (Alcon). Care was taken to avoid lens contact because this could induce cataract development. A coverslip coated with 0.3% hypromellose (Genteal gel; Novartis Pharmaceuticals) was placed on top of the eye to provide magnification and visualization of the back of the eye. A 33-gauge blunt needle attached to a 10- μ L syringe (Hamilton, Reno, NV) was inserted through the scleral puncture, guided lateral to the lens, and inserted through the retina. rAAV-XIAP or rAAV-GFP (2- μ L vol) combined with fluorescein tracer (50:1 vol/vol) was delivered to the subretinal space of the eye. The fluorescein allowed for immediate visualization and evaluation of the injection location, enabling ascertainment of a successful subretinal delivery. Injections were delivered in a consistent manner between the 12:00 and 2:00 positions. Postsurgical care consisted of administration of the antibiotic 0.3% ciprofloxacin hydrochloride (Ciloxan; Alcon) and a nonsteroidal anti-inflammatory drug, 0.03% flurbiprofen sodium (Ocufen; Allergan, Irvine, CA), for 5 days after injection.

Retinal Detachment

Approximately 2 weeks after viral injections, a retinal detachment was performed in the left eye of each rat, as previously described.² The detachments were created by injecting 10 mg/mL sodium hyaluronate (Healon; AMO, Santa Ana, CA) into the subretinal space near the site of the viral injection. Approximately one-third to one-half of the retina was detached, leaving the remaining attached portion to serve as an internal control. Animals were sampled at 24 hours, 3 days, and 2 months after detachment.

Caspase Assays (24 Hours after Detachment)

Twenty-four hours after creation of the detachment, the intact right retinas (internal control) and the detached portion of the left retinas were harvested from XIAP ($n = 15$) and GFP ($n = 15$) animals. Protein was extracted, as previously described.²⁶ Caspase 9 activity was

measured with an assay kit (Caspase 9 Colorimetric Assay Kit; BioVision Research Products, Mountain View, CA), in accordance with the manufacturer's instructions. This assay is based on the detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate LEHD-pNA. Caspase 3 activity was measured with another assay kit (Caspase 3 Colorimetric Assay Kit; Chemicon International, Billerica, MA), in accordance with the manufacturer's instructions. This assay is based on cleavage of the pNA-DEVD substrate by activated caspase 3.

Tissue Fixation and Processing

Each rat was administered a lethal injection (Euthansol; Schering-Plough Canada Inc., Pointe-Claire, QC, Canada) and was subsequently perfused with 4% paraformaldehyde (PFA) to preserve tissue structure. Left eyes were scored with a white hot needle to enable orientation during enucleation and embedment. Eyes were punctured with a needle to allow penetration of the fixative and were placed in 4% PFA overnight. Samples were then taken through a series of dehydration steps ending with embedment in paraffin. Eyes were sectioned at 10 μ m for histologic analysis.

Histologic Analysis

Hematoxylin and eosin staining was performed on 10- μ m sections to locate retinal detachments. Once a detachment was identified, subsequent slides were subjected to immunohistochemical analysis to confirm the presence of XIAP or GFP. XIAP was detected using an anti-HA mouse IgG primary antibody (Roche Applied Science, Laval, QC, Canada), followed by a goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). GFP was detected using an anti-GFP rabbit IgG (Invitrogen, Eugene, OR) followed by a goat anti-rabbit IgG (Invitrogen). Rhodopsin was detected with the B630 monoclonal antibody.²⁷ Slides were counterstained with the nuclear stain 4',6'-diamidino-2-phenylindole dihydrochloride. Images were obtained using a light microscope (Axioskop; Zeiss, Thornwood, NY) with a camera (AxioCam HRc; Zeiss).

TUNEL Staining

TUNEL was used to compare levels of apoptosis in detached regions of XIAP-treated versus GFP-treated samples 3 days after detachment. TUNEL-positive cells were detected with a commercial kit (Apoptag Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA). To eliminate observer bias, TUNEL-positive cells were detected using a program developed with mathematical software (Matlab, version R2007a; Mathworks, Natick, MA). The program analyzed digital images (Photoshop; Adobe Systems, San Jose, CA) of the outer nuclear layer (ONL) of detached retinas. A TUNEL-positive nucleus was identified, and its RGB values were recorded. The software scanned the image on a pixel-by-pixel basis to determine the number of pixels that fell within 2 SD of the RGB values of the positive nucleus. The number of "TUNEL-positive" pixels was then divided by the total tissue pixels to yield a ratio of TUNEL-positive pixels for that section.

Retinal Thickness Comparison

Eyes that were sampled 2 months after detachment were processed for histologic analysis. Images were taken of 10- μ m sections stained with hematoxylin and eosin. Thickness of the ONL was measured as a ratio of the number of nuclear layers across the ONL of the detached retina divided by the number of nuclear layers across the ONL of the attached portion of the same retina. Because retinal thickness varies with distance from the optic nerve, the thickness of the inner nuclear layer was used as a control to ensure that ONL measurements were taken at the same distance from the optic nerve head. For attached and detached regions, at least four counts were taken from each animal and averaged.

Results

The hypothesis that rAAV-XIAP infection would result in photoreceptor neuroprotection led to the development of three predictions that could be directly tested. The first prediction was that rAAV-XIAP-infected eyes would show less detachment-induced activation of caspases than rAAV-GFP-infected eyes. The second prediction was that the rAAV-XIAP eyes would show less TUNEL-positive staining, a marker for apoptotic death, than rAAV-GFP eyes. Finally, the hypothesis predicted that rAAV-XIAP infection would result in a significantly increased number of photoreceptors surviving extended periods of detachment in comparison with control eyes. Experimental results confirmed these predictions, strongly supporting the photoreceptor-protective properties of XIAP.

Caspase Activity Assays

rAAV-GFP served as an ideal vector and surgical control, showing rates of photoreceptor degeneration that were similar to our previously published retinal detachment data.^{2,26} There was no evidence that rAAV-GFP had a neuroprotective effect or accelerated photoreceptor degeneration. As seen in Figure 1, retinal detachment in the rAAV-GFP-infected eyes (GFP-OS) showed the expected elevation in caspase 3 and 9 activity compared with intact, nondetached retinas. In contrast, caspase activity levels in XIAP-treated retinas (XIAP-OS) showed no detachment-induced increase and were comparable to their contralateral attached controls. All caspase activities were measured at 24 hours after the detachment, which was previously shown to be the time of peak caspase activity.^{2,26}

TUNEL Analysis

Eyes were sampled 3 days after the creation of the retinal detachment and were embedded, sectioned, and processed for immunohistochemistry and TUNEL analysis. The 3-day time point was chosen because previous experimental studies in animal models demonstrated peak TUNEL-positive staining at 3 days after retinal detachment.^{2,8} Immunohistochemistry confirmed robust expression from both the rAAV-GFP and the rAAV-XIAP viral injections (Figs. 2A, B). Antibodies for GFP or XIAP identified strong staining in the cell bodies and inner and outer segments of the photoreceptors in the regions of the retinal detachments. This signal did not always cover the full detachment and was sometimes found in attached portions of the retina, suggesting that the viral infections and detachments did not always completely overlap. Automated, computer-based quantification of TUNEL staining showed that there were fewer TUNEL-positive cells in the rAAV-XIAP eyes than in the rAAV-GFP eyes (Fig. 2E-G). Although the XIAP-related decrease in the number of TUNEL-positive cells did not reach statistical significance compared with the GFP-treated eyes, the results do correlate well with the XIAP-related decrease in caspase activity shown in Figure 1.

Photoreceptor Survival in Chronic Detachment

To assess the long-term structural protection of photoreceptors, histologic examination was conducted on eyes sampled 2 months after retinal detachment. Immunohistochemistry against GFP (Figs. 3A, B) or XIAP (Figs. 3D, E) was used to visualize the site of rAAV virus injection and to correlate this with histologic studies. Morphologically, there were clear differences between XIAP-treated and GFP-treated retinas. Inner and outer segments of the XIAP-treated samples were generally more organized than those of the GFP-treated retinas (compare Figs. 3D, E with 3A, B), though less so than attached regions in the same eye. Rhodopsin staining confirmed that the preserved photoreceptors were viable and produced functional protein (Figs. 3C, F).

The layers of photoreceptor nuclei in the ONL were counted and compared between the detached retinas treated with rAAV-XIAP or rAAV-GFP and their normal attached

counterparts. Counts were always taken at the same distance from the optic nerve along the vertical meridian to account for the retinal thinning that naturally occurs toward the periphery of the eye. Overall, there was significant preservation of the ONL in the XIAP-treated detached retinas ($P < 0.05$; Figs. 3, 4). These retinas had four to eight nuclear layers (compared with 6–11 in the attached regions of the same eye). GFP-treated detached retinas had zero to seven nuclear layers (with 6–14 in the attached portions of the same eyes). For each animal, a ratio of ONL nuclei in the detached compared with the attached regions was calculated, and these values are presented in Figure 4E.

Discussion

Rapid reattachment is imperative to achieve a good visual outcome after retinal detachment. Animal studies, including the present study, have shown that caspases—the actual executors of apoptosis—are activated within 24 hours after detachment. Burton²⁸ conducted a detailed study showing that no patient could recover visual acuity of 20/20 if the duration of retinal detachment lasted 5 days or longer. Unfortunately, in many disease processes, the reapposition of the retina to the RPE cannot be achieved quickly, resulting in the continuous apoptotic death of photoreceptors. The use of photoreceptor-protective agents can potentially limit the extent of photoreceptor death until reattachment can occur.

The present study shows that XIAP can protect photoreceptors for at least 2 months of continual detachment. XIAP-treated retinas maintained larger numbers of nuclear layers in the ONL, and their inner and outer segments were better organized. In addition, they stained robustly with an antibody to rhodopsin, suggesting that they remained viable. In this study, complete neuroprotection was not achieved. On average, XIAP-treated retinas preserved 60% to 70% of the ONL compared with 30% to 40% in control GFP-treated animals. There may be various reasons for such incomplete protection by XIAP. As we have found in other studies, the procedure for injecting virus is often variable from one animal to the next, resulting in different levels of vector spread and viral gene expression.²³ In addition, in this study the subretinal injections did not always coincide with areas of full detachment, so regions of unprotected retina might have been included in the analysis.

Critics of antiapoptotic therapy argue that blocking caspases would be ineffective because once caspases are activated, the cell is committed to the death pathway and is beyond the so-called point of no return (for a review see Ref. 29). The protective effects of XIAP suggest that this is not so. However, we cannot rule out the possibility that XIAP has effects in addition to caspase inhibition. XIAP has been shown to suppress cell death through other mechanisms. Through its RING zinc finger domain, XIAP has E3 ubiquitin ligase activity and can promote the degradation of proapoptotic proteins (for a review see Ref. 10). XIAP is also involved in the transcriptional activation of prosurvival pathways through TAK1.^{10,30,31} TAK1 is a mitogen-activated protein kinase kinase kinase (MAP-KKK) involved in the activation of the NF- κ B and JNK1 pro-survival pathways.^{30,31} Thus, the ability of XIAP to protect photoreceptors for up to 2 months may be attributed, in part, to the activation or suppression of multiple pathways. The inhibition of caspase activity and the decreased TUNEL counts in XIAP-infected eyes, however, do support caspase inhibition as a principal mechanism through which XIAP exerts its photoreceptor-protective effects.

The present study provides proof-of-principle for XIAP efficacy in the treatment of retinal detachment. Rapid delivery of XIAP to the site of retinal detachment has the potential to limit the acute damage experienced by photoreceptors, thus buying the patient critical time until successful reattachment can be achieved. In this respect, AAV-mediated XIAP delivery will have limited success because the upregulation of XIAP will not be rapid enough to protect photoreceptors before the apoptotic cascade is initiated. Strategies for the rapid

delivery of XIAP to the target cells will have to be explored to make XIAP therapy clinically applicable. Further study will also be required to determine the effects of XIAP therapy on the remodeling of second-order neurons and the proliferation of retinal glial cells as well as on final visual outcome.

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Figure 1.

Caspase 3 and 9 assays after retinal detachment in GFP- and XIAP-treated retinas. Subretinal injections of rAAV-GFP or rAAV-XIAP were followed by retinal detachment in the left eye (OS) of the animal. Right eyes (OD) served as intact controls. Significantly increased caspase 3 and 9 activity ($P < 0.05$, Student's t -test) was observed in the GFP-treated detached retinas compared with the XIAP-treated retinas. There was no significant difference between XIAP-treated retinas and their contralateral intact controls.

Figure 2.

Immunohistochemistry with antibodies to GFP (**A**) and to the HA tag of XIAP (**B**) confirmed robust overexpression in the cell bodies and inner segments (IS) and outer segments (OS) of the photoreceptors from both rAAV constructs. Controls run without a primary antibody for (**C**) GFP or (**D**) XIAP. (**E, F**) TUNEL analysis confirmed that GFP-treated retinas had more apoptotic nuclei than XIAP-treated retinas (*brown pigment; black arrows, insets*). (**G**) TUNEL-positive pixel counts (*box plot*) supported the immunohistochemistry results. Each box contains the values between the 25th and 75th percentiles, and the line within the box represents the median value. Bar lines above and below each box indicate the 90th and 10th percentiles, respectively. The box plot was generated with graphing and data analysis software (SigmaPlot, version 8.0; SPSS, Inc., Chicago, IL). ONL, outer nuclear layer.

Figure 3.

Immunohistochemistry for (A, B) GFP and (D, E) XIAP confirmed sustained expression at 2 months after detachment. The GFP signal (*green*) was faint because many of the photoreceptors expressing the viral transgene had died. In contrast, XIAP signal (*red*) was bright and was accompanied by increased numbers of photoreceptors. Note that in retinal areas in which the XIAP signal was reduced (*arrowhead*), photoreceptor loss was considerable. Rhodopsin staining (*red*) in (C) GFP-injected and (F) XIAP-injected retinas shows that the preserved photoreceptors are able to synthesize functional protein. *Arrow*: ONL. Scale bar, 50 μm .

Figure 4.

Comparison between (A, C) attached and (B, D) detached retinas in XIAP- and GFP-treated animals. At 2 months after detachment, (D) XIAP-treated retinas were consistently thicker than (B) GFP-treated retinas and their inner and outer segments were more organized. (E) A ratio was obtained by dividing the number of nuclear layers in the ONL in a detached region of the retina by the number of nuclear layers in the ONL in the attached retina in the same eye. XIAP-treated detached retinas had significantly higher ratios than GFP-treated retinas ($P < 0.05$, Student's *t*-test). Scale bar, 50 μm .