PERSPECTIVE

Cell loss in retinal dystrophies by apoptosis – death by informed consent!

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For most inherited retinal dystrophies there is no satisfactory treatment and this represents a significant health problem. In recent years molecular genetic studies have had a marked impact on our understanding of inherited retinal disease, and a number of causative mutations have been identified in retina specific genes.^{1–6} The reversal of disease by gene therapy in the retinal degeneration (rd) and retinal degeneration slow (rds) mice by transgenic rescue,^{7,8} has given rise to great interest in the use of gene therapy for retinal dystrophies in humans. However, there are a number of problems to be overcome if this technology is to be practicable. These include limiting expression of the gene to the correct cell at an appropriate level, identifying suitability of vectors for targeting the gene, and perfecting the method of delivery.⁹

A particular dilemma has emerged in that there is increasing evidence that the metabolic defect caused by the mutation does not cause cell death directly in some disorders, in both humans and animals. In retinitis pigmentosa (RP) associated with mutations in the rod specific gene rhodopsin, there is also loss of cone photoreceptors.¹⁰ A mutation in the rod specific cGMP phosphodiesterase β subunit gene in Irish setter dogs with rod/cone dysplasia, results in the death of cones as well as rods despite normal activity of the cone specific cGMP phosphodiesterase.¹¹ Similarly, in the Royal College of Surgeons (RCS) rat photoreceptor degeneration occurs although the basic defect resides in the retinal pigment epithelium.¹² Furthermore, in a chimera created from an albino mouse transfected with a mutant rhodopsin gene and a wild type pigmented mouse there is regional distribution of pigmented and non-pigmented cells, yet photoreceptor cell death is diffuse, suggesting that the photoreceptors containing wild type and mutant rhodopsin genes degenerate simultaneously.¹³ Recently, it has been shown that in animal models of retinal degeneration, photoreceptor cell death occurs by a common mechanism known as apoptosis or programmed cell death,¹⁴ a process governed by gene expression. The importance of this discovery to the clinician is that the process may be amenable to therapeutic manipulation.^{15,16} If this holds true for human diseases, it might be possible to slow disease progression by inhibiting apoptosis in many diseases rather than by attempting to correct the primary genetic defect. Since the degenerative changes seen in progressive retinal diseases such as in RP and macular disease occur over several decades, a modest decrease in the rate of cell death through therapeutic intervention could have a major clinical impact by prolonging the years of useful vision.

What is apoptosis?

The word 'apoptosis' comes from Greek meaning 'falling off' as in leaves falling from trees.¹⁷ For a number of years

cell biologists have been fascinated by the phenomenon of apoptosis as an alternative mode of cell death due to necrosis. Apoptosis is observed during embryogenesis (termed histogenetic cell death) and normal cell turnover,¹⁸ and genetic regulation of the process has been implied.¹⁹ The purpose of apoptosis (or programmed cell death) is to remove cells no longer required or which are damaged in a controlled and coordinated manner. The mechanism for desirable cell death without detrimental harm to an organism has evolved from a diverse evolutionary background.²⁰ For example, cells that form the xylem in plants die to allow conductance of fluid, and this also accounts for loss of the tadpole's tail during metamorphosis. Similarly, neurons which fail to make connections in the brain will die without detriment to the brain. Specified cell death has not only evolved as part of development, but also as an organism's defence mechanism. It makes sense for a cell to kill itself when invaded by a virus, so that its own cellular machinery is not used for the production of new virus particles. However, some viruses have developed a mechanism to prevent host cell death allowing them to replicate.

Pathogenesis

For a specific cell to die by apoptosis, it must receive a signal which then causes the cell to execute a programme of genetically determined biochemical events leading to cell destruction (that is, the cell appears to 'commit suicide'). A cell committed to apoptosis withdraws from its neighbours, its chromatin is condensed, and then its DNA is cut into discrete fragments of regular size. Inability to repair multiple breaks in the DNA strands is the lethal event which probably kills the cell.²¹ The cell breaks down into membrane bound apoptotic bodies which are engulfed by neighbouring cells or sometimes by circulating macrophages, neatly removing them without leakage of the cell contents which may be detrimental to surrounding cells (as shown by inflammation in necrosis). Unlike necrosis, apoptotic cell death occurs in an asynchronous fashion in individual cells. Table 1 summarises the main differences between apoptosis and necrosis.

Table 1 Comparison of the general features of apoptosis versus necrosis

Characteristic	Apoptosis	Necrosis
Stimuli	Physiological	Pathological
Occurrence	Individual cells	Groups of cells
DNA breakdown	Internucleosomal	Randomised
Pathology	Apoptotic bodies	Swelling/lysis
Phagocytosis	Present	Absent
Lysosomal enzyme release	Absent	Present
Exudative inflammation	Absent	Present
Scar formation	Absent	Present

Detection

Apoptosis is biochemically characterised by cleavage of DNA (in the tissue of interest) at internucleosomal sites,²¹ which produces a ladder of fragments of 180–200 base pair multimers when resolved by agarose gel electrophoresis. DNA fragmentation can also be observed at the single cell level by in situ labelling of apoptotic cell nuclei using a histochemical technique known as TUNEL²² (TdT mediated dUTP biotin nick end labelling). In this technique the ends of the DNA fragments are free allowing a biotin moiety to be attached which can be visualised by avidin peroxidase staining. DNA fragmentation is almost always detected when the morphological changes associated with apoptosis are observed, except in some lower invertebrates.²¹

Regulation of apoptosis

The regulation, biochemical mechanisms, and identities of genes which regulate and execute this process are incompletely understood. However, some genetic and biochemical aspects of the process have been characterised.

GENETICS

Pivotal work in the nematode *Caenorhabditis elegans*²³ has identified 14 genes involved in programmed cell death at the stages of cell death including determination of which cell is going to die, and the killing, engulfment, and degradation of that cell. Two of the genes which must function for cells to undergo death are (i) *ced-3*,²⁴ where interleukin 1 β converting enzyme (ICE) is the human homologue²⁵ and (ii) *ced-4*,²⁴ which is a novel mammalian gene.²⁶ Mutations in either of these genes allow the survival of almost all cells that normally die and the surviving cells appear to be functional.^{24–27} The *ced-3* and *ced-4* genes are negatively regulated by a gene called *ced-9*,²⁸ where *bcl-2* is the human homologue.²⁹ Mutations in the *ced-9* gene cause increased activity of *ced-3* and *ced-4*, which kills the cells that would normally survive.²⁴

In addition to the genes identified in *C. elegans* there are a number of others including *c-myc*,³⁰ *p53*,³¹ *c-fos*,³² polyubiquitin,³³ and clusterin³⁴ which have been shown to be implicated in apoptosis in various cell types. *C-myc* is commonly associated with cell proliferation, but it is important in apoptosis in other systems.³⁰ The mechanism of action of *c-myc* may be associated with the levels of growth factors in the medium with cells in growth arrest being highly susceptible to apoptosis.¹⁷ *C-myc* and *bcl-2* are thought to act cooperatively to achieve immortalisation of tumour cells.³⁵ Wild type *p53* is suggested to function as a tumour suppressor gene³⁶ and is thought to keep the cell cycle for mitosis in phase G1 (resting phase before DNA replication) to allow DNA repair.¹⁷ If the damage is irreparable then apoptosis is triggered in the cell. Polyubiquitin has been shown to be involved in targeting protein for non-lysosomal degradation³³ and clusterin is a constitutively expressed glycoprotein which is involved in cellular differentiation and tissue remodelling especially in developing epithelia.³⁴

Since the discovery of *bcl-2* and ICE (IL1 β C)³⁷ there has been an explosion in the literature on the identification of their function and biochemistry. The *bcl-2* gene was first discovered because of its overexpression in non-Hodgkin's B cell malignant lymphomas.³⁸ *Bcl-2* appears to block events relatively early in apoptotic cell death in that none of the morphological characteristics of apoptotic cells are seen. The latest data indicate that *bcl-2* may prevent the activation of apoptosis genes or block the action of the apoptosis gene products.³⁹ The *bcl-2* gene is localised on

chromosome 18q21 and the *Bcl-2* protein (25–26 kD)³⁹ resides in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane of cells.⁴⁰ Mitochondrial localisation implies that oxidative phosphorylation in *Bcl-2* protein may be relevant to its function. However, mitochondrial DNA mutant cells which lack a functional respiratory chain are capable of undergoing apoptosis which can be blocked by *Bcl-2* expression.⁴⁰ In the nuclear envelope *Bcl-2* might be associated with nuclear pore complexes suggesting a role in nuclear transport.⁴¹ In the endoplasmic reticulum *Bcl-2* may be involved in Ca^{2+} regulation, since there is evidence supporting an important role for Ca^{2+} in apoptosis.⁴² Recently, a number of genes with sequence homology to *bcl-2* have been identified such as *bax*⁴³ and *bcl-x*⁴⁴ which have regulatory effects on apoptosis. *Bax* is a 21 kDa protein which exists as a homodimer and can form a heterodimer with *Bcl-2* in vivo. Since *Bax* can inhibit the death suppressor activity of *Bcl-2*, the ratio of these two proteins in a cell could determine whether the cell survives or dies on receipt of a potentially fatal stimulus. Additional genes with some sequence homology to *bcl-2* include *mcl-1*⁴⁵ and *A1*.⁴⁶ To date, the function of their gene products has not been identified.

Less is known about the gene for interleukin 1 β converting enzyme (ICE) which has been localised to chromosome 11q22.2–q22.3.³⁷ ICE is a substrate specific cysteine protease which cleaves the 33 kDa pro-interleukin 1 β (IL-1 β) protein into the biologically active 17.5 kDa interleukin 1 β . The active product is one of the primary mediators of the body's response to inflammation, immunological injury, and tissue injury.⁴⁷ High levels of IL-1 β have been detected in Alzheimer's disease, rheumatoid arthritis, septic shock, and head injury.⁴⁸ Abnormal regulation of ICE could result in cell death, suggesting the possibility of treating such diseases by inhibiting ICE. Overexpression of ICE in fibroblast cells in culture induces apoptosis and can be inhibited by *crmA*⁴⁹ and *bcl-2*,⁴⁸ implying a pathway of programmed cell death in vertebrates which may be similar to that seen in *C. elegans*. The substrate which ICE acts on to cause cell death is unknown, but it may cleave proteins that are essential for cell viability.

BIOCHEMISTRY

Ca^{2+} mobilisation has been implicated as a key event in the early signalling pathway leading to apoptosis from a number of studies.¹⁷ The specific changes induced by the raised Ca^{2+} levels that cause cell death are unknown. Ca^{2+} ionophores such as A23187 are potent inducers of apoptosis suggesting that Ca^{2+} flux can activate apoptosis itself. Another possible explanation is that Ca^{2+} directly activates the endonuclease which causes internucleosomal DNA fragmentation. The enzyme responsible for DNA cleavage has proved very elusive. A few groups have partially identified candidate endonucleases but the protein size and its location within the cell have yet to be determined.⁵⁰

Ca^{2+} may not be the only activator for this mechanism of cell death. A wide spectrum of other factors has been shown to induce apoptosis including cAMP, glucocorticoids, tumour necrosis factor, transforming growth factor β 1, X/γ irradiation, and withdrawal of growth factors.⁵¹ Furthermore, disruption of epithelial cell-matrix interactions induces apoptosis and has been termed 'anoikis'.⁵² A number of agents are known to inhibit cell death including Zn^{2+} and protein kinase C; however, the mechanism of action is unknown.⁵¹

Many of the inducers of apoptosis can be inhibited by RNA, protein synthesis inhibitors, or both suggesting

Table 2 Mediators of apoptosis in different types of retina

Cell/tissue	Mediator/gene	DNA fragmentation	Cell death	Reference
Retina rd mouse	Embryogenesis	Yes	Present	14, 55
	Retinal degeneration	Yes	Present	14, 55
	Clusterin expression	–	Present	69
	c-fos expression	–	Present	73
rds mouse	Retinal degeneration	Yes	Present	14, 55
Rhodopsin transgenic mice	Retinal degeneration	Yes	Present	14, 55
RCS rat	Retinal degeneration	Yes	Present	59
	Intermittent and continuous light	Yes	Present	63
Light damaged rat	Cycloheximide	–	Inhibition	64
	Flunarizine	–	Inhibition	64
	Aurintricarboxylic acid	–	Inhibition	65
	Phorbol ester	–	Inhibition	65
Normal rat	Lead	No	Present	66
Cat retina	Retinal detachment	Yes	Present	67
Monkey RPE cells	Clusterin expression	Not tested	Present	69
Human retina	Clusterin expression	–	Present	71
RP retina	Clusterin expression	–	Present	71
Retinoblastoma	Disease	Not tested	Present	59

RCS=Royal College of Surgeons.

RPE=retinal pigment epithelium.

RP=retinitis pigmentosa.

that there is synthesis of components of the cell death programme which are encoded by 'death' genes. However, more recent evidence suggests that *de novo* protein synthesis is not required by all cells types for apoptosis to be initiated. This has given rise to the notion that there is a protein independent pathway of apoptosis.⁵⁰ Some cells require synthesis of new macromolecules upon receipt of a death inducing stimulus, whereas other cells maintain control over constitutively expressed molecules by the presence of inhibitors.

Apoptosis in the retina

The evidence for apoptosis in the retina and the factors influencing this process are summarised in Table 2 and are discussed below in more detail.

EMBRYOGENESIS

During embryogenesis the retina develops as a layered entity with uniform density of different cells types. However, by the end of development there is spatiotemporal non-uniform distribution of cells as seen in the fovea and macula regions compared with the periphery.^{53 54} It has been suggested that retinal ganglion cell death in the periphery during retinal expansion is an important factor in retinal specialisation.⁵⁵ DNA fragmentation has been shown to occur,²⁰ confirming the concept that apoptosis is the mechanism of cell loss during retinal development.⁵⁶

ANIMAL MODELS OF RETINAL DEGENERATION

The biochemical landmarks of apoptosis (TUNEL labelling and DNA ladders) have been examined in a number of animal models of retinal degeneration and compared with histology of the retina at varying timepoints postnatally. In rd, rds, and transgenic mice expressing mutant rhodopsin genes (either Pro347Ser or Q344ter),^{14 57} apoptosis in photoreceptor nuclei was observed during normal development of the retina, but continued into the period of retinal degeneration. In rd/rd retina, TUNEL labelled cells were observed up to postnatal day 12 during normal retinal maturation. However, during the third postnatal week there was a dramatic increase in labelling of photoreceptor nuclei concomitant with histological cell loss. In rds/rds mice TUNEL labelled cells were observed 3–8 weeks postnatally but not in older animals, despite the continued loss of photoreceptors over

a 1 year period. In the transgenic animals TUNEL labelled nuclei were observed at 3–7 weeks postnatally. Although the initial genetic mutation in these three animal models are different, it is concluded that apoptosis is the final common pathway of photoreceptor cell death in these animal models.¹⁴ In the RCS rat model, where the expression of the primary genetic defect is in the retinal pigment epithelium (RPE) causing inability to phagocytose outer segments,^{12 58} apoptosis was observed in the photoreceptors.⁵⁹ At postnatal day 25 DNA fragmentation and TUNEL labelled nuclei were detected, with more abundant labelling at the posterior pole compared with the peripheral retina. Photoreceptors have been 'rescued' (cell degeneration delayed) in the RCS rat by either intravitreal injection of growth factors⁶⁰ or transplantation of RPE cells,⁶¹ which may be the result of suppression of apoptosis. The cellular interactions leading to targeting of the photoreceptors, as opposed to other neurons of the retina, for apoptosis in the RCS rat make this an interesting model to study.

Apoptosis is not limited to cell loss in genetically determined degenerations. A number of studies have evaluated light damage in normal animals to determine the role of apoptosis in photoreceptor cell loss. Using an anti-DNA antibody the photoreceptor nuclei of light damaged rat retinas showed chromatin condensation and cytoplasmic densification, which was prevented by cycloheximide treatment.⁶² Both intermittent and continuous light triggers DNA fragmentation and TUNEL staining of photoreceptors, but intermittent light induced apoptosis earlier and more extensively.⁶³ Light induced apoptosis at early post-exposure stages can be inhibited by cycloheximide (a protein synthesis inhibitor),⁶⁴ flunarizine (a Ca²⁺ channel blocker),⁶⁴ and aurintricarboxylic acid (an endonuclease inhibitor)⁶⁵ by intravitreal injection. Additionally, phorbol myristate (a protein kinase C activator) partially inhibited DNA fragmentation early after exposure to light.⁶⁵ These results suggest that protein synthesis and Ca²⁺ signalling are important early on in the induction of apoptosis. Furthermore, protein kinase C may be involved in the regulation of apoptosis. Developmental exposure to lead which produces the morphological features of apoptosis does not exhibit DNA fragmentation suggesting another mechanism of apoptosis.⁶⁶ The peak of TUNEL positive cells was observed 1–3 days after detachment and had decreased by 7 days. However, positive nuclei were still apparent at 28 days. Apoptosis has been shown to occur in some tumours including retinoblastoma.⁶⁸ It might therefore be possible to cause regression of the tumour if the apoptotic pathway is manipulated.

GENE EXPRESSION

Attempts have been made to identify gene expression during apoptosis in the retina. Clusterin (TRPM-2) is an apoptosis inducible gene preferentially expressed in peripheral retina of the rd mouse.⁶⁹ Clusterin expression is present during normal retinal development and increases with the onset of retinal degeneration, followed by a decrease paralleling the loss of photoreceptors in this model. The gene for clusterin maps to chromosome 8p.⁷⁰ In normal human retina clusterin mRNA expression is present in the ganglion cell layer, the inner nuclear layer, and outer plexiform layer with no signal in the photoreceptor layer. Specifically, there is intense immunoreactivity in the inner limiting membrane, Bruch's membrane, and the vascular endothelium.⁷¹ In contrast, the retina from an RP patient was more strongly labelled throughout, although the specific localisation of the signal was

reduced.⁷¹ Increased clusterin expression is also associated with neurodegenerative diseases such as Alzheimer's and Pick's disease.⁷² The expression of the immediate early gene *c-fos* has also been examined in *rd* mouse retina⁷³ since this gene has been shown to be switched on before apoptosis in other systems. *C-fos* expression was at its peak during the period of retinal cell death and it has been suggested that increased levels of cGMP may influence *c-fos* expression. Increased levels of cGMP have also been correlated with apoptosis in the *rd* mouse.⁷⁴

Apoptosis in other systems of the eye

Apoptosis has also been documented in the lens and cornea. In the wild type mouse lens nuclear apoptosis starts at embryonic day 16 with breakdown of DNA to nucleosides, which are expelled into the intracellular space and transported to the anterior and posterior surfaces.⁷⁵ Synthesis of crystallins continues during this process which finishes at postnatal day 14. It is suggested that this process leads to transparent and highly refractive lenses and that loss of a component of this process leads to primary fibres and juvenile cataract. Additionally, expression of the proto-oncogene *c-fos* has been observed in programmed cell death associated with cataract formation in the rat lens by treatment of the lens with hydrogen peroxide.⁷⁶ In corneal epithelial cells, endothelial cells and stromal fibroblasts, interleukin 1 α (IL-1 α) induces apoptosis.⁷⁷ It is suggested that IL-1 α could modulate functions in keratocytes, therefore agonists or antagonists could be used to modulate keratocyte activity during wound healing.

Future strategies and potential therapeutic roles

Since apoptosis seems to be an important event in the control of specific cell populations during development and normal cell turnover, influencing apoptosis could serve many therapeutic applications. Manipulation of factors which influence the apoptotic pathway could be a potential avenue to follow for ameliorating disease. For example, it has been demonstrated in neurons that withdrawal of growth factors induces apoptosis.⁵¹ Furthermore, intravitreal injection of various growth factors into the RCS rat and in a light damaged rat model delayed photoreceptor cell death for several months.^{60 78} One explanation for this rescue could be that injection of growth factors is preventing induction of the apoptotic pathway, prolonging the survival of photoreceptors. The loss of photoreceptor cells was delayed for 3 months in the RCS rat which is about an eighth of its lifespan. A similar approach for delaying progressive retinal disease in humans is obviously very attractive. However, before such an approach can be justified it would be necessary to establish the functional capacity of rescued photoreceptors, the longevity of effect, and efficacy of different growth factors.

Another avenue for therapy would be the genetic manipulation of apoptosis associated genes. For example, one route might be the exploitation of the inhibitory effect on apoptosis by expression of the *bcl-2* gene. Initial studies might be to introduce into *rd* or *rd*s mice a *bcl-2* transgene to determine if this would alter the disease phenotype by slowing down retinal cell death. If this was successful, use of either liposome or intraocular injections⁹ of an adenoviral⁷⁹ *bcl-2* construct to the retina may be a first step towards gene therapy for progressive disorders of the retina in humans. Other studies on the mechanism of inhibition of the apoptosis inducing genes could be also be investigated including the use of antisense oligonucleotides to block translation of apoptotic mRNAs.¹⁷ An important issue which needs to be addressed with such strategies, is

that it is critical to limit the scope of manipulation to the target tissue of interest. Any attempt at systemic correction of a genetic defect could have a negative impact on normal apoptotic changes occurring in tissues such as gastrointestinal and respiratory epithelium. One way in which this could be controlled is by using tissue specific promoters to drive the transgene – for example, the rhodopsin promoter for expression in photoreceptor cells.⁸

Manipulation of the apoptotic mechanism could have significant implications for the design of therapeutic strategies for human retinal dystrophies, for which no treatment is currently available. Schemes for gene therapy of individual mutations might well be an overwhelming task, whereas strategies aimed at manipulating apoptosis might be much more practical, as it would apply to multiple different mutations affecting different genes.

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