



uOttawa

L'Université canadienne
Canada's university

**FACULTÉ DES ÉTUDES SUPÉRIEURES
ET POSTDOCTORALES**



**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

Laura Zadro-Lamoureux
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)
GRADE / DEGREE

Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

**XIAP (X-Linked Inhibitor of Apoptosis) Gene Therapy Protects Photoreceptors
in an Animal Model of Retinal Detachment-induced Apoptosis**

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. C. Tsilfidis
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. D. Park

Dr. R. Korneluk

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

**XIAP (X-Linked Inhibitor of Apoptosis) Gene Therapy Protects
Photoreceptors in an Animal Model of Retinal Detachment-induced
Apoptosis**

Laura Zadro-Lamoureux

This thesis is submitted as a partial fulfillment of the M.Sc. program in
Cellular and Molecular Medicine

Submitted: November 10, 2008

Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

© Laura Zadro-Lamoureux, Ottawa, Canada, 2008



Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*
ISBN: 978-0-494-52365-0
Our file *Notre référence*
ISBN: 978-0-494-52365-0

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.


Canada

ABSTRACT

Retinal detachments cause photoreceptor apoptosis. XIAP (X-linked inhibitor of apoptosis) inhibits caspases-3, -7, and -9, which prevents the apoptotic cascade. This study evaluates XIAP gene therapy as a means to provide photoreceptor neuroprotection following retinal detachment.

Subretinal injections of virally-delivered XIAP or green fluorescent protein (GFP; injection control) were performed in rats. Two weeks later, retinal detachments were created at the viral injection site. Eyes were harvested 24 hours post-detachment to analyze caspase activity and at 3 days and 2 months for histological analysis.

Caspase assays indicated rises in caspase-3 and -9 activities in detached GFP-treated retinas, whereas XIAP-treated retinas behaved comparably to attached controls. Three day TUNEL analysis showed less apoptosis in XIAP-treated detachments. Two month histology confirmed preservation of photoreceptors in XIAP-treated detachments, whereas GFP-treated detached retinas had deteriorated significantly.

The results suggest that XIAP confers structural photoreceptor neuroprotection for at least two months following retinal detachment.

TABLE OF CONTENTS

Abstract.....	ii
Table of Contents.....	iii
List of Figures.....	v
List of Abbreviations.....	vi
Acknowledgments.....	viii
CHAPTER 1 - INTRODUCTION.....	1
1.1 The Retina.....	1
1.1.1 Retinal Morphology.....	1
1.2 Retinal Detachment.....	5
1.2.1 Rhegmatogenous Retinal Detachment.....	6
1.2.2 Tractional Retinal Detachment.....	8
1.2.3 Exudative Retinal Detachment.....	8
1.3 Current Treatments for Retinal Detachment.....	9
1.4 Apoptosis.....	12
1.4.1 Extrinsic vs. Intrinsic Pathway of Apoptosis.....	13
1.5 The Inhibitors of Apoptosis.....	17
1.5.1 The Inhibitor of Apoptosis Family of Proteins.....	17
1.5.2 Regulation of the Inhibitors of Apoptosis Proteins.....	18
1.5.3 XIAP (X-linked Inhibitor of Apoptosis).....	19
1.5.4 The Therapeutic Potential of XIAP.....	23
1.6 Retinal Degeneration and XIAP Gene Therapy.....	24
1.6.1 Applicability of XIAP Gene Therapy to Retinal Degeneration.....	24
1.6.2 Retinal Detachment-induced Apoptosis.....	27
1.7 Thesis Experiments and Hypothesis.....	27
1.7.1 Rationale.....	27
1.7.2 Objectives and Hypothesis.....	28
CHAPTER 2 - MANUSCRIPT:	
XIAP protects photoreceptors from retinal detachment-induced apoptosis.....	29
CHAPTER 3 - GENERAL DISCUSSION.....	51
3.1 Cataract Development.....	51
3.2 Possible Reasons for Incomplete Protection of Photoreceptors.....	52
3.2.1 Injection Procedure.....	52
3.2.2 Caspase-Independent Cell Death.....	53
3.2.3 Necrosis.....	55
3.3 Safety and Efficacy of AAV-mediated Gene Therapy.....	56
3.3.1 Safety and Efficacy of AAV Vectors.....	56
3.3.2 Safety and Efficacy of XIAP - Is over-expression dangerous?.....	58
3.4 Alternate Modes of XIAP delivery.....	60
3.5 Future Directions.....	61
4. CONCLUSION.....	62

5. REFERENCES.....	63
Appendix 1 - Troubleshooting.....	73
Appendix 2 - Basic Protocols.....	79

LIST OF FIGURES

Figure 1. Gross Anatomy of the Eye.....	3
Figure 2. Structure, Function, and Histology of the Mammalian Retina.....	4
Figure 3. Pathogenesis of a Rhegmatogenous Retinal Detachment.....	7
Figure 4. Surgical Options for Retinal Detachment.....	11
Figure 5. Intrinsic and Extrinsic Activation of Apoptosis.....	16
Figure 6. Functional Domains of the XIAP Protein.....	21
Figure 7. XIAP Inhibits both Intrinsic and Extrinsic Activation of Apoptosis.....	22
Figure 8. Caspase-3 and -9 assays following retinal detachment in GFP- and XIAP-treated retinas.....	41
Figure 9. 3 Day Immunohistochemistry.....	43
Figure 10. 2 Month Immunohistochemistry.....	45
Figure 11. Retinal Thickness Comparison.....	47

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AIF	apoptosis-inducing factor
AMD	age-related macular degeneration
Apaf-1	apoptotic protease activating factor-1
ARVO	Association for Research in Vision and Ophthalmology
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIR	baculoviral IAP repeat
BSA	bovine serum albumin
CBA	chicken beta-actin
cDNA	chromosomal deoxyribonucleic acid
CHEORI	Children's Hospital of Eastern Ontario Research Institute
CICD	Caspase-independent cell death
CIHR	Canadian Institutes of Health Research
DAPI	diamindino-2-phenylindole dihydrochloride
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
EndoG	endonuclease G
ERG	electroretinogram
FADD	Fas-associated death domain
FasL	Fas ligand
GFP	green fluorescent protein
GDNF	glial cell line-derived neurotrophic factor
HA	hemagglutinin
H&E	hematoxylin and eosin
IAP	inhibitor of apoptosis
IgG	immunoglobulin G
INL	inner nuclear layer
IP	intraperitoneal
LCA	Leber's congenital amaurosis
MAPKKK	mitogen-activated protein kinase kinase kinase
MOMP	mitochondrial outer membrane permeabilization
MNU	<i>N</i> -methyl- <i>N</i> -nitrosourea
MPTP	1-methyl-phenyl-1,2,3,6-tetrahydropyridine
NaBH ₄	sodium borohydride
NBT	nitro blue tetrazolium
NF-κB	nuclear factor-kappa B
NH ₄ Cl	ammonium chloride
NIH	National Institutes of Health
NSE	neuron-specific enolase
NSERC	Natural Sciences and Engineering Council of Canada
Omi/HtrA2	Omi/high temperature requirement protein A2
OMM	outer mitochondrial membrane
ONL	outer nuclear layer
PD	Parkinson's disease

PFA	paraformaldehyde
pNA	p-nitroanilide
PVD	posterior vitreous detachment
rAAV	recombinant adeno-associated virus
RGC	retinal ganglion cell
RING	really interesting new gene
RNA	ribonucleic acid
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
RRD	rhegmatogenous retinal detachment
Smac/DIABLO	second mitochondria-derived activator of caspase/direct IAP-binding protein with low propidium iodide
SNpc	substantia nigra pars compacta
TBS	tris buffered saline
Tdt	terminal deoxynucleotidyl transferase
TNF- α	tumour necrosis factor- α
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
XAF1	X-linked inhibitor of apoptosis (XIAP)-associated factor-1
XIAP	X-linked inhibitor of apoptosis

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. Catherine Tsilfidis for agreeing to mentor me as a student in her lab. I have learned so much and am extremely fortunate to have been part of such a cutting edge project.

Secondly, I would like to thank Adam Baker for his patience in guiding me through my experiments and answering my never-ending list of questions. I would also like to thank all members of the Tsilfidis lab for their support and friendship.

I would have never arrived at this point in my life without the encouragement and support of my two wonderful parents and my big brother Matthew. They set the bar high and reminded me during rough times that my hard work would pay off.

Finally, I would like to thank my phenomenal husband and best friend Marc with whom I have experienced the wonders of science since a young age. Thank you for being there for me every step of the way and helping me through. You have helped to shape me into who I am today.

CHAPTER 1 - INTRODUCTION

Retinal degeneration is common in various inherited visual diseases. In addition to genetics, trauma to the eye can also lead to retinal degeneration. Injuries involving the exertion of mechanical forces on the eye can cause a retinal detachment leading to the death of the photoreceptor cells contained within the retina that are necessary for vision. Although the molecular mechanisms by which retinal degeneration occurs have not been fully elucidated, some facts are known. In particular, past research has found that a final common pathway exists for many forms of retinal disease, including retinal detachment, which involves cell death via apoptosis (Travis, 1998). The prospect of limiting the susceptibility of retinal cells to apoptotic death could therefore represent a therapeutic approach for preventing or delaying retinal degeneration, regardless of its cause.

1.1 The Retina

1.1.1 Retinal Morphology

The retina is a layer of tissue comprised of neurosensory cells located at the back of the eye (Figure 1). Its function is to convert light into electrical impulses to be sent to the brain. The retina is divided into several distinct layers (Figure 2). The outermost layer is the retinal pigment epithelium (RPE). Adjacent to the RPE is the outer nuclear layer (ONL), which contains the photoreceptors (rods and cones). The cell bodies of the photoreceptors are contained within the ONL (Figure 2). The outer segments of the photoreceptors are composed of stacks of membranes containing the visual pigment molecules while the inner segments contain mitochondria, ribosomes, and are the site of assembly of the visual pigment molecules (Kolb *et al.*, 2007). Microscopically, the

photoreceptors appear as a brushed border flush with the RPE. Rods make up 97% of the ONL of the human retina and mediate vision in dim light (van Soest *et al.*, 1999). Cones are responsible for detailed visual acuity and colour vision and are found primarily in the centre of the retina, the macula (Figure 1). The ONL is the site where the initial response to light is received and translated into an electrical signal. This signal, which will ultimately reach the brain, is transmitted from the photoreceptor cells in the ONL to horizontal and bipolar cells in the inner nuclear layer (INL). Synapses between the photoreceptors and the horizontal and bipolar cells occur in the outer plexiform layer. Cells of the INL in turn synapse with cells in the ganglion cell layer at the inner surface of the retina. The inner plexiform layer consists of the dendrites of the cells from the ganglion cell layer synapsing with the axons of the INL. The axons from the ganglion cell layer project to the brain via the optic nerve.

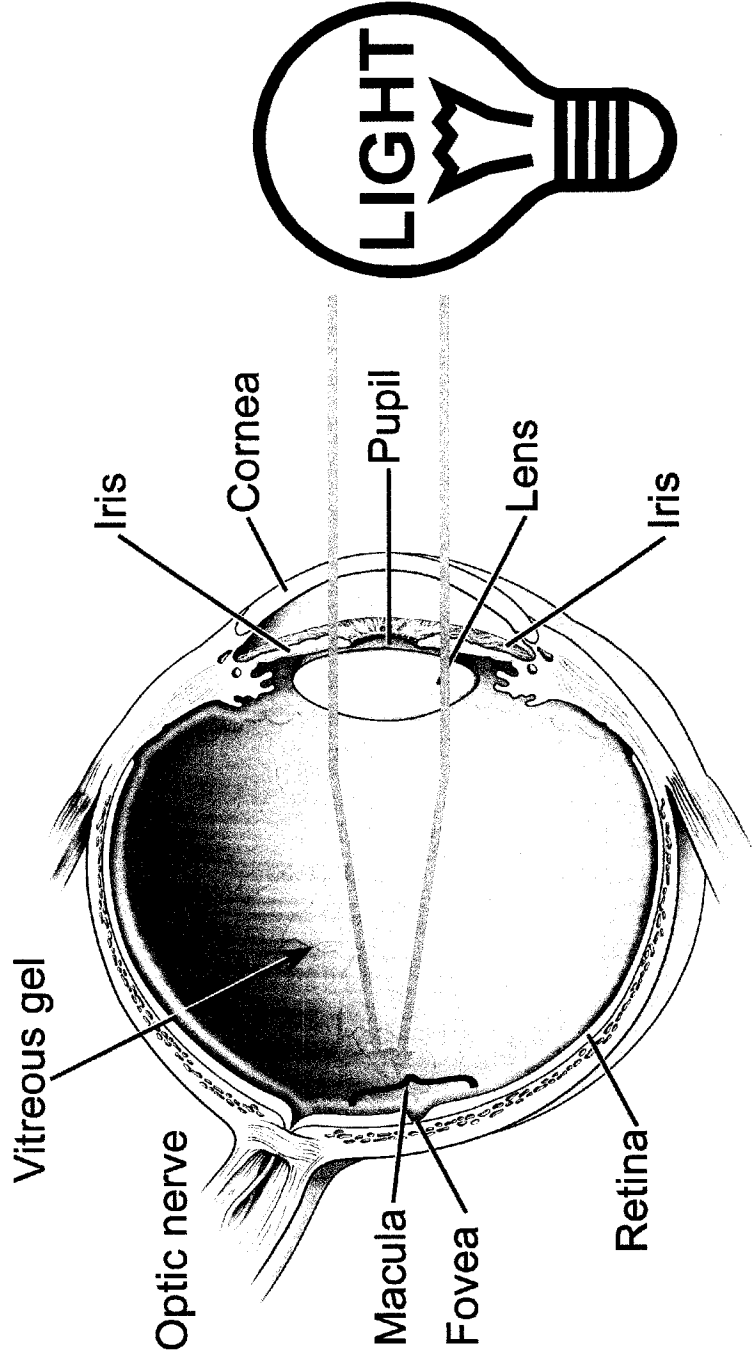


Figure 1: Gross Anatomy of the Eye – Light enters the eye through the cornea, the transparent outer portion of the eye. The amount of light that can enter the eye is controlled by the pupil, an opening at the centre of the iris, the coloured circular part of the eye immediately in front of the lens. The lens, together with the cornea, focus light onto the retina at the back of the eye. Photoreceptor cells (rods and cones) within the retina receive light and convert it into electrical impulses. The macula is a small portion of the retina responsible for sharp, central vision. The fovea is a small spot located within the macula which contains densely packed cones. It is accountable for the clearest vision of the retina. The optic nerve is the converging point at which all retinal axons meet and send their axons to the brain. The vitreous gel is a colourless gel substance that fills the eye and maintains its structure.

(Modified from: The National Eye Institute, National Institutes of Health)

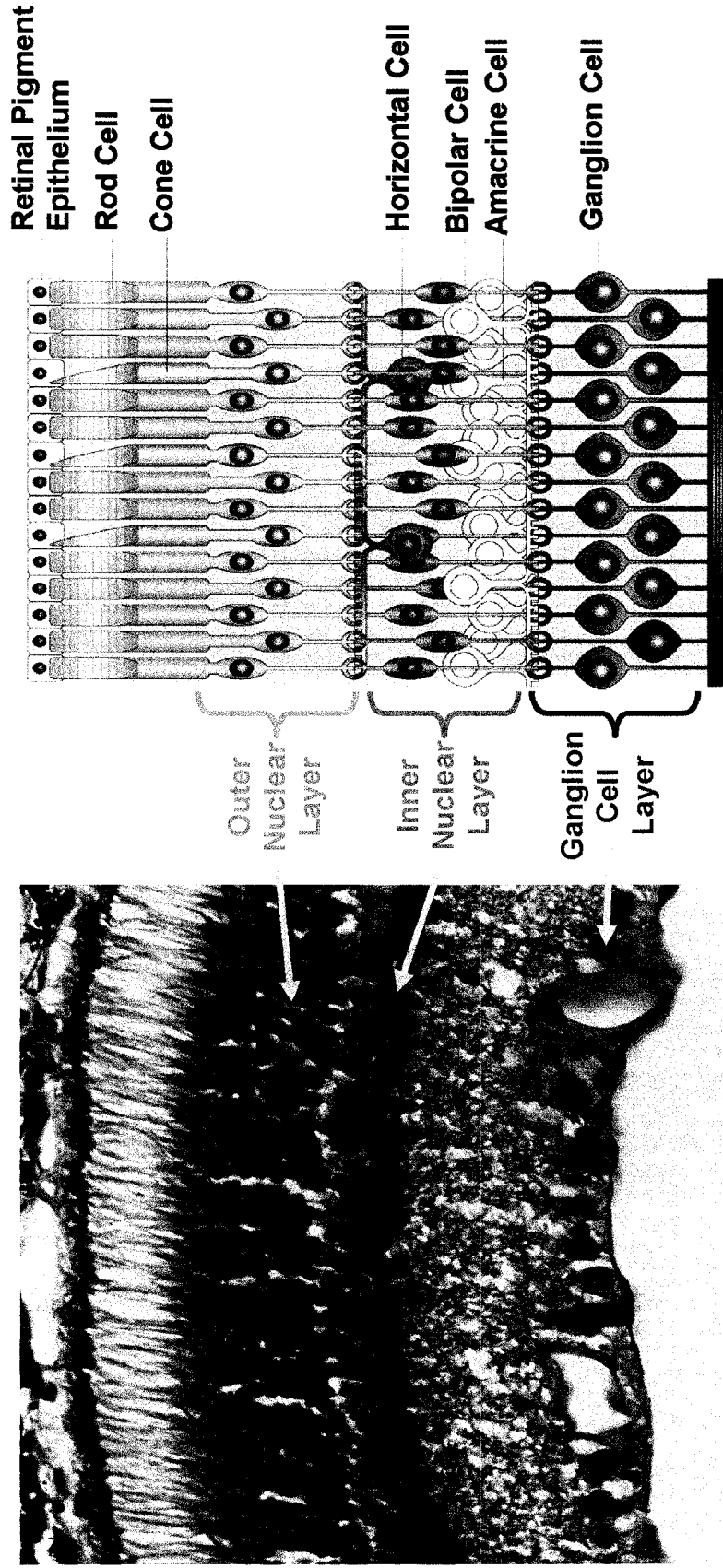


Figure 2: Structure, Function, and Histology of the Mammalian Retina – *Left* – A hematoxylin and eosin stained section of a rat retina depicting the nuclear layers of the retina responsible for the detection and transmission of light. *Right* - The retina is comprised of three main layers of neurons. Light generates an electrical signal in the photoreceptors (rods and cones) of the outer nuclear layer which is in turn transmitted to neurons in the inner nuclear layer. The neurons of the inner nuclear layer synapse with neurons in the ganglion cell layer which project axons to the brain via the optic nerve.

(Modified from Livesey and Cepko, 2001)

1.2 Retinal Detachment

Retinal detachment is a serious clinical problem characterized by the separation of the neurosensory retina from the RPE (Cook *et al.*, 1995). Visual symptoms of retinal detachment include the appearance of a cloudy haze or shadow across the vision of the eye or the onset of sudden bright flashes accompanied by showers of black dots that progress to strand and cobweb like structures (Kang and Luff, 2008). These symptoms are frequently termed as flashes and floaters.

The risk of retinal detachment in otherwise normal eyes is 1 in 10 000 per year with a lifetime risk of 1 in 300 (Gariano and Kim, 2004). Some of the more common risk factors for retinal detachment include increasing age, cataract surgery, myopia, and trauma. In all cases, the consequence of a detachment is a loss of metabolic support to the retina which shortly leads to apoptotic photoreceptor cell death (Zacks *et al.*, 2003). Although an initial detachment may be localized, it is imperative that a patient see a physician as soon as possible as without treatment the detachment can spread. Visual loss is generally extensive if left untreated, especially if the detachment involves the central macula (Zacks *et al.*, 2003). Recovery of vision depends on the severity and duration of the detachment and a lack of treatment can lead to permanent visual impairment or blindness.

Retinal detachments are caused by a variety of factors. They can be classified into three types: tractional, exudative, and rhegmatogenous. Scarring of the vitreous or retina (tractional), leakage of fluid into the subretinal space (exudative), as well as breaks in the retina (rhegmatogenous) can all lead to a detachment (Kang and Luff, 2008). The treatment for each type of detachment can vary.

1.2.1 Rhegmatogenous Retinal Detachment

Rhegmatogenous retinal detachment (RRD) is the most common type of retinal detachment. This condition is also often referred to as a primary retinal detachment or an idiopathic detachment of the retina (Li, 2003). It manifests as the result of retinal breaks caused by vitreoretinal traction (Gariano and Kim, 2004). Posterior vitreous detachment (PVD), whereby the vitreous separates from the retina as part of the normal aging process results in retinal breaks (Kang and Luff, 2008). This allows fluid from the vitreous cavity to enter the subretinal space (Figure 3). Although PVD is relatively rare in people younger than 30, it affects as many as 63% of those over the age of 70 (Foos and Wheeler, 1982). In most people, PVD is benign and goes unnoticed; however, those with symptoms carry a 10-15% risk of developing retinal breaks (Hikichi and Trempe, 1994; Sharma *et al.*, 1999). Rhegmatogenous retinal detachments are most often associated with age, myopia, cataract surgery, trauma, degenerative retinal lesions, Stickler's syndrome, Juvenile X-linked retinoschisis, and Marfan's syndrome (Kang and Luff, 2008).

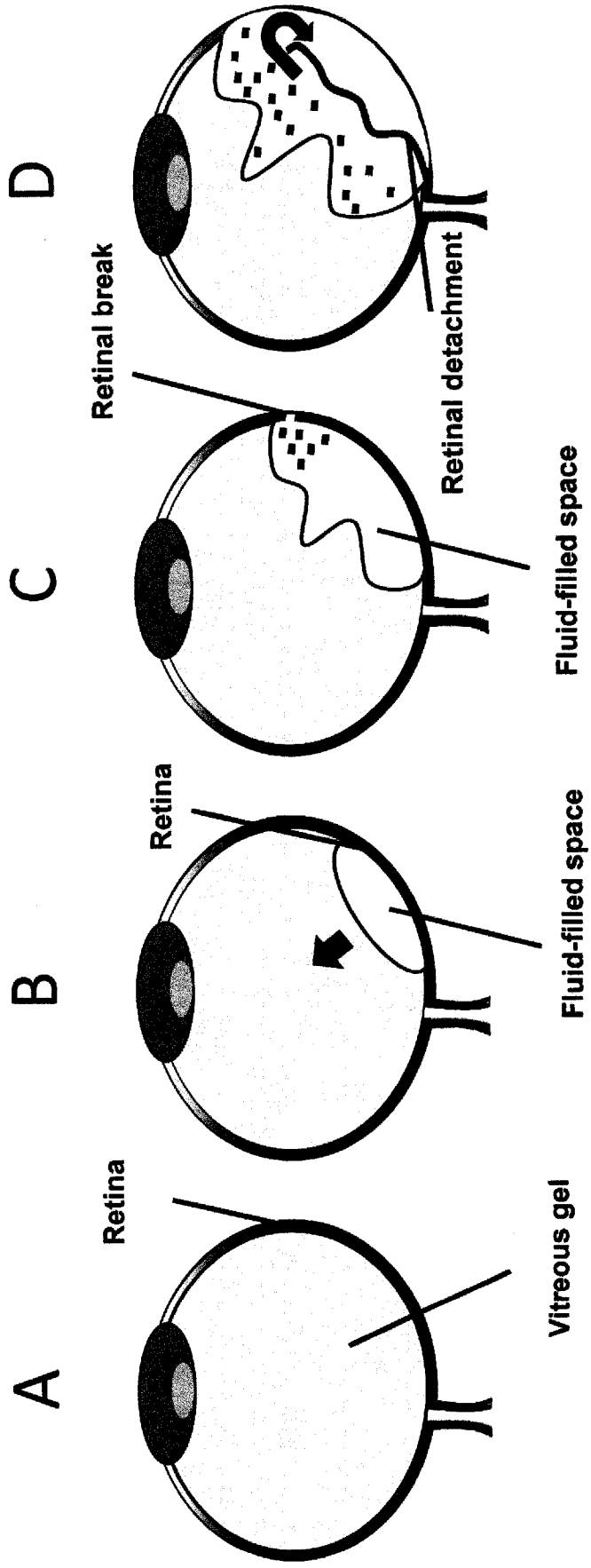


Figure 3: Pathogenesis of a Rhegmatogenous Retinal Detachment - A) In a healthy eye, the vitreous gel fills the vitreous cavity of the eye and remains adherent to the retina. **B)** Factors such as age or trauma cause the vitreous gel to shrink which in turn exerts a centripetal traction force on the retina (arrow), eventually leading to the separation of the gel from the retina. This condition is called posterior vitreous detachment (PVD). As the result of this separation, fluid accumulates between the gel and the retina. **C)** In vulnerable patients, vitreoretinal traction and PVD cause a retinal break. Consequently, blood and retinal pigment epithelium cells can then enter the vitreous cavity. These are commonly known as "floaters". **D)** Liquid from the fluid filled space can spill through the retinal break (curved arrow), forcing the detachment of the retina from the underlying retinal pigment epithelium.

(Modified from: Gariano and Kim, 2004)

1.2.2 Tractional Retinal Detachment

Tractional retinal detachments are the direct result of the exertion of centripetal mechanical forces on the retina, usually mediated by fibrotic tissue present from a previous hemorrhage, injury, surgery, infection, or inflammation (Gariano and Kim, 2004). In order to treat a tractional detachment, these forces must be lifted by unfastening the scar tissue from the retinal surface (Gariano and Kim, 2004). Tractional retinal detachments are most often associated with proliferative diabetic retinopathy, proliferative vitreoretinopathy, retinopathy of prematurity, penetrating eye injury, sickle cell retinopathy, and retinal vein occlusion (Kang and Luff, 2008). Unfortunately, the final visual outcomes in patients who suffer from this type of detachment are generally poor (Gariano and Kim, 2004).

1.2.3 Exudative Retinal Detachment

An exudative (or serous) detachment occurs following the accumulation of serous and/or hemorrhagic fluid in the subretinal space (Gariano and Kim, 2004). Exudative detachments usually result from injury, inflammation, or vascular abnormalities that cause fluid to gather beneath the retina. In contrast to rhegmatogenous retinal detachments, exudative detachments do not usually present with a hole, tear, or break. They are most commonly associated with inflammation (e.g. uveitis and scleritis), neoplasia (e.g. choroidal melanoma, haemangioma, and metastasis), vascular disorders (e.g. retinal macroaneurysm), maculopathy (e.g. neovascular macular degeneration) and congenital disorders such as optic disc pit (Kang and Luff, 2008). With successful

treatment of the underlying condition causing the exudative detachment, the prospects for visual recovery are excellent (Gariano and Kim, 2004).

1.3 Current Treatments for Retinal Detachment

Generally, if caught early enough, a retinal detachment can be repaired successfully. In some cases, retinal detachment can be discouraged by treating the retinal breaks caused by PVD before they lead to a detachment. The best opportunity for prevention is to treat an individual as soon as possible following PVD and retinal tear formation as there is often a variable interval between retinal break and detachment (Byer, 1994). Retinal breaks can be treated using laser therapy or cryotherapy to fuse the retina and the RPE by creating a scar adhesion (Kang and Luff, 2008). The chorioretinal scar that is generated prevents fluid access into the subretinal space (Gariano and Kim, 2004). This treatment is almost 100% successful, but does not prevent the development of breaks in other locations (Pollack and Oliver, 1981).

Once the retina becomes detached, further surgical procedures are required. In general, surgical correction aims to relieve vitreoretinal traction, and to close retinal tears and holes (Gariano and Kim, 2004). The two most common surgical procedures for repairing a detachment are scleral buckling and vitrectomy with gas tamponade (Kang and Luff, 2008). In the scleral buckling procedure, a pliable silicone element is positioned beneath the rectus muscles of the eye and is sutured to the sclera (Figure 4) (Gariano and Kim, 2004). The inward indentation (buckling) pushes the wall of the eye towards the detached retina and reduces internal vitreoretinal traction (Gariano and Kim, 2004).

The vitrectomy approach (Figure 4) involves removing some of the vitreous through sclerotomies, small incisions made in the sclera, the outer connective tissue of the eye. The sclerotomies are usually made in the pars plana, a region of the outer eye close to where the sclera meets the iris. During the procedure, subretinal fluid is drained and laser therapy or cryotherapy is applied around the flattened retinal break (Kang and Luff, 2008). Next, the vitreous cavity is filled with a tamponade (usually a gas but occasionally silicone oil) that holds the retina in place while scarring develops around the break (Kang and Luff, 2008).

Finally, an alternate form of treatment is pneumatic retinopexy. This method is less invasive and is carried out by injecting a bubble of gas into the vitreous cavity. The patient's head is then positioned so that the bubble is situated on the retinal break (Kang and Luff, 2008). As soon as the retina is flattened, the break can be treated with laser therapy or cryotherapy.

Despite the variety of techniques available to treat retinal detachment, the successful reattachment of a retina does not always translate into a good visual outcome, and patients who initially present with poorer vision are less likely to achieve good final visual acuity (Abouzeid and Wolfensberger, 2006). Results are best through earliest possible diagnosis and treatment.

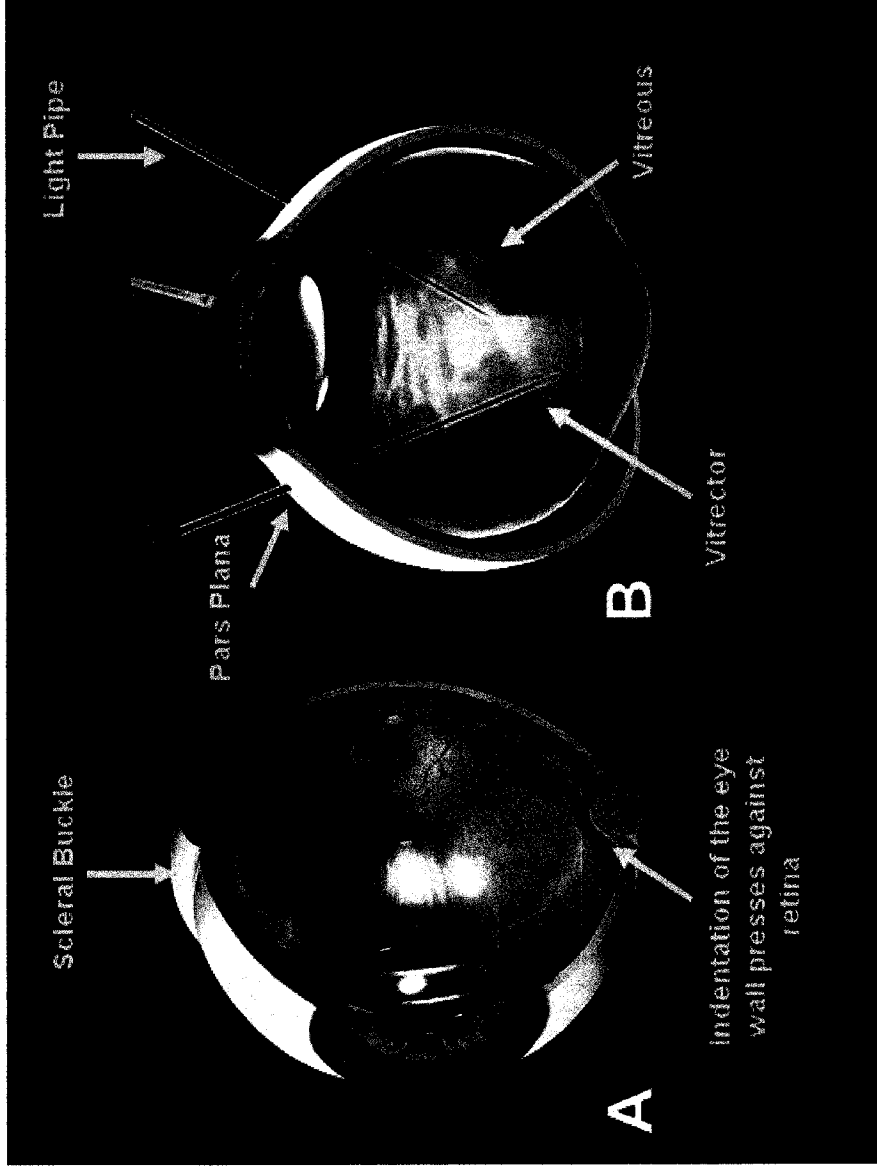


Figure 4: Surgical Options for Retinal Detachment – A) Scleral Buckle - A silicone band is placed around the eye and sutured. This brings the detached retina in contact with the indented eye wall which relieves the traction of the vitreous on the tear and reduces the amount of fluid that can enter the hole. **B) Vitrectomy** - Sclerotomies are created in the pars plana so that a fibre-optic light and vitrector can be inserted into the eye. The eye is illuminated and some of the vitreous is removed. Subretinal fluid is drained and laser therapy or cryotherapy is used to flatten the retinal break. The vitreous is replaced with a compatible tamponade (usually a gas but occasionally silicone oil) which holds the retina in place so that scarring can develop to seal the break. (Modified from: Kang and Luff, 2008)

1.4 Apoptosis

Under normal conditions, apoptosis is a regulated process by which an organism can safely remove unwanted, damaged, or dangerous cells. This may include but is not limited to cells that are infected with viruses, cells with DNA damage, and cells of the immune system that have fulfilled their immunological function. Various physiological cellular processes are dependent on apoptosis, particularly the organization of organs and tissues during embryonic development (Holcik and Korneluk, 2001). It is essential that apoptosis remain tightly controlled within an organism to avoid disease. Uncontrolled cell death is often associated with the destruction of healthy cells and tissue as is seen in neurodegenerative disorders and autoimmune disease (Holcik and Korneluk, 2001). In contrast, inadequate cell death can lead to uninhibited proliferation as in the case of cancer.

Apoptosis is accomplished through a complex cascade of events that involves the interaction of various proteins. The initiation of apoptosis can be triggered by a multitude of signalling mechanisms. Apoptosis occurs in two phases, an initial commitment phase followed by an execution phase that involves the actual breakdown of cellular components (Sun *et al.*, 1999). During the execution phase, the following occur: condensation and fragmentation of nuclear chromatin, dilation of the endoplasmic reticulum, and alterations to the cell membrane that result in the recognition and subsequent phagocytosis of the cell (Arends and Wyllie, 1991; Takahashi and Earnshaw, 1996). One of the highly recognized features of the execution phase of apoptosis is the activation of caspases, proteolytic enzymes that when activated, initiate cell death. Caspases are constitutively expressed in cells; however, to prevent the unintentional

cleavage of their targets, they exist as pro-enzymes (procaspases) (Holcik and Korneluk, 2001). It has been suggested that "initiator" caspases such as caspase-8, either directly or indirectly activate downstream "effector" caspases, such as caspase-3 and -7 (Cohen, 1997) which cleave vital cellular proteins. This activation is achieved through their proteolytic cleavage which releases subunits that reconstitute an active caspase heterodimer (Holcik and Korneluk, 2001). Caspase activation is often considered as the "point of no return" in apoptotic pathways as activated caspases cleave essential proteins, destroy key components of cellular infrastructure, and activate factors that mediate cell damage. Caspases have historically been considered as the key executioners of apoptosis, and in many instances this is the case (Doonan and Cotter, 2004); however other factors also play a role. Two principal pathways, an intrinsic and an extrinsic pathway, are responsible for the initiation of apoptosis. Both of these pathways involve the activity of caspases.

1.4.1 Extrinsic vs. Intrinsic Pathway of Apoptosis

The extrinsic or receptor-mediated pathway of apoptosis typically involves the interaction of a death ligand (e.g. FasL or TNF- α) with a death receptor (e.g. Fas or TNF-R1 or -R2). For example, both Fas and FasL are surface membrane proteins that are members of the tumour necrosis factor- α (TNF- α) superfamily of proteins (Love, 2003). The interaction of Fas and FasL results in the formation of the death-inducing signaling complex (DISC) (Wajant, 2002). This complex contains the adaptor protein Fas-associated death domain (FADD), as well as caspases-8 and -10, which together initiate apoptosis (Wajant, 2002) through further caspase activation, thereby providing a direct

link between cell death receptors and the caspases (Cohen, 1997). Cells undergoing apoptosis via the extrinsic pathway carry out the death programme by activating a hierarchy of caspases, with caspase-8 and caspase-10 being at or near the peak of this apoptotic cascade (Cohen, 1997). Caspase-8 is an initiator caspase and is the most apical caspase in extrinsic pathway-induced apoptosis (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Its activation leads to further propagation of the apoptotic cascade as downstream effector caspases become subsequently activated (Figure 5).

The intrinsic pathway is regulated by the mitochondria. Mitochondria are best known for their role in aerobic respiration, however, they also function in suppressing apoptosis by compartmentalizing proapoptotic factors (Doonan and Cotter, 2004). The intrinsic pathway of apoptosis involves the mitochondrial release of cytochrome-c which interacts with caspase-9 and apoptotic protease activating factor-1 (Apaf-1) resulting in the formation of an apoptosome which then orchestrates cell death (Slee *et al.*, 1999) (Figure 5). The initiator caspase-9, once activated, can then activate effector caspases-3, -6, and -7 leading to the progressive breakdown of the cell (Zou *et al.*, 1997; Srinivasula *et al.*, 1998). The release of cytochrome c can be chemical- or irradiation-induced. In addition, it has been proposed that mitochondria serve as an amplifier in receptor-mediated apoptosis as the activation of caspase-8 cleaves a cytosolic substrate that leads to the release of cytochrome c (Kuwana *et al.*, 1998; Scaffidi *et al.*, 1998). Intrinsically-activated apoptosis requires an initial permeabilization of the outer mitochondrial membrane (OMM) in order to permit the release of cytochrome c, an event which is partially controlled by the Bcl-2 family of proteins (Doonan and Cotter, 2004). The

Bcl-2 family of proteins is comprised of both death antagonists (e.g. Bcl-2) and death agonists (e.g. Bax and Bid) (Doonan and Cotter, 2004).

Although different pathways and signals lead to apoptosis, the process of cell death is carried out by activated proteolytic caspases and both the intrinsic and extrinsic pathways of apoptosis converge at the point of caspase-3 and -7 activation. Therefore, there are at least two major mechanisms by which apoptosis can be activated: one involving caspase-8 (extrinsic) and the other involving caspase-9 (intrinsic) as the most apical caspase (Sun *et al.*, 1999) (Figure 5).

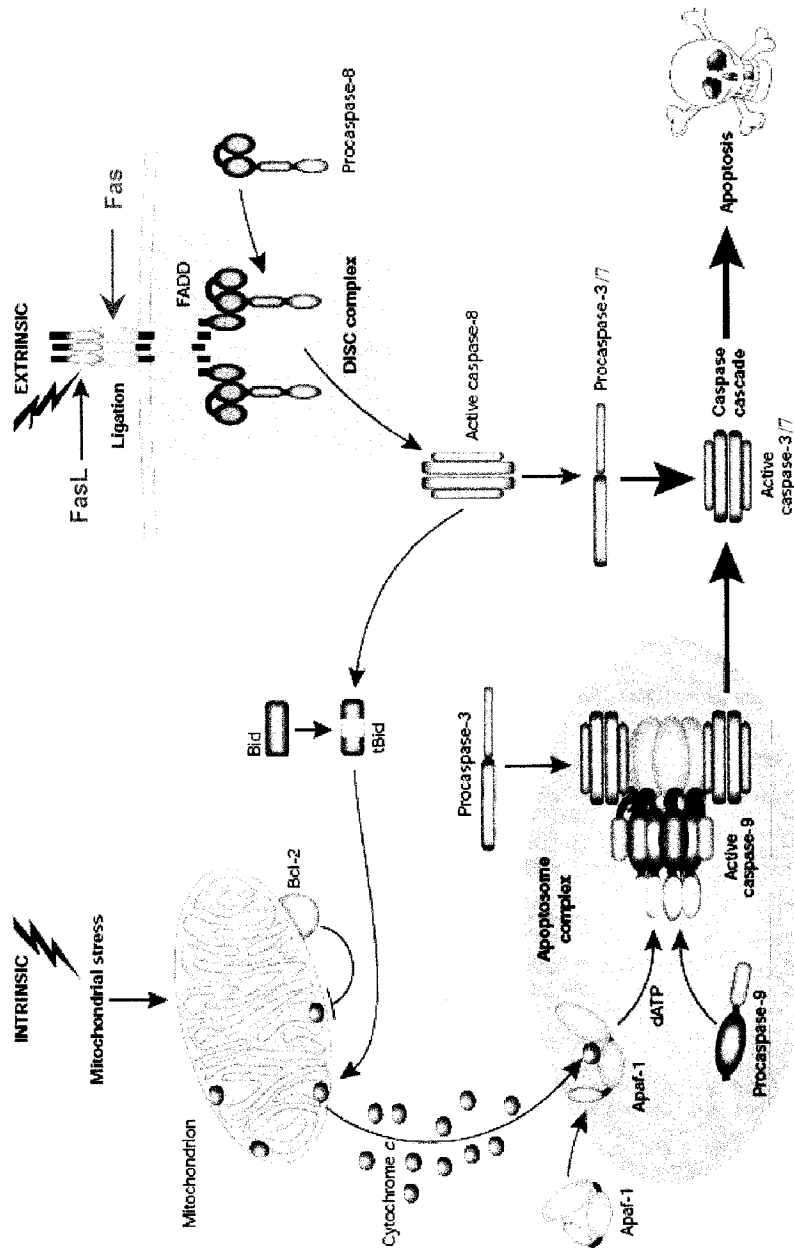


Figure 5: Intrinsic and Extrinsic Activation of Apoptosis – Apoptosis is activated via two major pathways: one activated by death receptor activation, such as Fas (extrinsic) and the other by stress induced stimuli (intrinsic). **Extrinsic activation** results in the activation of the initiator caspase-8 following its recruitment to the DISC complex as mediated by the adaptor molecule FADD. **Intrinsic activation** results from mitochondrial stress that leads to permeabilization of the outer mitochondrial membrane causing the release of cytochrome c. This release is regulated in part by the Bcl-2 family of proteins which consists of both pro-apoptotic members (e.g. Bax and tBid) as well as anti-apoptotic members (e.g. Bcl-2), which promote or inhibit the release of cytochrome c. Once released, cytochrome c forms a complex with Apaf-1 and pro-caspase-9 resulting in the formation of the apoptosome. The two pathways converge as both initiator caspases-8 and -9 subsequently activate downstream effector caspases which in turn execute the cleavage of critical cellular substrates. (Modified from: Macfarlane and Williams, 2004)

1.5 The Inhibitors of Apoptosis

1.5.1 The Inhibitors of Apoptosis Family of Proteins

The destructive abilities of the caspases are controlled by two classes of cellular apoptotic inhibitors: the Bcl-2 and inhibitor of apoptosis (IAP) gene families, which encode anti-apoptotic proteins that regulate apoptotic pathways (Holcik and Korneluk, 2001; Deveraux *et al.*, 2001). The difference between the two families is that the Bcl-2 proteins are only able to block the intrinsic pathway of apoptosis by inhibiting the release of cytochrome c (Holcik and Korneluk, 2001). In comparison, the IAP proteins are the only endogenous inhibitors that are able to block both the intrinsic and extrinsic pathway by directly binding to and inhibiting the action of both the initiator and effector caspases (Holcik and Korneluk, 2001). The IAP proteins regulate programmed cell death in a wide range of organisms ranging from insects to humans (Deveraux *et al.*, 1997). Their function is achieved through the inhibition of the final caspase cascade leading to apoptosis (Renwick *et al.*, 2006).

The IAP proteins contain at least one (and up to three) motif approximately 80-amino-acids long termed a baculoviral inhibitor of apoptosis repeat (BIR), which has a conserved cysteine and histidine core sequence (C_{X2}C_{X6}W_{X3}D_{X5}H_{X6}C) (Holcik *et al.*, 2001). The BIR domains in conjunction with their linker regions bind to and suppress caspase activity (Clem, 2001; Lacasse *et al.*, 1998) and are consistently present at the amino-terminus of the protein (Petrin *et al.*, 2003b). These BIR domains in addition to their linker regions are vitally important for anti-apoptotic activity as they are each directly responsible for the inhibition of distinct caspases (Takahashi *et al.*, 1998; Deveraux *et al.*, 1999). Several IAPs also possess a carboxy-terminal RING (really

interesting new gene) zinc finger, which has been recognized to have E3 ubiquitin ligase activity (Yang *et al.*, 2000). Evidence suggests that members of the IAP family that possess E3 ligase activity can ubiquitinate the caspases that they bind, thus targeting them for proteasome degradation (Suzuki *et al.*, 2001b and Yang and Li, 2000).

Due to their ability to inhibit the final steps of the caspase cascade, the IAPs present as a promising therapeutic means by which a variety of diseases involving apoptosis could be treated.

1.5.2 Regulation of the Inhibitors of Apoptosis Proteins

Just as the IAP proteins regulate caspase function, there are other proteins that exist to control the function of IAPs themselves. The inhibition of IAP family members is mediated by a collection of proteins that includes Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low propidium iodide), Omi/HtrA2 (Omi/high temperature requirement protein A2), and XAF1 (X-linked inhibitor of apoptosis (XIAP)-associated factor-1) (Straszewski-Chavez *et al.*, 2007). Smac/DIABLO is a mitochondrial protein that regulates the caspase-inhibiting activity of the IAP proteins. Mature Smac/DIABLO is released from mitochondria at the same time as cytochrome c, during intrinsically-activated apoptosis (Holcik and Korneluk, 2001). The binding of Smac/DIABLO to an IAP protein disrupts the ability of the IAP to bind to caspases thereby promoting apoptosis (Holcik and Korneluk, 2001). Both Smac/DIABLO and Omi/HtrA2 have been shown to function as mitochondria-associated proteins that are released from the mitochondria in response to apoptotic

stimuli in order to inhibit the function of XIAP, a potent IAP (Suzuki *et al.*, 2001a; Verhagen *et al.*, 2000; Du *et al.*, 2000).

In addition to Smac/DIABLO, XAF1 is a proapoptotic protein that antagonizes the caspase inhibition ability of XIAP. Although the exact mechanisms by which XAF1 accomplishes its pro-apoptotic effect are still not fully known, several possibilities exist. In a study by Liston *et al.*, the expression of XAF1 has been shown to cause the redistribution of XIAP from the cytosol to the nucleus (Liston *et al.*, 2001). More recently, XAF1 has been shown to facilitate receptor-mediated apoptosis by translocating to the mitochondria, activating the mitochondrial pathway and promoting XIAP inactivation (Straszewski-Chavez *et al.*, 2007). Interestingly, XAF1 is ubiquitously expressed in normal tissues but is present at low to undetectable levels in many different cancer cell lines suggesting that its influence may be critical in controlling the apoptotic resistance of certain cancer cells (Liston *et al.*, 2001).

1.5.3 XIAP (*X-linked Inhibitor of Apoptosis*)

XIAP protein is a member of the IAP family that directly interacts with and inhibits the catalytic activity of caspases-3, -7, and -9 (Holcik *et al.*, 2001). It was given its name as it was first discovered on the X chromosome (Liston *et al.*, 1996). XIAP is the most potent member of the IAP family as it prevents the activation of effector caspases that function late in apoptosis past the convergence point of most apoptotic pathways (Emamaullee *et al.*, 2005). XIAP possesses three BIR domains. Although sequence similarities exist between the BIR domains, they each exhibit different affinities with respect to the binding and inhibition of caspases (Liston *et al.*, 2001). The BIR2

domain is responsible for the caspase-3- and caspase-7-inhibiting activity; however, it requires the assistance of residues found in the linker region that precedes BIR2 (Holcik and Korneluk, 2001; Takahashi *et al.*, 1998). The BIR3 domain potently inhibits caspase-9 (Sun *et al.*, 2000). In addition to binding with caspases, XIAP can also interact with Smac/DIABLO and Omi/HtrA2 through both its BIR2 and BIR3 domains (Chai *et al.*, 2000; Liston *et al.*, 2001) (Figure 6).

XIAP is a promising and effective therapeutic option as an anti-apoptotic therapy as it is capable of blocking both the intrinsic and extrinsic pathways of apoptotic activation (Figure 7).

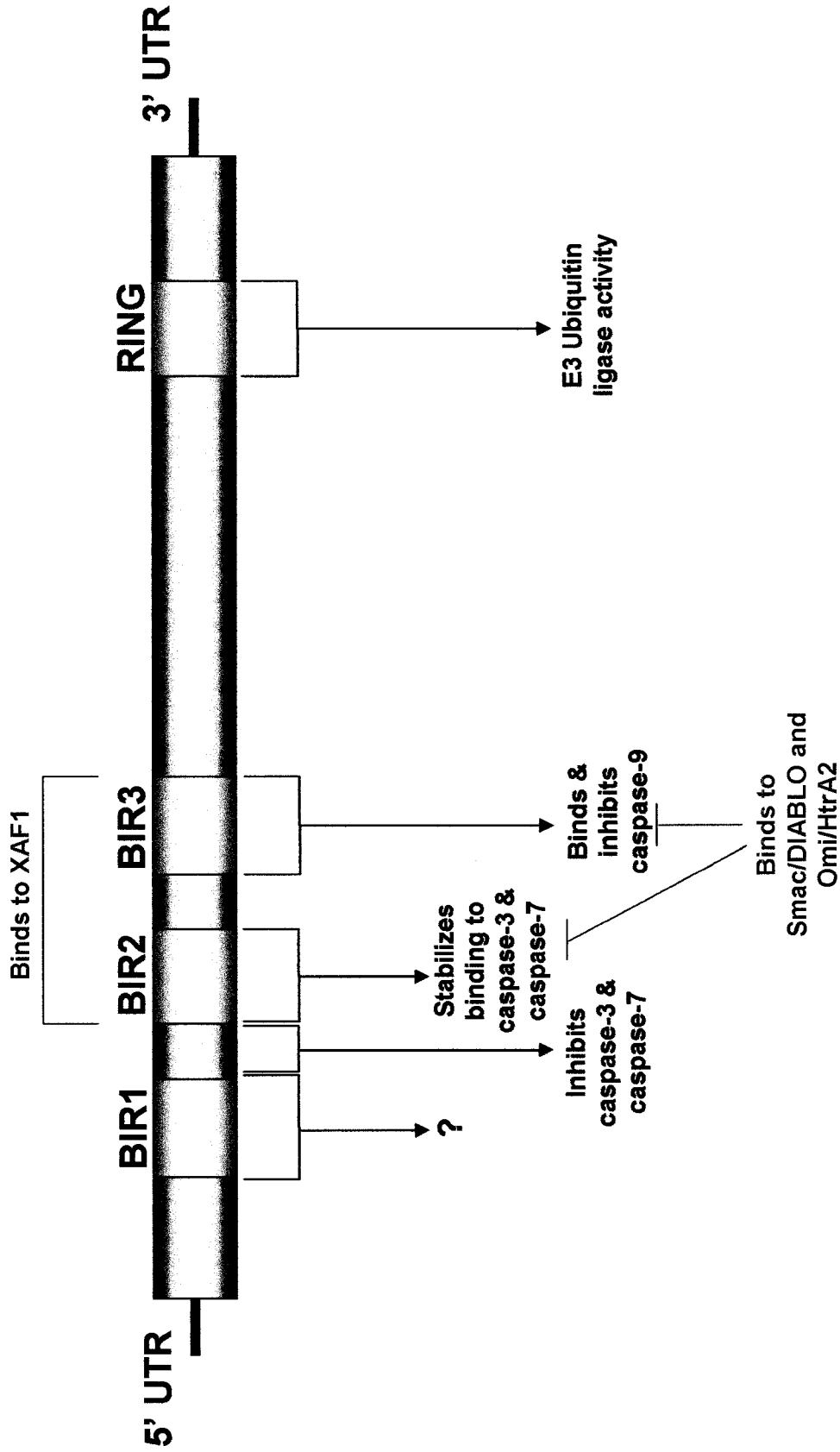


Figure 6: Functional Domains of the XIAP Protein - XIAP contains three BIR domains as well as a RING-zinc finger domain. The function of the BIR1 domain remains unclear. The BIR2 domain in concert with the linker region that precedes it is responsible for the inhibition of caspases-3 and -7. The BIR3 domain binds to and inhibits caspase-9. These functions are inhibited by the release of Smac/DIABLO and Omi/HtrA2 from the mitochondria. XAF1 is also a recognized inhibitor of XIAP that interacts with the BIR2 and BIR3 domains. (Modified from: Holcik and Korneluk, 2001).

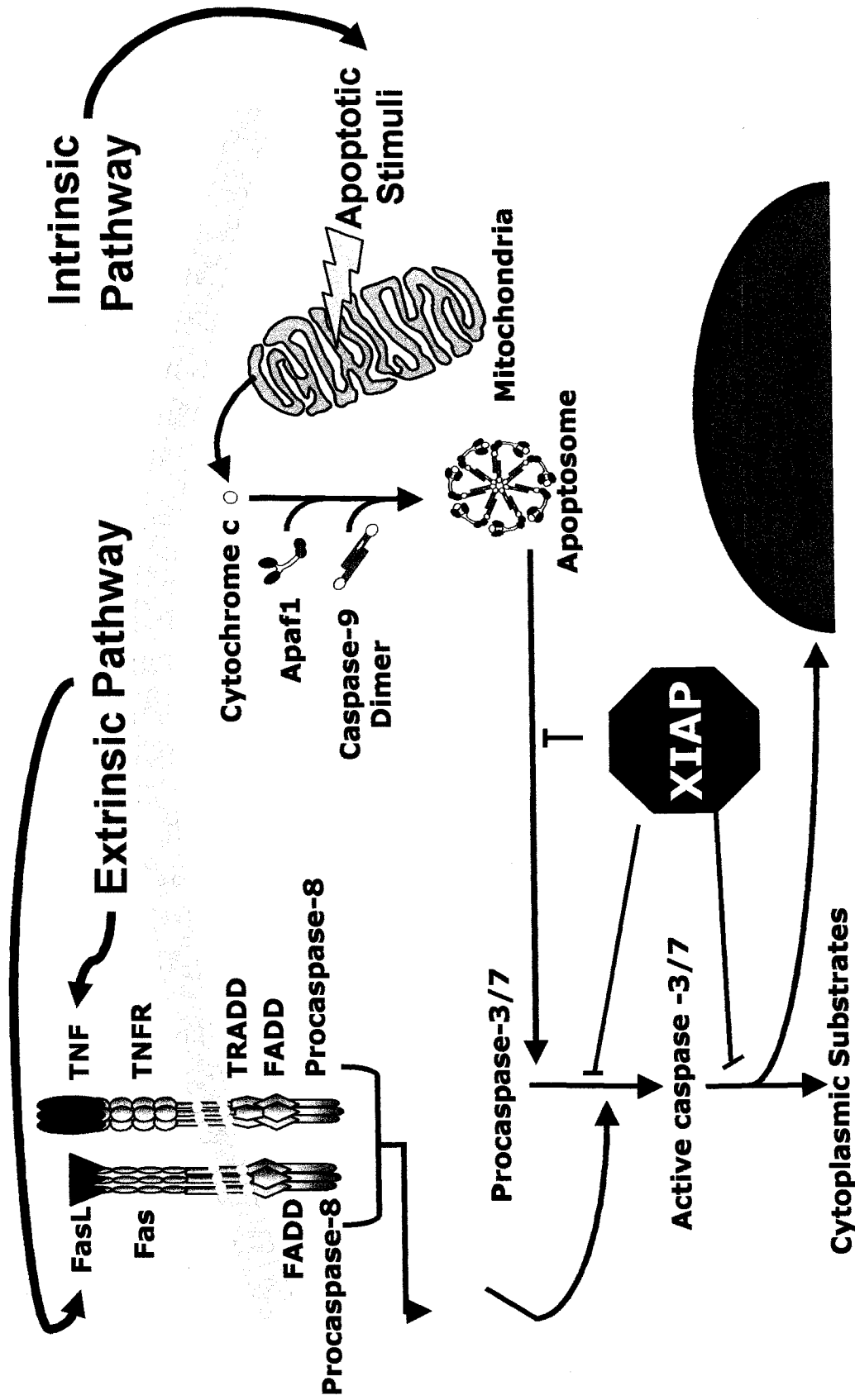


Figure 7: XIAP Inhibits both Intrinsic and Extrinsic Activation of Apoptosis - XIAP blocks the activity of caspases-3, -7, and -9, thereby regulating both the extrinsic (exogenous) pathway and the intrinsic (endogenous) pathway of the activation of apoptosis. The inhibition of both initiator caspase-9 and effector caspases 3- and -7 puts XIAP in a position to inhibit either route of activation. (Modified from: Liston *et al.*, 2003)

1.5.4 The Therapeutic Potential of XIAP

Substantial evidence has amassed in recent years reinforcing XIAP's therapeutic potential of conferring resistance to apoptosis in a variety of *in vivo* disease models. In a study by Xu *et al.*, virally mediated over-expression of XIAP prevented the degeneration of CA1 neurons in the hippocampus following transient forebrain ischemia in adult male Wistar rats (Xu *et al.*, 1999). In addition to structural protection, functional protection was also observed in this study as XIAP over-expression prevented deficits in spatial learning and neuronal activity.

XIAP has also proven to be effective in experimental models of Parkinson's disease (PD) when combined with a neurotrophic factor. In a study by Eberhardt *et al.*, a PD model was created by administering the neurotoxin 1-methyl-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into the substantia nigra pars compacta (SNpc) of mice (Eberhardt *et al.*, 2000). The clinical, biochemical, and neuropathological effects produced by MPTP are irreversible and mimic those observed in idiopathic PD (Bloem *et al.*, 1990). Prior to this study, it had been shown that chronic administration of MPTP, daily for a period of 5 days, was able to induce apoptotic cell death in the SNpc of mice (Tatton and Kish, 1997). In addition, previous studies showed that MPTP toxicity involves the activation of caspases (Yang *et al.*, 1998). The results Eberhardt *et al.* provided the first evidence that adeno-virally delivered XIAP is effective in an *in vivo* model of PD and that in conjunction with the neurorestorative glial cell-derived neurotrophic factor (GDNF), it may provide a promising therapy to work against the progressive cell loss and functional impairment of PD (Eberhardt *et al.*, 2000).

These examples among many others have laid the framework to continue testing XIAP as a therapeutic agent capable of treating a wide range of disorders resulting from apoptotic cell death.

1.6 Retinal Degeneration and XIAP Gene Therapy

Retinal degeneration is a serious problem that affects millions of people worldwide. It can manifest in response to trauma to the eye or as the result of various retinal disorders. In disorders such as glaucoma, retinitis pigmentosa (RP), retinal ischemia, age-related macular degeneration (AMD), and diabetic retinopathy, cell death via apoptosis is the final common pathway to degeneration (Travis, 1998). Given this knowledge, any approach that could limit the apoptotic cascade would potentially be widely applicable to a variety of forms of retinal degeneration.

1.6.1 Applicability of XIAP Gene Therapy to Retinal Degeneration

Various retinal degenerations both inherited and induced are characterized by a loss of vision linked to photoreceptor cell death (Doonan and Cotter, 2004). The genetics of many retinal disorders are complicated and involve a multitude of different genes with numerous mutations existing for any one gene. To target a specific mutation would be a daunting task. Targeting apoptosis, however, which is a highly regulated, final common pathway to photoreceptor cell death has been suggested as a more practical approach (Doonan and Cotter, 2004). Anti-apoptotic therapy is also a more widely applicable therapy as it would encompass a variety of different disorders, both genetic and trauma related.

Past studies have demonstrated the effectiveness of XIAP gene therapy in the eye. Kugler *et al.* transduced retinal ganglion cells (RGCs) using an adenoviral vector expressing XIAP and showed that XIAP was capable of preventing RGC death following axotomy of the optic nerve of adult rats (Kugler *et al.*, 2000). In a similar fashion, Straten *et al.*, built on this evidence through the discovery that the coadministration of XIAP with GDNF acted synergistically to protect axotomized RGCs (Straten *et al.*, 2002). This combination treatment was shown to have greater efficacy than the administration of either treatment independently (Straten *et al.*, 2002).

Further evidence to support the application of XIAP gene therapy to the eye has been documented in models of glaucoma. Glaucoma is a chronic optic neuropathy that in rats, rabbits, monkeys, and humans involves RGC death by apoptosis (Garcia-Valenzuela *et al.*, 1995; Quigley *et al.*, 1995; Kerrigan *et al.*, 1997). In a study by McKinnon *et al.*, rats were given unilateral intravitreal injections of an adeno-associated virus expressing XIAP driven by the chicken beta-actin (CBA) promoter. Ocular hypertension was induced in the same eye to mimic glaucoma. Following chronic exposure to elevated intraocular pressure, optic nerve axon counts were conducted to determine the neuroprotective effects of the administration of XIAP. Based on the results of this study, XIAP gene therapy was found to promote optic nerve axon survival (McKinnon *et al.*, 2002), suggesting that this approach may also be promising for clinical use in humans.

Apoptotic cell death triggered by retinal ischemia as seen in acute glaucoma is also common to several other ocular diseases including diabetic retinopathy, and retinal vascular occlusion (Renwick *et al.*, 2005). In a study by Renwick *et al.*, XIAP-mediated

gene therapy was shown to convey both functional and structural neuroprotection to the retina following an episode of transient ischemia.

The studies by Kugler *et al.* (2000), Straten *et al.* (2002), McKinnon *et al.* (2002), and Renwick *et al.* (2005) all showed XIAP efficacy in protecting RGCs from apoptotic cell death. Additional studies have been conducted which show XIAP neuroprotection of other cell types in the eye, namely the photoreceptors. In a study by Petrin *et al.*, a chemotoxic model of retinal degeneration was created using *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent that cause DNA methylation and results in rapid destruction of the photoreceptor layer. Rats were first injected subretinally with a recombinant adeno-associated virus (rAAV) encoding XIAP followed six weeks later by an intraperitoneal (IP) injection of MNU. The findings demonstrated that the rAAV-XIAP injected eyes exhibited both structural and functional protection for up to one week following MNU injection as compared to complete destruction of the photoreceptor layer in the control eyes (Petrin *et al.*, 2003b). Petrin *et al.* suggest that as XIAP is able to promote potent protection in an MNU model, one of the most severe retinal degeneration models available, it shows great potential for the treatment of less severe and slower progressing forms of inherited retinal degeneration (Petrin *et al.*, 2003b).

With respect to inherited retinal disorders, RP is a common cause of inherited blindness with a worldwide prevalence of about 1 in 4000 for a total of more than one million affected individuals (Hartong *et al.*, 2006). Leonard *et al.* (2007) demonstrated that the administration of rAAV-XIAP to P23H and S334ter rhodopsin transgenic rat models of RP exerted long term structural and functional neuroprotection of photoreceptors (Leonard *et al.*, 2007).

As is evident from the studies conducted to date, XIAP gene therapy is a promising and attractive therapy with a broad range of applications for the management of retinal degeneration.

1.6.2 Retinal Detachment-induced Apoptosis

XIAP gene therapy may also be effective in preventing visual loss associated with retinal detachment. Following a retinal detachment, apoptosis is triggered in the photoreceptors due to their loss of metabolic support. The photoreceptors are the first to die as they rely on oxygen and nourishment from the underlying choroid, which is beneath the RPE with which the retina has lost contact. Animal studies have shown that photoreceptors die through the activation of caspases-3, -7, and -9 (Zacks *et al.*, 2003).

1.7 Thesis Experiments and Hypothesis

1.7.1 Rationale

Previous studies have provided proof of principle for anti-apoptotic gene therapy in the treatment of retinal diseases. These studies have demonstrated that gene therapy with XIAP protects retinal neurons at both structural and functional levels, and helps to preserve vision in animal models of retinal disease. The current study examines the use of XIAP, which functions by inhibiting the activity of caspases-3, -7, and -9, as a therapy to provide protection from apoptosis in an animal model of retinal detachment.

1.7.2 Objectives and Hypothesis

Under normal circumstances, if left untreated, photoreceptors within the retina will fall victim to apoptosis following retinal detachment. The objective of this project is to determine whether a gene therapy approach targeting XIAP to the retina prior to retinal detachment will show similar neuroprotective effects as those from previous studies of retinal degeneration. This will be accomplished by determining whether XIAP decreases the susceptibility of retinal cells to apoptotic death following retinal detachment.

Based on the results of previous experiments which demonstrate the protective abilities of XIAP, it is hypothesized that administration of XIAP prior to retinal detachment will have a neuroprotective effect on the cells of the ONL following detachment.

CHAPTER 2 - MANUSCRIPT

Accepted for publication by **Investigative Ophthalmology and Visual Science**:

XIAP protects photoreceptors from retinal detachment-induced apoptosis

Invest. Ophthalmol. Vis. Sci. First published on December 5, 2008 as doi:10.1167/iovs.08-2855

Laura A. Zadro-Lamoureux,^{1,2} David N. Zacks,³ Adam N. Baker,¹ Qiong-Duan Zheng,³
William W. Hauswirth,⁴ and Catherine Tsilfidis^{1,2}

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

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

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

⁴University of Florida College of Medicine, Department of Ophthalmology, Gainesville, Florida, U.S.A.

Supported by grant MOP-79509 from the Canadian Institutes of Health Research (CIHR) to CT and by a fellowship to LAZ from the Natural Sciences and Engineering Research Council of Canada (NSERC). DNZ was supported by grant EY14705 from the NIH and a Career Development Award from Research to Prevent Blindness, Inc. We acknowledge NIH grants EY13729, EY11123, NS36302, EY08571, and grants from the Macular Vision Research Foundation, Foundation Fighting Blindness, Juvenile Diabetes Research Foundation, Hope for Vision and Research to Prevent Blindness, Inc. for partial support of this work. WWH and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.

Contribution of Authors:

This manuscript was written by Laura Zadro-Lamoureux (LAZ) and Dr. Catherine Tsilfidis (CT) and edited by Dr. David Zacks (DNZ) and Dr. William Hauswirth (WWH). CT and DNZ conceived and designed the experiments. All of the experiments and figures are the work of LAZ with the exception of Figure 8, which was carried out by Qiong-Duan Zheng (QDZ) at the Kellogg Eye Center, University of Michigan. The surgical procedures were performed by LAZ, Adam Baker (ANB) and DNZ. The recombinant adeno-associated virus was supplied by WWH.

ABSTRACT

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

Methods. Subretinal injections of a recombinant adeno-associated 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 analyzed for caspase-3 and -9 activity. Histological 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 rise 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 in comparison to GFP-treated detachments. At 2 months after the detachment, histology and immunohistochemistry confirmed the preservation of the ONL at sites of XIAP over-expression, 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 following retinal detachment.

INTRODUCTION

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 (Cook *et al.*, 1995; Zacks *et al.*, 2003), 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 (Gariano and Kim, 2004). Risk increases dramatically when factors such as trauma, myopia, cataract surgery, previous retinal detachment or family history are considered (Kang and Luff, 2008). 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 which allows vitreal fluid to leak into the subretinal space and detach the retina from the underlying RPE. In tractional detachment, fibrovascular tissue caused by an 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 (Erickson *et al.*, 1983), and opsin redistribution (Lewis *et al.*, 1991). In addition, there is remodelling of the synapses of second-order neurons and proliferation of retinal glial cells (Lewis *et al.*, 1998). Some of these changes are significant obstacles to

recovery but may be somewhat reversible after reattachment of the retina (Lewis *et al.*, 2002). However, the primary cause of visual loss is most likely photoreceptor death by apoptosis (Cook *et al.*, 1995; Chang *et al.*, 1995). In animal studies, apoptosis is initiated within 24 hours after retinal detachment and peaks at 3 days (Zacks *et al.*, 2003; Lewis *et al.*, 2002). 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 (Zacks *et al.*, 2003).

XIAP (the X-linked inhibitor of apoptosis) 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 3 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 (Liston *et al.*, 2003). XIAP also contains a carboxy-terminal RING zinc finger domain that possesses E3 ubiquitin ligase activity. This domain determines the fate of XIAP and/or the cell depending on the severity of the cellular insult. Under conditions of severe cellular stress, XIAP will auto-ubiquitinate 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 (Liston *et al.*, 2003).

In disease models, XIAP has been shown to confer protection in forebrain ischemia (Xu *et al.*, 1999), in MPTP-induced Parkinson's disease (Crocker *et al.*, 2003; Eberhardt *et al.*, 2000), and in cisplatin-induced ototoxicity (Chan *et al.*, 2007; Cooper *et*

al., 2006; Tabuchi *et al.*, 2007). In gene therapy studies in the eye, XIAP protects ganglion cells following axotomy of the optic nerve (Kugler *et al.*, 2000; Straten *et al.*, 2002), increased intraocular pressure (McKinnon *et al.*, 2002), and retinal ischemia (Renwick *et al.*, 2006). It also protects photoreceptors from chemotoxic insult (Petrin *et al.*, 2003a; Petrin *et al.*, 2003b), and in genetic models of retinitis pigmentosa (Leonard *et al.*, 2007). The current study examines the protective effects of XIAP delivered via an adeno-associated 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 (Zacks *et al.*, 2003).

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 with both 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 (rAAV) 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 post-transcriptional regulatory element (WPRE) in the 3' untranslated region of the construct. Serotype 5 rAAV was generated (Hauswirth *et al.*, 2000; Zolotukhin *et al.*, 2002) and purified (Leonard *et al.*, 2007) 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 (Mydriacyl; Alcon Canada, Mississauga, ON, Canada) and 2.5% phenylephrine hydrochloride (Mydfrin; 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 (Gentel gel; Novartis Pharmaceuticals Inc., Mississauga, ON, Canada) was applied to the eye. Subretinal injections were performed by creating a sclerotomy approximately 2mm posterior to the limbus with a 20-gauge V-

lance knife (Alcon). Care was taken to avoid lens contact as this could induce cataract development. A cover slip coated with Genteal 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 Company, Reno, NV) was inserted through the scleral puncture, guided lateral to the lens, and inserted through the retina. A 2 μ L volume of rAAV-XIAP or rAAV-GFP combined with fluorescein tracer (50:1 v/v) was delivered to the subretinal space of the eye. The fluorescein allowed for immediate visualization and evaluation of the injection location, allowing ascertainment of a successful subretinal delivery. Injections were delivered in a consistent manner between the 12:00 and 2:00 position. Post-surgical care consisted of administration of the antibiotic 0.3% ciprofloxacin hydrochloride (Ciloxan; Alcon), and a non-steroidal anti-inflammatory drug 0.03% flurbiprofen sodium (Ocufen; Allergan, Irvine, CA) for five days post-injection.

Retinal Detachment

Approximately two weeks following viral injections, a retinal detachment was performed in the left eye of each rat as previously described (Zacks *et al.*, 2003). The detachments were created by injecting 10mg/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 the 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 (Zacks *et al.*, 2004). Caspase-9 activity was measured using a Caspase-9 Colorimetric Assay Kit (BioVision Research Products, Mountain View, CA), as per the manufacturer's instructions. This assay is based on the detection of the chromophore p-nitroanilide (pNA) following cleavage from the labeled substrate LEHD-pNA. Caspase-3 activity was measured using a Caspase-3 Colorimetric Assay kit (Chemicon International, Billerica, MA), as per manufacturer's instructions. This assay is based on cleavage of the pNA-DEVD substrate by activated caspase-3.

Tissue Fixation and Processing

Rats were administered a lethal injection of Euthansol and were subsequently perfused with 4% paraformaldehyde (PFA) in order 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. The samples were then taken through a series of dehydration steps ending with embedment in paraffin. Eyes were sectioned at 10 μ m for histological analysis.

Histological Analysis

Hematoxylin and eosin (H&E) staining was performed on 10 μ m sections to locate retinal detachments. Once a detachment was identified, subsequent slides were subjected to immunohistochemical analysis in order to confirm the presence of XIAP or GFP. XIAP was detected using an anti-HA mouse IgG primary antibody (Roche Applied Science, Laval, QC), followed by a goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). GFP was detected using an anti-GFP rabbit IgG (Invitrogen, Eugene, OR) followed by a goat anti-rabbit IgG (Invitrogen, Eugene, OR). Rhodopsin was detected with the B630 monoclonal antibody (Rohlich *et al.*, 1989). Slides were counterstained with the nuclear stain 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). Images were obtained using a Zeiss Axioskop light microscope with a Zeiss AxioCam HRc camera.

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (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 using the Apoptag Peroxidase *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA). In order to eliminate observer bias, TUNEL-positive cells were detected using a program developed with the mathematical software MATLAB (version R2007a, The Mathworks Inc.). The program analyzed Photoshop images (Adobe Systems Inc.) 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 two standard

deviations 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 at 2 months after the detachment were processed for histological analysis. Images were taken of 10 μ m sections stained with H&E. 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. Since retinal thickness varies with distance from the optic nerve, the thickness of the inner nuclear layer (INL) was used as a control to ensure that ONL measurements were taken at the same distance from the optic nerve head. For both attached and detached regions, at least four counts were taken from each animal and averaged.

RESULTS

The hypothesis that rAAV-XIAP transfection would result in photoreceptor neuroprotection led to the development of three predictions that could be directly tested. The first prediction was that rAAV-XIAP transfected eyes would show less detachment-induced activation of caspases than rAAV-GFP transfected 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 transfection would result in a significantly increased number of photoreceptors

surviving extended periods of detachment in comparison to control eyes. The 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 (Zacks *et al.*, 2003 and Zacks *et al.*, 2004). There was no evidence that rAAV-GFP had a neuroprotective effect or accelerated photoreceptor degeneration. As seen in Figure 8, retinal detachment in the rAAV-GFP transfected eyes (GFP-OS) showed the expected elevation in caspase-3 and -9 activity as compared to intact, non-detached 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 (Zacks *et al.*, 2003; Zacks *et al.*, 2004).

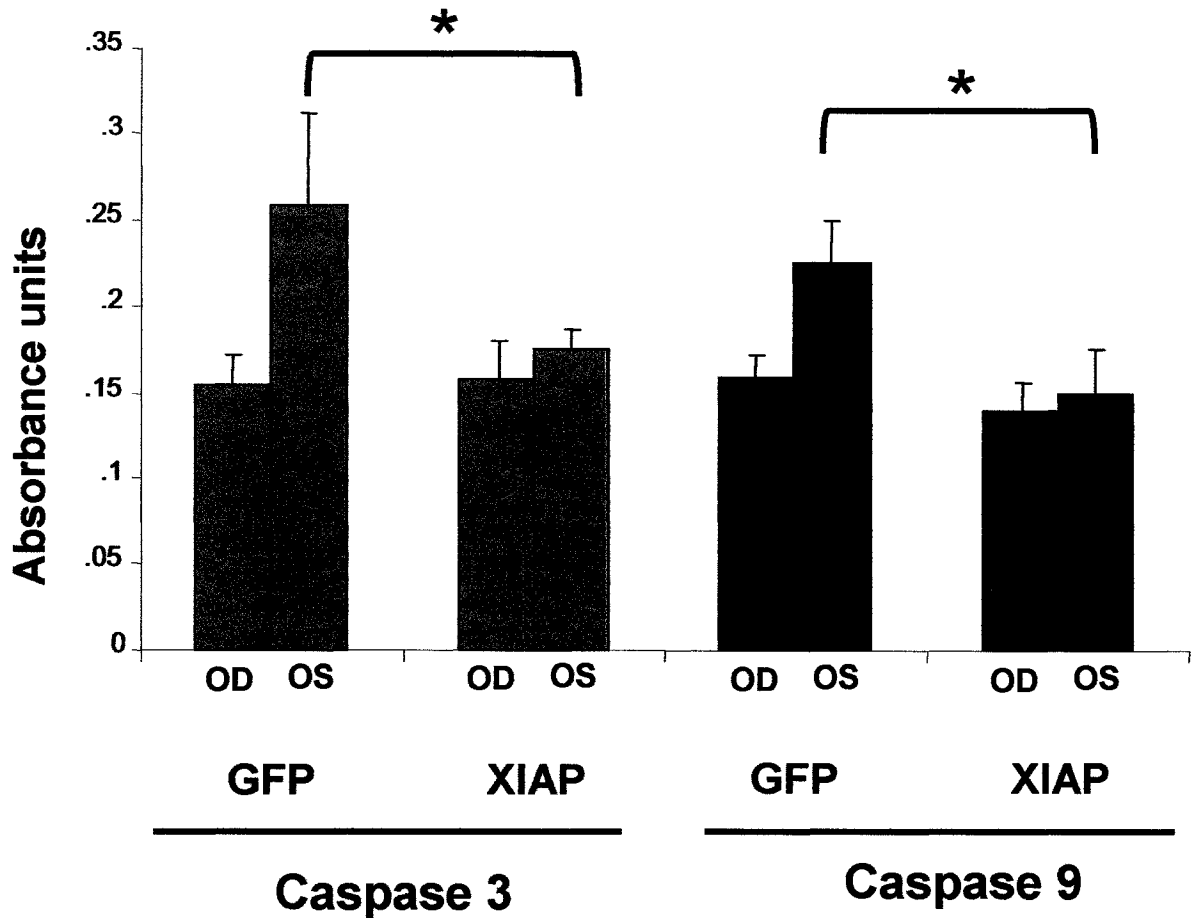


Figure 8: Caspase-3 and -9 assays following retinal detachment in GFP and XIAP-treated retinas – Subretinal injections of rAAV-GFP or rAAV-XIAP were followed by retinal detachment in the left eye (OS) of the animal. Right eyes (OD) served as intact controls. Significantly increased caspase-3 and -9 activity ($p < 0.05$, student t-test) was observed in the GFP-treated detached retinas in comparison to the XIAP-treated retinas. There was no significant difference between XIAP-treated retinas and their contralateral intact controls.

TUNEL Analysis

Eyes were sampled at 3 days following 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 (Zacks *et al.*, 2003; Lewis *et al.*, 2002). Immunohistochemistry confirmed robust expression from both the rAAV-GFP and rAAV-XIAP viral injections (Figure 9A, 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 transfections and the detachment 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 (Figure 9E-G). Although the XIAP-related decrease in the number of TUNEL-positive cells did not reach statistical significance as compared to the GFP-treated eyes, the results do correlate well with the XIAP-related decrease in caspase activity shown in Figure 8.

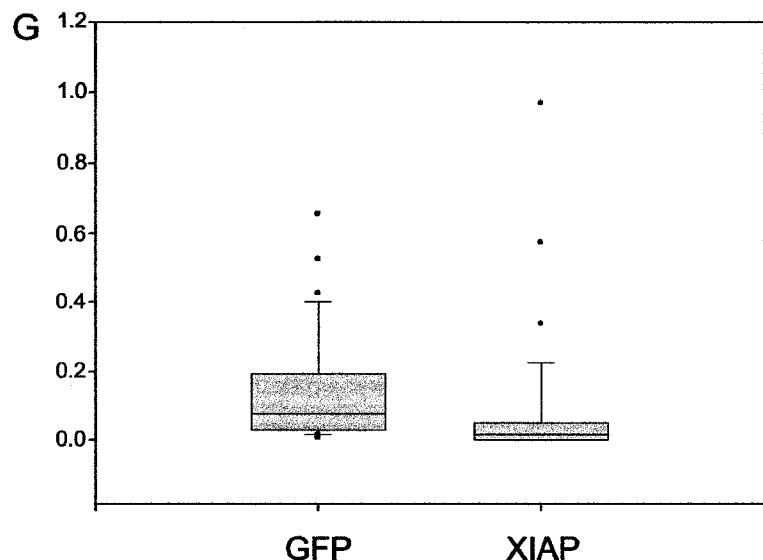
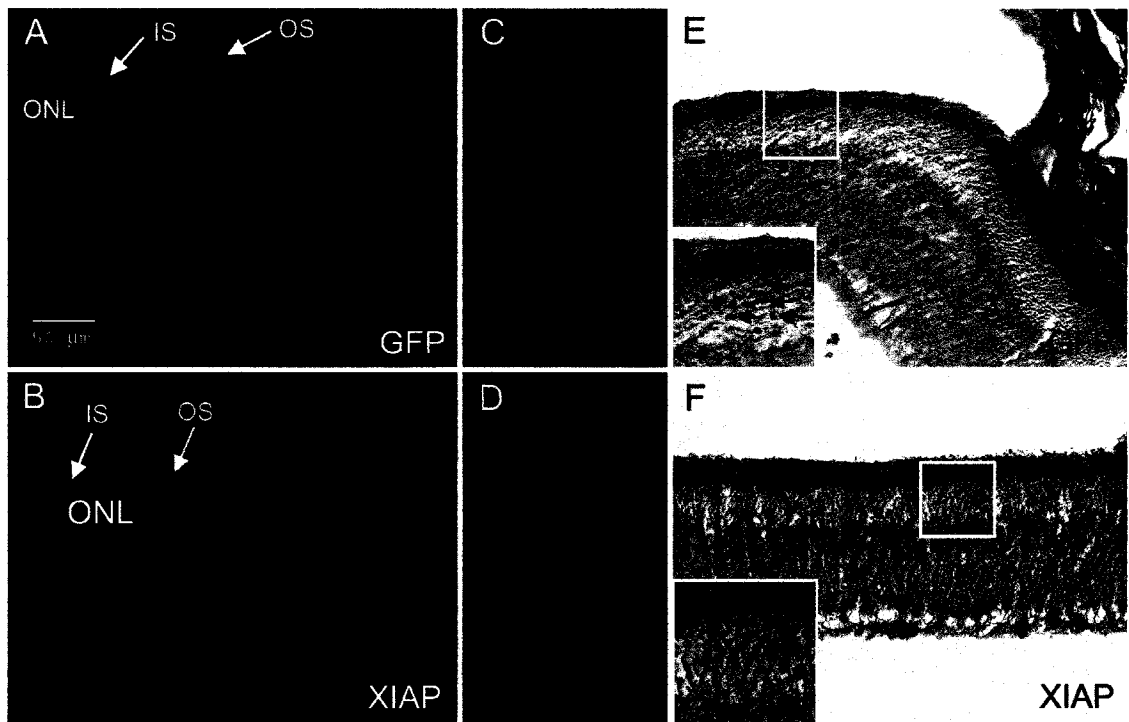


Figure 9: 3 Day Immunohistochemistry – antibodies to GFP (A) and to the HA tag of XIAP (B) confirmed robust over-expression in the cell bodies and inner (IS) and outer (OS) segments of the photoreceptors from both rAAV constructs. Controls run without primary antibody for GFP or XIAP are shown in C and D respectively. TUNEL analysis confirmed that GFP-treated retinas had more apoptotic nuclei than XIAP-treated retinas (brown pigment in E, F and black arrows in insets). TUNEL-positive pixel counts (boxplot, G) supported the immunohistochemistry results. Each box contains the values between the 25th and 75th percentile, 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 boxplot was generated with SigmaPlot, version 8.0 (SPSS, Inc.). *ONL*, outer nuclear layer.

Photoreceptor Survival in Chronic Detachment

To assess long-term structural protection of photoreceptors, histology was conducted on eyes sampled at 2 months after the retinal detachment.

Immunohistochemistry against GFP (Fig. 10A, B) or XIAP (Fig. 10D, E) was employed to visualize the site of rAAV virus injection and to correlate this with histological studies.

Morphologically, there were clear differences between XIAP-treated and GFP-treated retinas. The inner and outer segments of the XIAP-treated samples were generally more organized than those of the GFP-treated retinas (compare Figure 10D, E with 10A, B), although less so than attached regions in the same eye. Rhodopsin staining confirmed that the preserved photoreceptors were viable and produced functional protein (Figure 10C, F).

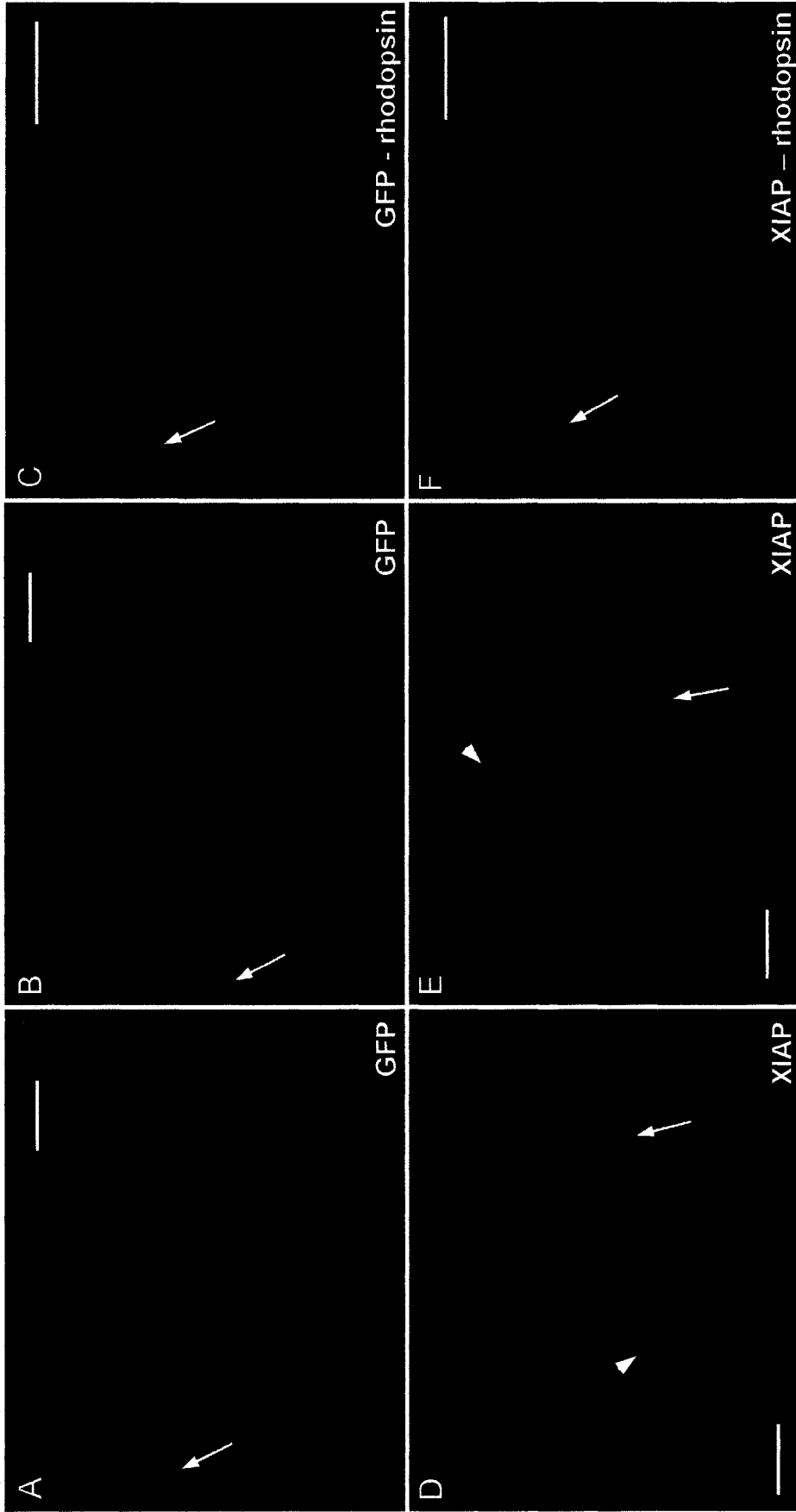


Figure 10: 2 Month Immunohistochemistry – for GFP (A, B) and XIAP (D, E) confirmed sustained expression at 2 months after the detachment. The GFP signal (green) was faint because many of the photoreceptors expressing the viral transgene had died. In contrast, XIAP signal (red) was bright, and accompanied by increased numbers of photoreceptors. Note that in retinal areas where XIAP signal was reduced (arrowhead), photoreceptor loss was considerable. Rhodopsin staining (red) in GFP-injected (C) and XIAP-injected (F) retinas shows that the preserved photoreceptors are able to synthesize functional protein. Outer nuclear layer identified by arrow. Magnification bar = 50µm.

The layers of photoreceptor nuclei in the ONL were counted and compared between the detached retinas treated with either 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 as one moves towards the periphery of the eye. Overall, there was significant preservation of the ONL in the XIAP-treated detached retinas ($p < 0.05$) (Figures 10 and 11). These retinas had from 4 to 8 nuclear layers (compared to 6 to 11 in the attached regions of the same eye). GFP-treated detached retinas had from 0 to 7 nuclear layers (with 6 to 14 in the attached portions of the same eyes). For each animal, a ratio of ONL nuclei in the detached relative to the attached regions was calculated, and these values are presented in Figure 11E.

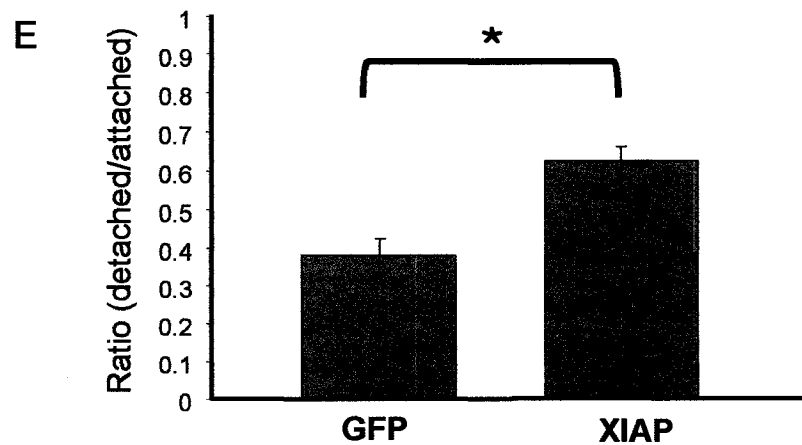
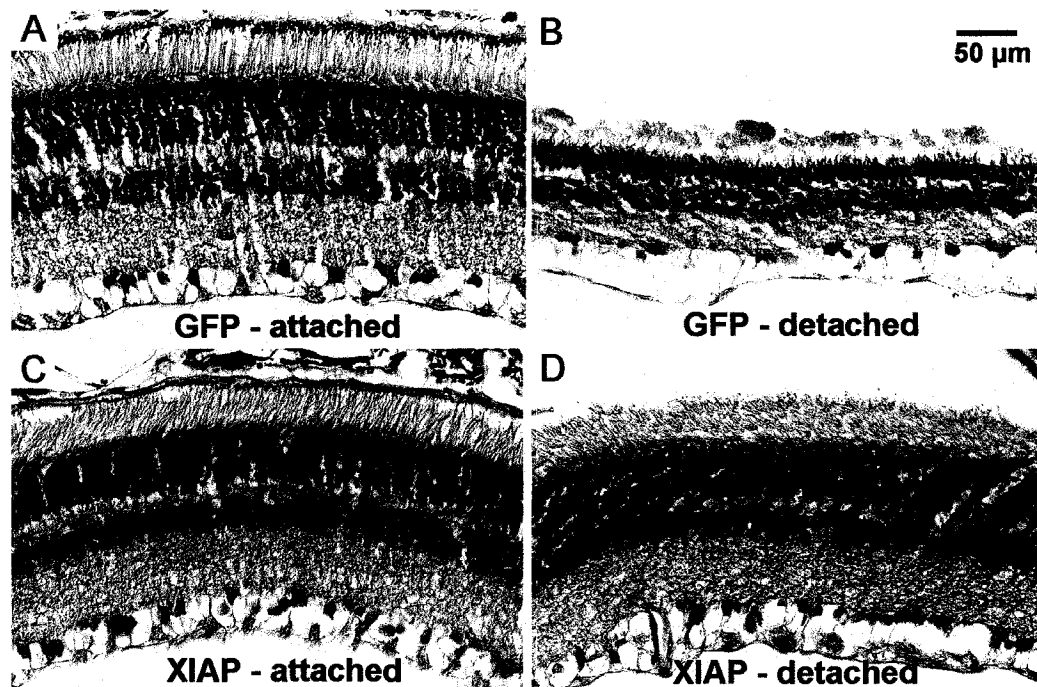


Figure 11: Retinal Thickness Comparison – between attached (A, C) and detached (B, D) retinas in XIAP and GFP-treated animals. At two months after detachment, XIAP-treated retinas (D) were consistently thicker than GFP-treated retinas (B) and their inner and outer segments were more organized. A ratio was obtained by dividing the number of nuclear layers in the ONL in a detached region of the eye by the number of nuclear layers in the ONL in the attached retina in the same eye (E). XIAP-treated detached retinas had significantly higher ratios than GFP-treated retinas ($p < 0.05$, student t-test).

DISCUSSION

Rapid re-attachment is imperative in order to achieve a good visual outcome following retinal detachment. Animal studies, including the current study, have shown that caspases – the actual executors of apoptosis – are activated within 24 hours after a detachment. Burton (Burton, 1982) 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 re-apposition 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 re-attachment can occur.

The current 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 60-70% of the ONL, compared to 30-40% 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 (Leonard *et al.*, 2007). In addition, in this study the subretinal injections did not always completely coincide with areas of full detachment, so there may have been regions of unprotected retina that were also included in the analysis.

Critics of anti-apoptotic 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 'point of no return' (reviewed in Boatright and Salvesen, 2003). The protective effects of XIAP suggest that this is not so. However, we cannot rule out the possibility that XIAP is having other effects in addition to caspase inhibition. XIAP has been shown to suppress cell death via other mechanisms. Through its RING zinc finger domain, XIAP has E3 ubiquitin ligase activity and can promote the degradation of pro-apoptotic proteins (reviewed in Liston *et al.*, 2003). XIAP is also involved in the transcriptional activation of prosurvival pathways through TAK1 (Liston *et al.*, 2003; Hofer-Warbinek *et al.*, 2000; Sanna *et al.*, 2002). TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) involved in the activation of both the NF- κ B and JNK1 prosurvival pathways (Hofer-Warbinek *et al.*, 2000; Sanna *et al.*, 2002). 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-transfected eyes, however, does 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 suffered by photoreceptors, thus buying the patient critical time until successful re-attachment can be achieved. In this respect, AAV-mediated XIAP delivery will have limited success since upregulation of XIAP will not be rapid enough to protect the 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 remodelling of second order neurons and reduced proliferation of retinal glial cells, as well as on final visual outcome.

ACKNOWLEDGEMENTS

We would like to thank Irena Szymanska, Jennifer Cameron and Kevin Leonard for technical assistance, Valerie Wallace for reagents and Marc Lamoureux for developing the TUNEL detecting software.

CHAPTER 3 - GENERAL DISCUSSION

3.1 Cataract Development

Viral delivery to the retina of Brown Norway rats was achieved through a subretinal injection. A small puncture was made in the sclera and a needle was advanced through the vitreous and the retina into the subretinal space. Care was taken to avoid contact with the lens. Nevertheless, one of the complicating factors experienced at the outset of this experiment was the incidence of cataracts seen in the animals under study. Despite what seemed to be generally flawless injections, a large proportion of the rats developed moderate to severe cataracts a few days following the subretinal injections. It is recognized that any contact made with the lens during the injection procedure can lead to the development of a cataract. Even though an injection appeared to be perfect, there remains the possibility that the slightest nick of the lens could have occurred leading to cataract formation. As a result, not all animals could be used for the subsequent detachments as it was impossible to accurately detach the retina when the view of the retina was obscured by the opacity of the cataract.

Subsequent to this study, subretinal injections of a mix of fluorescein and water were conducted in the eyes of Long Evans rats in an attempt to determine what had caused the cataracts. Various modes and angles of entry to the eye were attempted in order to determine the best injection procedure. Interestingly, the results of this side-study indicated that cataract incidence was substantially decreased in the Long Evans breed of rat as compared to the Brown Norway rats used in the current study, even in animals that had their lenses intentionally nicked. As a follow-up, an additional study was conducted in which 10 Long Evans rats were injected subretinally with either rAAV-

XIAP or rAAV-GFP, to rule out that the virus could have influenced cataract development. Up to six months following the injections, only two animals developed cataracts (1 XIAP and 1 GFP). The results suggest that a breed-specific sensitivity may exist toward the development of cataracts post-surgery. Further investigation into the cause of cataract development should be considered in order to reduce the number of animals required for future experimentation.

3.2 Possible Reasons for Incomplete Protection of Photoreceptors

3.2.1 Injection Procedure

XIAP gene therapy does not completely confer resistance to cell death in the retinal detachment model. Despite the increased survival rates in the XIAP-treated detached retina group, at best, only 60-70% of the ONL was preserved. There are several possible reasons for this result. It may be that not all of the cells affected by the detachment were transduced by the subretinal injection with rAAV-XIAP. Despite the fact that injection locations were kept relatively consistent and were recorded, it is impossible to say with certainty that the detachments were created in the exact same location as the injections. Therefore, there were likely regions in some of the eyes where the injection location and the subsequent detachment did not overlap. In these cases, the cells may not have over-expressed XIAP adequately to confer protection, which could account for the partial protection exhibited.

It is also possible that there was some variability in the injection procedure from one eye to the next, which would result in variable levels of expression of XIAP. This variability was seen in previous studies from our lab (Leonard *et al.*, 2007). This might

account for increased levels of protection in some eyes and reduced levels of protection in others.

3.2.2 Caspase-Independent Cell Death

Yet another possibility as to why we did not observe complete protection is that perhaps not all cell death resulting from retinal detachment is apoptotic death. It is well documented that mammalian cells can die via other pathways which are biochemically and morphologically distinct from the apoptotic pathways (Kroemer and Martin, 2005). Although apoptosis was clearly involved in the cell death seen in the current experiment, as evidenced by the activation of caspases-3, -7, and -9 in the caspase assays, and the presence of TUNEL positive staining in the detached retinas, the contribution of other forms of programmed cell death cannot be ruled out. Recent evidence suggests the existence of multiple non-apoptotic, regulated cell death mechanisms, some of which overlap or are mutually exclusive with apoptosis (Degterev and Yuan, 2008). Past research suggests that caspase-inhibition often does not prevent cell death but causes a shift to caspase-independent self-destruction processes (Kroemer and Martin, 2005). The basis of this argument lies in the fact that many of the stimuli that lead to caspase-dependent apoptosis also promote mitochondrial damage that cannot be reversed by blocking caspase activity (Kroemer and Martin, 2005). Perhaps following retinal detachment, caspase-independent cell death (CICD), a form of non-apoptotic cell death, could also be contributing to the death of photoreceptors.

Even though CICD has similar features as classical apoptosis (e.g. mitochondrial outer membrane permeabilization [MOMP], diffusion of proteins from the intermembrane space of the mitochondria, and DNA fragmentation), it does not lead to the activation of the caspase cascade (Okada and Mak, 2004). Therefore, CICD is considered as a non-apoptotic form of cell death. Apoptosis and CICD are initiated similarly by the permeabilization of the outer mitochondrial membrane followed by the subsequent release of proteins into the cytosol that triggers the implementation of death programs (Blank and Shiloh, 2007). CICD involves the release of caspase-independent death effectors including apoptosis-inducing factor (AIF), endonuclease G (EndoG), and Omi/HtrA2 (Garrido and Kroemer, 2004). Each of these proteins is capable of contributing to cell death through different mechanisms. The release of AIF leads to its translocation from the cytosol to the nucleus where it promotes DNA degradation and chromatin condensation (Galluzzi *et al.*, 2008 and Ye *et al.*, 2002). EndoG is a mitochondrial nuclease that once liberated into the cytosol, translocates to the nucleus where it has been shown to cause oligonucleosomal DNA fragmentation, even in the presence of caspase inhibitors (Li *et al.*, 2001). Omi/HtrA2 is a protease that also resides in the mitochondrial intermembrane space. Upon its release into the cytosol, it promotes cell death through two mechanisms. The first involves IAP inhibition which leads to increased caspase activity while the second involves its serine protease activity, which is caspase-independent (Susin *et al.*, 1999 and Hedge *et al.*, 2002).

CICD is a complex process that encompasses several different mechanisms of cell death. It is possible that any number of these mechanisms could be at work in conjunction with apoptosis following a retinal detachment. This suggests a potential

explanation as to why we do not see complete protection from cell death by using a caspase inhibitor following a retinal detachment.

3.2.3 Necrosis

In spite of the fact that past studies have provided evidence showing that photoreceptors die via apoptosis following a retinal detachment (Zacks *et al.*, 2003), there remains some debate as to the contribution of necrosis. Apoptosis can be distinguished from necrosis by the occurrence of internucleosomal DNA fragmentation, as can be visualized by *in situ* labeling with terminal deoxynucleotidyl transferase (Tdt)-mediated incorporation of biotinylated nucleotides into the 3' ends of DNA fragments (terminal dUTP nick end labeling [TUNEL]) (Cook *et al.*, 1995). The results of the TUNEL experiments conducted for the current study further corroborate the existing data that retinal detachment-induced cell death does in fact occur by apoptosis. In comparison, cells that die by necrosis often exhibit changes in nuclear morphology but not the organized chromatin condensation and fragmentation of DNA that is characteristic of apoptotic cell death (Edinger and Thompson, 2004). Furthermore, apoptotic bodies are recognized and removed by phagocytic cells, therefore apoptosis is notable for the absence of inflammation surrounding the dying cell (Edinger and Thompson, 2004).

Necrosis is a form of unregulated cell death that normally occurs as the result of overwhelming stress that is incompatible with cell survival (Degterev and Yuan, 2008). It is characterized by organelle swelling, mitochondrial dysfunction, massive oxidative stress, and rapid plasma-membrane permeabilization (Degterev and Yuan, 2008). Necrosis is typically associated with inflammation. Interestingly, evidence of

inflammation was observed in the current study as macrophages were seen in some of the retinal sections analysed, suggesting that necrosis could be occurring concurrently with apoptosis. This therefore represents another basis for which XIAP-gene therapy does not completely confer resistance to cell death in a retinal detachment model and supports the hypothesis that another mechanism must be influencing cell death. It has been suggested that milder insults to the cell cause apoptosis, while more severe insults induce uncontrollable necrosis (McConkey, 1998). Perhaps some of the more severe detachments exhibited higher levels of necrosis than those that were less damaged.

Taking into consideration all of the factors that may have influenced cell death and the degree of protection exhibited by treatment with rAAV-XIAP, it is likely that more than one type of cell death occurs following retinal detachment. Despite the influence of these factors, statistically significant protection was observed in the treated group, thereby indicating that rAAV-XIAP treatment exerts a protective effect on cells of the ONL following a retinal detachment.

3.3 Safety and Efficacy of AAV-mediated Gene Therapy

3.3.1 Safety and Efficacy of AAV Vectors

Historically, gene therapy studies have raised concerns about the safety and efficacy of viral vectors. AAV vectors, such as those used in the current study, appear to be promising for ocular gene therapy as they are relatively non-toxic even at high doses and initiate only a minimal immune response. In fact, AAVs (in contrast to adenovirus), although capable of eliciting a modest immune response, have shown no toxicity subretinally in several large animal species, including monkeys (Hauswirth and

Beaufriere, 2000). Additionally, 60% to 80% of humans are seropositive for AAV (Hauswirth and Beaufriere, 2000). Furthermore, rAAVs remain episomal, eliminating threats of insertional mutagenesis.

To date, AAVs have been used in several Phase I clinical trials in humans and appear to be completely safe. Three groups (in Europe and the United States) have initiated Phase I clinical trials employing the use of AAV to deliver RPE65 to patients with Leber's congenital amaurosis (LCA). LCA comprises a group of recessively inherited, severe blinding diseases caused by rod-cone dystrophies (Bainbridge *et al.*, 2008). One type of this disease, LCA2, results from mutations in the RPE-specific 65 kDa protein gene RPE65 (Maguire *et al.*, 2008). This protein is responsible for the production of 11-*cis*-retinal, a natural ligand and chromophore of the opsins, a group of light-sensitive membrane-bound G protein-coupled receptors found in photoreceptor cells of the retina (Maguire *et al.*, 2008). Without 11-*cis*-retinal, the opsins are unable to capture light and transduce it into the electrical signals required for vision (Perrault *et al.*, 1999). Published results from the Phase I clinical studies using AAV-RPE65 are very promising and demonstrate improvement in vision (Bainbridge *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008). In considering vector safety, these studies demonstrate that AAVs were well tolerated with almost no evidence of complications or adverse effects (Bainbridge *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008), warranting that further trials in ocular gene therapy will be expanded in the future.

In order for a gene therapy to be effective for the treatment of chronic retinal degeneration, it is critical that the desired transgene expression persist for years to decades. The longevity of transgene expression has been validated in canine models of

LCA treated with AAV gene therapy vectors. In these models, transgene expression is maintained well into the fifth year following a single subretinal injection (Acland *et al.*, 2005). Furthermore, these dogs continue to exhibit significant preservation of vision as assessed by electroretinograms (ERGs), obstacle avoidance, and visual tracking behaviour. To build on support for persistence in canine models, studies conducted in monkeys have also shown that expression can persist for at least several years post-delivery (Bennett *et al.*, 1999). In addition, rAAV delivered to the subretinal space did not cause any adverse functional effects.

Although the current mode of delivery for XIAP using the rAAV vector has been effective in *in vivo* studies, further exploration of safer delivery strategies will be necessary to prepare XIAP for clinical use.

3.3.2 Safety and Efficacy of XIAP - Is over-expression dangerous?

One of the obvious concerns in promoting the over-expression of an IAP is that the prevention of apoptotic cell death could result in uncontrolled cell proliferation. In healthy cells, the over-expression of XIAP does not appear to have any adverse effects. In fact, researchers at the Children's Hospital of Eastern Ontario Research Institute (CHEORI) in Ottawa have generated transgenic mice with XIAP driven by the ubiquitin promoter. These mice have high levels of expression in the brain and remain phenotypically indistinguishable from wild-type mice (R. Korneluk, personal communication). Additionally, transgenic mice over-expressing XIAP driven by the neuron-specific enolase (NSE) promoter also had high levels of expression in the brain without any evidence of abnormalities (Crocker *et al.*, 2003).

In contrast to these results, concern for the safety of XIAP has been raised in recent studies that suggest that transgenic mice over-expressing XIAP in mature Purkinje cells and in retinal bipolar cells experience neurodegeneration (Korhonen *et al.*, 2008). In separate studies, it has also been suggested that the over-expression of XIAP in malignant cell-lines induces chemoresistance (Wilkinson *et al.*, 2004; Berezovskaya *et al.*, 2005; Tong *et al.*, 2005). Similarly, some studies of patients with malignancies including acute myeloid leukemia demonstrated that increased XIAP expression is associated with a poor outcome (Tamm *et al.*, 2004a and Tamm *et al.*, 2004b). Currently, Aegea, under license from Idera Pharmaceuticals, is developing an antisense oligonucleotide targeting XIAP messenger RNA, as a potential cancer therapy (Tamm, 2008). This therapy would seemingly work by preventing the translation of XIAP. Additionally, small molecule inhibitors of XIAP that bind and inhibit the BIR3 and BIR2 domains are also currently under development (Schimmer, 2007).

The conflicting effects of the over-expression of XIAP described in various studies may be the result of different levels of expression in the various studies, or could also be due to differences in the cell types over-expressing the transgene. Based on results from animal studies to date, a therapy utilizing the over-expression of XIAP to treat retinal degeneration does not appear to put a healthy individual at risk. Its impact on a cancer patient, however, could be very different, especially if the mode of delivery risks the possibility that XIAP over-expression could be driven in cells other than those targeted for treatment. An ideal vector for XIAP gene therapy should drive transgene expression only in the desired target cells (in the case of the current study, the photoreceptors). Additionally, this expression must be suitably high to provide

neuroprotection without inducing toxicity. The current study employs the use of a rAAV vector driven by chicken beta-actin, a general promoter that expresses in many different cell types. For the purposes of the study, this vector works well to transduce the photoreceptor cells, however, from a clinical standpoint, this promoter would not be appropriate for human therapy as its target for expression is not specific enough. The threat remains that the virus could infect other cells in the body should it somehow travel outside the subretinal space. Future studies should investigate the effectiveness of other promoters more specific to the photoreceptor cells. Another option would be to consider the use of an inducible promoter. These promoters have fewer safety concerns as they permit the regulation of gene expression.

XIAP therapy holds tremendous potential; however, it is important that all safety precautions be taken into consideration before administering this therapy in a clinical setting. A list of contraindicating conditions highlighting patients who may not be suitable candidates for XIAP gene therapy will no doubt be a likely requirement prior to its use clinically.

3.4 Alternate Modes of XIAP delivery

Retinal detachment as well as other acute disorders such as retinal ischemia requires immediate intervention to prevent cell loss. Although gene therapy studies to date have provided proof-of-principle for the anti-apoptotic protection exerted by rAAV-XIAP, it is imperative that alternate modes of delivery be considered for clinical use as acute trauma such as ischemia or detachment can often occur without warning. Gene

therapy is ineffective in response to an acute insult as the upregulation of the therapeutic gene cannot occur quickly enough to prevent an apoptotic response to injury.

Alternate delivery options include the use of a Tat-XIAP fusion protein that could rapidly transport XIAP in its protein form to the site of injury. The Tat-peptide is an 11 amino-acid peptide derived from the 47-57 residues of the HIV-1 Tat protein. Proteins that are fused to the Tat-peptide are capable of efficiently crossing cell membranes and entering cells. These proteins could be used in conjunction with rAAV-XIAP gene therapy in order to provide an immediate dose of XIAP for initial protection from apoptosis as well as the upregulation of the XIAP transgene a few days later to maintain its over-expression.

Tat-XIAP protein delivery could also be combined with delivery of biodegradable nanoparticles encapsulating XIAP. This approach would provide immediate delivery of XIAP protein and then a more sustained release over the next month or two as the nanospheres degrade. This transient upregulation of XIAP would be sufficient to treat acute disease but would limit any lasting possible harmful effects of XIAP should they exist.

3.5 Future Directions

Now that a proof-of-principle has been established demonstrating that XIAP is effective at protecting photoreceptors from apoptotic cell death following retinal detachment, further studies are required to move this therapy closer to the clinic. First and foremost, it will be important to conduct further tests in larger animal models where the retina can be reattached following retinal detachment. Experiments are currently

being designed to re-create these experiments in a non-human primate model. This model will more accurately resemble a human retinal detachment. The current studies show structural protection of photoreceptors by XIAP. The non-human primate model will allow both structural and functional assessments following re-attachment to determine whether XIAP therapy confers functional protection, similar to that observed in studies of retinitis pigmentosa (Leonard *et al.*, 2007) and retinal ischemia (Renwick *et al.*, 2006). Functional protection could be easily assessed through ERG analysis. Once structural and functional protection is established in a non-human primate model, it is expected that XIAP will be developed for clinical use in humans.

4. CONCLUSION

Based on the findings of the current study in addition to the various others described, XIAP gene therapy represents a promising therapeutic strategy for the treatment of a multitude of diseases that involve cell death by apoptosis. The ability to limit the progression of retinal degeneration could provide an improved quality of life for individuals and families affected by blindness or vision loss. The goal of the current project was to establish proof-of principle that XIAP is effective at providing structural protection following retinal detachment. This was indeed verified based on the results described above. Pending further research into the safety and efficacy of this treatment it is hoped that this therapy will be adapted for clinical use.

5. REFERENCES

- Abouzeid H, Wolfensberger TJ. Macular recovery after retinal detachment. *Acta Ophthalmol.Scand.* 2006;84:597-605.
- Acland GM, Aguirre GD, Bennett J, et al. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol.Ther.* 2005;12:1072-1082.
- Arends MJ, Wyllie AH. Apoptosis: Mechanisms and roles in pathology. *Int.Rev.Exp.Pathol.* 1991;32:223-254.
- Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual function in leber's congenital amaurosis. *N.Engl.J.Med.* 2008;358:2231-2239.
- Bennett J, Maguire AM, Cideciyan AV, et al. Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc.Natl.Acad.Sci.U.S.A.* 1999;96:9920-9925.
- Berezovskaya O, Schimmer AD, Glinskii AB, et al. Increased expression of apoptosis inhibitor protein XIAP contributes to anoikis resistance of circulating human prostate cancer metastasis precursor cells. *Cancer Res.* 2005;65:2378-2386.
- Blank M, Shiloh Y. Programs for cell death: Apoptosis is only one way to go. *Cell.Cycle* 2007;6:686-695.
- Bloem BR, Irwin I, Buruma OJ, et al. The MPTP model: Versatile contributions to the treatment of idiopathic parkinson's disease. *J.Neurol.Sci.* 1990;97:273-293.
- Boatright KM, Salvesen GS. Caspase activation. *Biochem.Soc.Symp.* 2003;(70):233-242.
- Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996;85:803-815.
- Burton TC. Recovery of visual acuity after retinal detachment involving the macula. *Trans.Am.Ophthalmol.Soc.* 1982;80:475-497.

Byer NE. Natural history of posterior vitreous detachment with early management as the premier line of defense against retinal detachment. *Ophthalmology* 1994;101:1503-13; discussion 1513-4.

Chai J, Du C, Wu JW, Kyin S, Wang X, Shi Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 2000;406:855-862.

Chan DK, Lieberman DM, Musatov S, Goldfein JA, Selesnick SH, Kaplitt MG. Protection against cisplatin-induced ototoxicity by adeno-associated virus-mediated delivery of the X-linked inhibitor of apoptosis protein is not dependent on caspase inhibition. *Otol.Neurotol.* 2007;28:417-425.

Chang CJ, Lai WW, Edward DP, Tso MO. Apoptotic photoreceptor cell death after traumatic retinal detachment in humans. *Arch.Ophthalmol.* 1995;113:880-886.

Chang GQ, Hao Y, Wong F. Apoptosis: Final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron* 1993;11:595-605.

Clem RJ. Baculoviruses and apoptosis: The good, the bad, and the ugly. *Cell Death Differ.* 2001;8:137-143.

Cohen GM. Caspases: The executioners of apoptosis. *Biochem.J.* 1997;326 (Pt 1):1-16.

Cook B, Lewis GP, Fisher SK, Adler R. Apoptotic photoreceptor degeneration in experimental retinal detachment. *Invest.Ophthalmol.Vis.Sci.* 1995;36:990-996.

Cooper LB, Chan DK, Roediger FC, et al. AAV-mediated delivery of the caspase inhibitor XIAP protects against cisplatin ototoxicity. *Otol.Neurotol.* 2006;27:484-490.

Crocker SJ, Liston P, Anisman H, et al. Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. *Neurobiol.Dis.* 2003;12:150-161.

Degterev A, Yuan J. Expansion and evolution of cell death programmes. *Nat.Rev.Mol.Cell Biol.* 2008;9:378-390.

Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J.* 1999;18:5242-5251.

- Deveraux QL, Schendel SL, Reed JC. Antiapoptotic proteins. the bcl-2 and inhibitor of apoptosis protein families. *Cardiol.Clin.* 2001;19:57-74.
- Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 1997;388:300-304.
- Doonan F, Cotter TG. Apoptosis: A potential therapeutic target for retinal degenerations. *Curr.Neurovasc Res.* 2004;1:41-53.
- Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33-42.
- Eberhardt O, Coelln RV, Kugler S, et al. Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of parkinson's disease. *J.Neurosci.* 2000;20:9126-9134.
- Edinger AL, Thompson CB. Death by design: Apoptosis, necrosis and autophagy. *Curr.Opin.Cell Biol.* 2004;16:663-669.
- Emamaullee J, Liston P, Korneluk RG, Shapiro AM, Elliott JF. XIAP overexpression in islet beta-cells enhances engraftment and minimizes hypoxia-reperfusion injury. *Am.J.Transplant.* 2005;5:1297-1305.
- Erickson PA, Fisher SK, Anderson DH, Stern WH, Borgula GA. Retinal detachment in the cat: The outer nuclear and outer plexiform layers. *Invest.Ophthalmol.Vis.Sci.* 1983;24:927-942.
- Foos RY, Wheeler NC. Vitreoretinal juncture. synchysis senilis and posterior vitreous detachment. *Ophthalmology* 1982;89:1502-1512.
- Galluzzi L, Joza N, Tasdemir E, et al. No death without life: Vital functions of apoptotic effectors. *Cell Death Differ.* 2008;15:1113-1123.
- Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp.Eye Res.* 1995;61:33-44.
- Gariano RF, Kim CH. Evaluation and management of suspected retinal detachment. *Am.Fam.Physician* 2004;69:1691-1698.

- Garrido C, Kroemer G. Life's smile, death's grin: Vital functions of apoptosis-executing proteins. *Curr.Opin.Cell Biol.* 2004;16:639-646.
- Hamasaki DI, Machemer R, Norton EW. Experimental retinal detachment in the owl monkey. VI. the ERG of the detached and reattached retina. *Albrecht Von Graefes Arch.Klin.Exp.Ophthalmol.* 1969;177:212-221.
- Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet* 2006;368:1795-1809.
- Hauswirth W, Aleman TS, Kaushal S, et al. Phase I trial of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: Short-term results. *Hum.Gene Ther.* 2008.
- Hauswirth WW, Beaufriere L. Ocular gene therapy: Quo vadis? *Invest.Ophthalmol.Vis.Sci.* 2000;41:2821-2826.
- Hegde R, Srinivasula SM, Zhang Z, et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J.Biol.Chem.* 2002;277:432-438.
- Hikichi T, Trempe CL. Relationship between floaters, light flashes, or both, and complications of posterior vitreous detachment. *Am.J.Ophthalmol.* 1994;117:593-598.
- Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J.Biol.Chem.* 2000;275:22064-22068.
- Holcik M, Gibson H, Korneluk RG. XIAP: Apoptotic brake and promising therapeutic target. *Apoptosis* 2001;6:253-261.
- Holcik M, Korneluk RG. XIAP, the guardian angel. *Nat.Rev.Mol.Cell Biol.* 2001;2:550-556.
- Kang HK, Luff AJ. Management of retinal detachment: A guide for non-ophthalmologists. *BMJ* 2008;336:1235-1240.
- Kerrigan LA, Zack DJ, Quigley HA, Smith SD, Pease ME. TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch.Ophthalmol.* 1997;115:1031-1035.

Kolb H, Fernandez E, Nelson R. Part II. Anatomy and Physiology of the Retina. *Webvision: The Organization of the Retina and Visual System*, 2007; <http://webvision.med.utah.edu>.

Korhonen L, Hansson I, Maugras C, et al. Expression of X-chromosome linked inhibitor of apoptosis protein in mature purkinje cells and in retinal bipolar cells in transgenic mice induces neurodegeneration. *Neuroscience* 2008;156:515-526.

Kroemer G, Martin SJ. Caspase-independent cell death. *Nat.Med.* 2005;11:725-730.

Kugler S, Straten G, Kreppel F, Isenmann S, Liston P, Bahr M. The X-linked inhibitor of apoptosis (XIAP) prevents cell death in axotomized CNS neurons in vivo. *Cell Death Differ.* 2000;7:815-824.

Kuwana T, Smith JJ, Muzio M, Dixit V, Newmeyer DD, Kornbluth S. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. *J.Biol.Chem.* 1998;273:16589-16594.

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 1998;17:3247-3259.

Leonard KC, Petrin D, Coupland SG, et al. XIAP protection of photoreceptors in animal models of retinitis pigmentosa. *PLoS ONE* 2007;2:e314.

Lewis GP, Charteris DG, Sethi CS, Leitner WP, Linberg KA, Fisher SK. The ability of rapid retinal reattachment to stop or reverse the cellular and molecular events initiated by detachment. *Invest.Ophthalmol.Vis.Sci.* 2002;43:2412-2420.

Lewis GP, Erickson PA, Anderson DH, Fisher SK. Opsin distribution and protein incorporation in photoreceptors after experimental retinal detachment. *Exp.Eye Res.* 1991;53:629-640.

Lewis GP, Linberg KA, Fisher SK. Neurite outgrowth from bipolar and horizontal cells after experimental retinal detachment. *Invest.Ophthalmol.Vis.Sci.* 1998;39:424-434.

Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001;412:95-99.

- Li X, Beijing Rhegmatogenous Retinal Detachment Study Group. Incidence and epidemiological characteristics of rhegmatogenous retinal detachment in Beijing, China. *Ophthalmology* 2003;110:2413-2417.
- Liston P, Fong WG, Kelly NL, et al. Identification of XAF1 as an antagonist of XIAP anti-caspase activity. *Nat. Cell Biol.* 2001;3:128-133.
- Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: There is more to life than Bcl2. *Oncogene* 2003;22:8568-8580.
- Liston P, Roy N, Tamai K, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996;379:349-353.
- Love S. Apoptosis and brain ischaemia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 2003;27:267-282.
- MacFarlane M, Williams AC. Apoptosis and disease: A life or death decision. *EMBO Rep.* 2004;5:674-678.
- Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 2008;358:2240-2248.
- McConkey DJ. Biochemical determinants of apoptosis and necrosis. *Toxicol. Lett.* 1998;99:157-168.
- McKinnon SJ, Lehman DM, Tahzib NG, et al. Baculoviral IAP repeat-containing-4 protects optic nerve axons in a rat glaucoma model. *Mol. Ther.* 2002;5:780-787.
- Muzio M, Chinnaiyan AM, Kischkel FC, et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996;85:817-827.
- Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer.* 2004;4:592-603.
- Perrault I, Rozet JM, Gerber S, et al. Leber congenital amaurosis. *Mol. Genet. Metab.* 1999;68:200-208.
- Petrin D, Baker A, Brousseau J, et al. XIAP protects photoreceptors from n-methyl-n-nitrosourea-induced retinal degeneration. *Adv. Exp. Med. Biol.* 2003a;533:385-393.

- Petrin D, Baker A, Coupland SG, et al. Structural and functional protection of photoreceptors from MNU-induced retinal degeneration by the X-linked inhibitor of apoptosis. *Invest.Ophthalmol.Vis.Sci.* 2003b;44:2757-2763.
- Pollak A, Oliver M. Argon laser photocoagulation of symptomatic flap tears and retinal breaks of fellow eyes. *Br.J.Ophthalmol.* 1981;65:469-472.
- Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest.Ophthalmol.Vis.Sci.* 1995;36:774-786.
- Renwick J, Narang MA, Coupland SG, et al. XIAP-mediated neuroprotection in retinal ischemia. *Gene Ther.* 2006;13:339-347.
- Rohlich P, Adamus G, McDowell JH, Hargrave PA. Binding pattern of anti-rhodopsin monoclonal antibodies to photoreceptor cells: An immunocytochemical study. *Exp.Eye Res.* 1989;49:999-1013.
- Sanna MG, da Silva Correia J, Ducrey O, et al. IAP suppression of apoptosis involves distinct mechanisms: The TAK1/JNK1 signaling cascade and caspase inhibition. *Mol.Cell.Biol.* 2002;22:1754-1766.
- Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 1998;17:1675-1687.
- Schimmer AD. Novel therapies targeting the apoptosis pathway for the treatment of acute myeloid leukemia. *Curr.Treat.Options Oncol.* 2007;8:277-286.
- Sharma S, Walker R, Brown GC, Cruess AF. The importance of qualitative vitreous examination in patients with acute posterior vitreous detachment. *Arch.Ophthalmol.* 1999;117:343-346.
- Slee EA, Harte MT, Kluck RM, et al. Ordering the cytochrome c-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J.Cell Biol.* 1999;144:281-292.
- Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by apaf-1-mediated oligomerization. *Mol.Cell* 1998;1:949-957.

Straszewski-Chavez SL, Visintin IP, Karassina N, et al. XAF1 mediates tumor necrosis factor-alpha-induced apoptosis and X-linked inhibitor of apoptosis cleavage by acting through the mitochondrial pathway. *J.Biol.Chem.* 2007;282:13059-13072.

Straten G, Schmeer C, Kretz A, et al. Potential synergistic protection of retinal ganglion cells from axotomy-induced apoptosis by adenoviral administration of glial cell line-derived neurotrophic factor and X-chromosome-linked inhibitor of apoptosis. *Neurobiol.Dis.* 2002;11:123-133.

Sun C, Cai M, Meadows RP, et al. NMR structure and mutagenesis of the third bir domain of the inhibitor of apoptosis protein XIAP. *J.Biol.Chem.* 2000;275:33777-33781.

Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J.Biol.Chem.* 1999;274:5053-5060.

Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;397:441-446.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol.Cell* 2001a;8:613-621.

Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in fas-induced cell death. *Proc.Natl.Acad.Sci.U.S.A.* 2001b;98:8662-8667.

Tabuchi K, Pak K, Chavez E, Ryan AF. Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage. *Neuroscience* 2007;149:213-222.

Takahashi A, Earnshaw WC. ICE-related proteases in apoptosis. *Curr.Opin.Genet.Dev.* 1996;6:50-55.

Takahashi R, Deveraux Q, Tamm I, et al. A single BIR domain of XIAP sufficient for inhibiting caspases. *J.Biol.Chem.* 1998;273:7787-7790.

Tamm I. AEG-35156, an antisense oligonucleotide against X-linked inhibitor of apoptosis for the potential treatment of cancer. *Curr.Opin.Investig Drugs* 2008;9:638-646.

Tamm I, Richter S, Oltersdorf D, et al. High expression levels of x-linked inhibitor of apoptosis protein and survivin correlate with poor overall survival in childhood de novo acute myeloid leukemia. *Clin.Cancer Res.* 2004a;10:3737-3744.

Tamm I, Richter S, Scholz F, et al. XIAP expression correlates with monocytic differentiation in adult de novo AML: Impact on prognosis. *Hematol.J.* 2004b;5:489-495.

Tatton NA, Kish SJ. In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* 1997;77:1037-1048.

Tong QS, Zheng LD, Wang L, et al. Downregulation of XIAP expression induces apoptosis and enhances chemotherapeutic sensitivity in human gastric cancer cells. *Cancer Gene Ther.* 2005;12:509-514.

Travis GH. Mechanisms of cell death in the inherited retinal degenerations. *Am.J.Hum.Genet.* 1998;62:503-508.

van Soest S, Westerveld A, de Jong PT, Bleeker-Wagemakers EM, Bergen AA. Retinitis pigmentosa: Defined from a molecular point of view. *Surv.Ophthalmol.* 1999;43:321-334.

Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;102:43-53.

Wajant H. The fas signaling pathway: More than a paradigm. *Science* 2002;296:1635-1636.

Wilkinson JC, Cepero E, Boise LH, Duckett CS. Upstream regulatory role for XIAP in receptor-mediated apoptosis. *Mol.Cell.Biol.* 2004;24:7003-7014.

- Xu D, Bureau Y, McIntyre DC, et al. Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *J.Neurosci.* 1999;19:5026-5033.
- Yang L, Matthews RT, Schulz JB, et al. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity is attenuated in mice overexpressing bcl-2. *J.Neurosci.* 1998;18:8145-8152.
- Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000;288:874-877.
- Yang YL, Li XM. The IAP Family: Endogenous caspase inhibitors with multiple biological activities. *Cell Res.* 2000;10:169-177.
- Ye H, Cande C, Stephanou NC, et al. DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat.Struct.Biol.* 2002;9:680-684.
- Zacks DN, Hanninen V, Pantcheva M, Ezra E, Grosskreutz C, Miller JW. Caspase activation in an experimental model of retinal detachment. *Invest.Ophthalmol.Vis.Sci.* 2003;44:1262-1267.
- Zacks DN, Zheng QD, Han Y, Bakhru R, Miller JW. FAS-mediated apoptosis and its relation to intrinsic pathway activation in an experimental model of retinal detachment. *Invest.Ophthalmol.Vis.Sci.* 2004;45:4563-4569.
- Zolotukhin S, Potter M, Zolotukhin I, et al. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* 2002;28:158-167.
- Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997;90:405-413.

APPENDIX 1

Troubleshooting

Histological Analysis - 3 days following Retinal Detachment

Hematoxylin & Eosin Staining

Initially, H&E staining was carried out on retinal sections to determine probable areas of detachment. Although the detached regions were visible following sectioning, the H&E staining helped locate areas with actual detachments as opposed to areas with ripped retinas, which may have resulted from histological processing. Ripped areas were distinguished from detached regions as the outer segments of the photoreceptors remained attached to the retinal pigment epithelium while the ONL remained with the rest of the retina.

Immunohistochemistry and Autofluorescence

Although in some cases immunofluorescence experiments worked well, in the majority of cases autofluorescence was a major problem. A number of different protocols were attempted to block autofluorescence, aside from the usual technique of blocking using 1% bovine serum albumin (BSA) and 5% goat serum in tris buffered saline (TBS). At first, slides were quenched using 50mM ammonium chloride (NH_4Cl) for 30 minutes prior to treatment. Following this step, background fluorescence was somewhat reduced; however, debris fluorescing within the RPE as well as fluorescence in the outer segments were still apparent in the control slides which were treated without primary antibody.

As a second option, quenching with sodium borohydride (NaBH_4) was attempted. Slides were quenched in 0.5% NaBH_4 for 10 minutes immediately following fixation in

4% PFA. The slides were subsequently blocked using 1% BSA and 5% goat serum in TBS. Once again, autofluorescence persisted.

In a separate experiment, a series of slides were de-paraffinized and mounted solely in TBS to determine the level of autofluorescence without the presence of antibodies. Despite the lack of fluorescent antibodies, the slides continued to fluoresce.

Finally, a new protocol was attempted using PowerBlock™ (Biogenex) solution. This method worked the best of all protocols attempted; however, debris within the retina and RPE continued to fluoresce which in turn interfered with accurate exposure times for pictures of the retina.

In addition to attempts to modify blocking protocols, a separate group of experiments were conducted using proteinase K as an antigen retrieval mechanism. Proteinase K unmask antigenic sites by breaking protein cross-links that often occur as the result of formalin or other aldehyde fixation. It is thought that proteinase K can unmask antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thereby enhancing the staining intensity of the applied antibodies. For these experiments, tissue samples were immersed in proteinase K for 15 minutes followed by a series of washes. Slides were subsequently immersed in PowerBlock™ for 7 minutes. Once again, autofluorescence continued to be an issue, and the antigen retrieval step did not appear to enhance staining effectiveness.

The most confusing aspect of the autofluorescence problem was that it did not happen all of the time. In order to determine if autofluorescence was animal specific, an experiment was conducted in which one slide from each animal was de-paraffinized and mounted in TBS, without the presence of fluorescent antibodies. The results of this study

indicated that all slides demonstrated autofluorescence. It was therefore concluded that the autofluorescence was not animal specific.

As it had been shown that the autofluorescence problem was likely not animal specific it was thought that whatever was causing the problem had to involve something that each tissue sample had been subjected to. Questions began to arise as to whether the problem may have been due to the fact that the eyes were fixed in 4% PFA for 24 hours following enucleation. Despite the fact that this protocol had worked without problems in the past, it was considered as a possible factor. A side study was conducted in which eyes were harvested from rats and perfused in the same manner as mentioned previously. For the fixation step, two eyes were fixed in 4% PFA for 24 hours while the other two eyes were fixed in 4% PFA for 1 hour. The results of the study indicated that fixation time was not an issue as all eyes continued to autofluoresce.

Despite the significant difficulty experienced with autofluorescence, the difference between background fluorescence and real XIAP and GFP signal was still clear, allowing tracking of the virus in detached regions of retina.

Alkaline Phosphatase Staining

As an alternative to immunofluorescence, alkaline phosphatase colorimetric staining was attempted as a method to identify XIAP-positive tissues. It was thought that this method would be more accurate as autofluorescence would not be an issue. Once again, background staining was a concern. In a few experiments, the technique worked relatively well; however, the samples had to be developed overnight as opposed to the 5-10 minutes suggested in the protocol for the BCIP/NBT (5-bromo-4-chloro-3-indolyl

phosphate/nitro blue tetrazolium) development kit. After overnight development some areas of the sections appeared to be positive for XIAP. The problem, however, was ruling out whether the signal was positive or simply overdeveloped. The results were encouraging, nevertheless, given that within a given section there were areas that were clearly positive.

Histological Analysis - 2 months following Retinal Detachment

Hematoxylin and eosin staining was conducted on 2 month samples to make morphological comparisons between the XIAP- and GFP-treated detached retinas. At this time point it was easier to notice the difference in the level of protection between each of the groups. Detached portions of GFP-treated retinas exhibited cell loss in the ONL, whereas XIAP-treated retinas exhibited minimal cell loss, up to 2 months post-detachment.

Immunohistochemistry experiments were also conducted on samples from the 2 month time point. As with the 3 day time point, similar problems were encountered with autofluorescence; however, some suitable sections were obtained (Figure 10). In some instances, areas were identified in XIAP-treated retinas where the ONL thickness diminished with decreasing expression of XIAP (Figure 10 - D and E). Additionally, in some of the GFP-treated detachments, the ONL was completely absent (Figure 10 - A and B).

Electroretinograms

At the beginning of the study, baseline electroretinograms (ERGs) were taken following the subretinal injections and prior to retinal detachment surgery. Further ERGs

were conducted on the animals 2 months post-detachment in order to compare retinal function prior to retinal detachment versus after retinal detachment. In addition, function was compared between the left (treated) and right (untreated) eye to determine whether the left eye experienced decreased function following surgery.

Following the subretinal injections, both the left (injected) and right (intact) eye performed comparably in the ERG tests. However, as expected, the results following detachment showed a significantly reduced ERG in the left detached eye of both XIAP- and GFP-treated animals. This was not surprising, as a previous study found that a corneal electrode could not detect ERGs from a detached retina (Hamasaki *et al.*, 1969). A relatively uniform reduction of function was seen in both the XIAP-treated and GFP-treated animals indicating that both groups received adequate and equal retinal detachments. As mentioned above, it would be advantageous to re-create this experiment in a larger animal model where the retina could be reattached to monitor function.

APPENDIX 2

Basic Protocols

Subretinal Injections

1. Rats were anaesthetized by isofluorane gas inhalation.
2. Injections were conducted under a dissecting microscope.
3. The analgesic Buprenorphine (0.04mg/kg) was given prior to surgery by animal care staff.
4. Dilating drops (mydriacyl and mydfrin) were given several minutes prior to surgery.
5. Alcaine was also given prior to surgery as a local anaesthetic.
6. Genteal (0.2% hypromellose) lubricant gel was applied to the eye for hydration and magnification with a coverslip.
7. GFP rats were designated with the numbers 1-25 and 51-75 and XIAP rats were designated with the numbers 26-50 and 76-100.
8. A V-lance was used to make an initial incision through the sclera. The incision was made a few millimetres below the iris at approximately the 11-12 o'clock position. The V-lance was inserted to the bevel.
9. A cover slip was applied to the eye for visualization of the injection.
10. Injection was delivered in most cases between 12-2 o'clock position.
11. A 32 gauge blunt (Hamilton syringes) was used for subretinal delivery of a 2 μ L volume of rAAV-CBA-XIAP or rAAV-CBA-GFP. Fluorescein tracer was combined with the virus to allow the immediate visualization and evaluation of the injection location.
12. Post-surgical care was carried out by the animal care staff. The post-operative treatment consisted of the antibiotic Ciprofloxacin HCl (Alcon Canada), and a non-steroidal anti-inflammatory (Flurbiprofen sodium, Allergan, Irvine, CA) for five days post-injection.

**Alkaline Phosphatase Staining using Streptavidin-Biotin Conjugates
(Paraffin Slides)**

1. De-wax slides up to PFA step.
2. Wash sections in 1x TBS – 3x – 3 min each.
3. Prepare humidified chamber by placing strips of paper towel in wells. Saturate each strip with ~1mL of TBS.
4. Block sections with 1% BSA and 5% Goat serum in TBS for 15-30min.

Blocking Solution Recipe (4 slides):

30 μ L BSA

150 μ L Goat Serum

3mL 1x TBS

Cover each slide with 250 μ L blocking solution, and place in humidified chamber.

5. Remove block solution by dabbing slide edge on paper towel.
6. Cover slides with 200 μ L of 1 $^{\circ}$ Ab solution and cover with parafilm squares. Make sure to leave one slide as a control. A GFP-treated section was used for the purpose of this experiment (i.e. no HA tag).

1 $^{\circ}$ Ab Solution (1:500) Recipe for 4 slides:

2 μ L Mouse Anti-HA Ab

3 μ L Triton X (0.3%)

1 mL Blocking Solution

(You want 1 μ L Ab for each 500 μ L of Ab solution)

7. Cover humidified chambers with lid, wrap in tinfoil and incubate overnight at 4 $^{\circ}$ C.
8. Unwrap and remove parafilm.
9. Add 2 $^{\circ}$ Ab diluted in 1x TBS for 60 minutes at room temperature in a humidified chamber (use 200 μ L per slide).

2 $^{\circ}$ Ab Solution (1:1000) Recipe for 4 slides:

1 μ L Biotin-XX goat anti-mouse IgG (2mg/mL) – (Invitrogen – B2763)

1mL 1x TBS

10. Wash 3 x 15 min. in 1x TBS.
11. Add 3 $^{\circ}$ Ab diluted in 1x TBS for 60 minutes at room temperature in a humidified chamber (use 200 μ L per slide).

3 $^{\circ}$ Ab Solution (1:200) Recipe for 4 slides:

5 μ L AP-Streptavidin (3mg/2mL) – (Invitrogen 43-4322)

1mL 1x TBS

12. Wash 4 x 5 min in 1x TBS.
13. Use BCIP/NBT kit to develop slides.

Image Subtraction Protocol
(Immunofluorescent Microscopy using Northern Eclipse and Adobe Photoshop)
(Courtesy of Dr. William Staines, University of Ottawa)

1. Northern Eclipse - Take a picture of the same image using light filters with two different wavelengths (e.g. FITC and Rhodamine). Try to get similar levels of background (autofluorescence) in both images. Your desired image, taken using the appropriate filter, will be designated as **Image A**. The duplicate picture taken in an alternate filter (and showing endogenous fluorescence) will be designated as **Image B**.
2. Open both images in Adobe Photoshop and adjust the brightness/contrast of **Image B** so that the endogenous fluorescence stands out. Make both images "grayscale" by clicking on each individually and then going to the "Image" menu and selecting "mode→grayscale".
3. Click on **Image A**. Go to the "Image" menu and select "Apply Image". Apply **Image B** to **Image A**. Apply the following settings:

Channel:	Black
Blending:	Subtract
Opacity:	100%
4. To check if it worked, create a new RGB image. Copy and paste **Image A** into one layer (the colour of the image when observed through the microscope), and **Image B** into another. The layer without an image should be black. The RGB image will give an idea of how much endogenous fluorescence was removed.