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The Cell and Molecular Biology of Glaucoma: Mechanisms of Retinal Ganglion Cell Death

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Glaucomatous optic neuropathies are characterized by the progressive degeneration of the optic nerve and retinal ganglion cells. Conceptually, the process of ganglion cell loss can be considered a series of autonomous, self-destructing pathways.[1] Retinal ganglion cells, for example, can be divided into basic regions, including the axon and synapse, the dendritic arbor, and the cell body. Each of these compartments, although contiguous with one another, can independently degenerate. A current model of glaucomatous pathogenesis suggests that the first compartment that is affected by intraocular pressure (IOP)–induced damage is the axon. The site of this damage is the laminar region of the eye, where the axons of the retinal ganglion cells exit the globe as they enter the optic nerve.

The process of the damage to the axons is a matter of great speculation and ongoing research efforts and is not the principal subject of this essay. Basically, however, the strain placed on this region by elevated IOP causes molecular and functional changes to the resident cell population of the tissue. The principal cell population is made up of astrocytes, but lamina cribrosacytes, which have molecular profiles that are distinct from those of astrocytes, are also thought to reside there. One mechanism of axonal damage may be loss of support functions from the resident glial cells. Loss of energy support, for example, may lead to axons becoming unable to sustain energy-dependent processes such as axonal transport. Whatever the mechanism, rodent models of chronic glaucoma suggest that axon dysfunction and subsequent degeneration, are early events in the pathology of retinal ganglion cells.[2,3] The level of damage to most axons is probably subacute, meaning that they are likely to retain connection and some level of communication with the ganglion cell soma. The evidence for subacute damage is that axons in glaucomatous mice degenerate by the dieback phenomenon at least in one model of glaucoma,[2] although several studies have suggested that a proportion of axons also exhibit more severe Wallerian degeneration.[3,4] Dieback is the process of degeneration that begins at the distal most point of the axon (the synapse) and progresses toward the cell body. This process could occur even if the initial site of damage is proximal to the cell body. A more acute injury, such as axotomy, would result in simultaneous degeneration of the axon throughout its length by Wallerian degeneration.

Loss of axonal function leads to the activation of degenerative atrophy of the retinal ganglion cell bodies. The popular theory for this is that ganglion cell somas require neurotrophic input from target neurons in the brain, and failure of axonal transport to deliver these molecules results in a recapitulation of the apoptosis program, similar to what is believed to occur to supernumerary ganglion cells during development. This mechanism is still not proven, although there is clear evidence that trophic factor receptors, such as the TrkB receptor bound to brain-derived neurotrophic factor (BDNF), cannot be transported into the retina in a glaucomatous condition. A caveat regarding this notion is that molecules such as BDNF are produced directly by other cells in the retina, and so it is unclear why loss of transport of factors from remote neurons could be damaging. One possibility is that different molecular pathways are activated by identical trophic factors interacting with receptors in different compartments of a neuron.[5] Thus, BDNF-binding in the synapse most likely activates a different signaling pathway from BDNF-binding receptors in the soma. Alternatively, in recent studies, Harder and Libby[6] have clearly demonstrated that the initiator molecules necessary for developmental ganglion cell programmed cell death are different from those that regulate ganglion cell apoptosis after injury, suggesting that there may be distinct activation pathways.

Once the cell death pathway is activated, ganglion cell somas undergo the intrinsic apoptosis program. This program is mediated by interactions of proteins of the *Bcl2* gene family. Members of this family share similar amino acid domains (BCL homology or BH domains), which allows them to interact with each other.[7] Some members, such as BCL-X and BCL2, have antiapoptotic roles. Others, such as BAX or BAK, have the apposing role of promoting apoptosis. The BH3-only domain–containing proteins are a third member of the family. These smaller peptides are involved in regulating the interaction between pro- and antiapoptotic *Bcl2* family members. Speaking in a most general sense, the committed step of apoptosis requires activation of one or more BH3-only proteins, which then facilitate the activation of BAX and/or BAK, presumably by both direct interactions with the prodeath members and by sequestering antiapoptotic members and blocking their activity. Apoptosis is executed in cells when proapoptotic proteins can translocate and aggregate at the surface of the mitochondrial outer membrane. This aggregation event leads to membrane instability and/or pore formation, which in turn leads to the release of the electron transporter cytochrome *c*. Cytochrome *c*, once present in the cytoplasm, interacts with other protein complexes that lead to the activation of cysteine proteases, which selectively digest the cell from within. Activation of proapoptotic BAX and/or BAK is the committed step in the intrinsic apoptosis pathway, since this also leads to mitochondrial dysfunction, rendering these organelles unable to generate energy for the cell (loss of ATP production), and reactive oxygen species are typically generated in toxic amounts. Thus, even if caspases are inhibited, a cell in this condition is likely to die a slower, more necrotic death.

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Recent studies suggest that in healthy cells BCL-X and BAX are in a delicate balance that prevents BAX from aggregating at the surface of the mitochondria. The exact mechanism of how BCL-X is able to antagonize BAX function is not well understood, but recent studies[8] suggest that in healthy cells BAX is present in an equilibrium, with an abundance of protein in the cytosol and a small fraction of protein bound to the mitochondrial outer membrane. The off-rate of BAX from the mitochondria is influenced by BCL-X, such that overexpression of this antiapoptotic protein can increase the off-rate by as much as 80%. Induction of BH3-only proteins appears to redistribute the equilibrium between mitochondrial and cytosolic BAX, by sequestering antiapoptotic proteins to decrease the mitochondrial off-rate, or by directly interacting with BAX to increase the on-rate, or perhaps by a combination of both. Several studies showing that multiple BH3-only proteins must be eliminated to replicate the *Bax/Bak* double-knockout phenotype ($Bax^{-/-}$ in neurons; see below) suggest that compete activation of BAX is dependent on a coordinated interaction of these activator proteins.

Early-Onset Atrophy in Dying Retinal Ganglion Cells: Epigenetic Events

Evidence from multiple laboratories has helped establish a hypothetical time-line of molecular events that are executed in dying ganglion cells (Fig. 1). The basic changes can be distinguished by whether they occur before BAX protein activation and mitochondrial involvement (see below). Pre-BAX events include degenerative atrophy of the cell soma and dendritic arbor, an initial phase of nuclear condensation, and the silencing of normal ganglion cell–specific gene expression. These events are most likely precipitated by a variety of cell signaling molecules, and there is evidence that p38 MAP kinase, Jun N-terminal kinases (JNK2 and JNK3), and the JUN kinases are

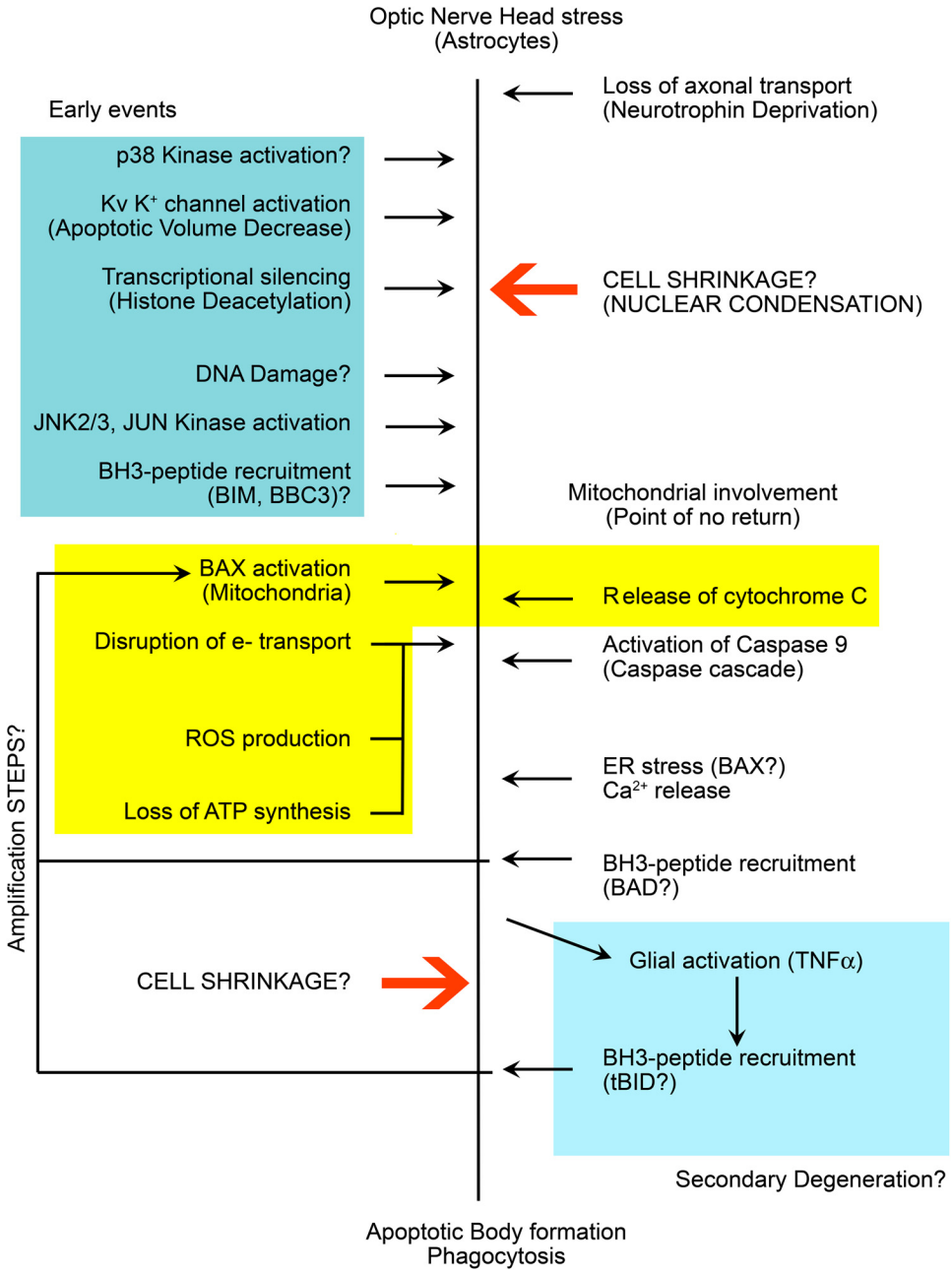

Figure 1. A hypothetical timeline of molecular events activated in retinal ganglion cells after axonal damage. The events precipitated by BAX activation (*yellow background*) center on dysfunction of the mitochondria and represent a point at which rescue of the cell is not possible. Early activation events (*teal background*) include the activation of several kinases including p38, JNK2/3, and JUN, as well as cell atrophy leading to shrinkage and the recruitment of some members of the BH3-only peptide family, which likely facilitate BAX activation. Later events are likely to include caspase activation and possibly endoplasmic reticulum (ER) stress, but the ordering of this latter event is not conclusive. Once some ganglion cells begin to die, it is also possible that secondary degenerative events are set in motion, which include the activation of both macro- and microglia and the production of the inflammatory cytokine TNFα (*light blue background*). TNFα may result in the death of adjacent unaffected ganglion cells or may exacerbate the death of affected cells by amplifying their level of damage and BAX activation.

critical early activators of the pre-BAX events.[9–11] How these kinase pathways are activated, and whether additional regulatory pathways are also involved are some of the critical unanswered questions of how axonal damage is transmitted to the cell soma.

In addition to atrophy events, the activation of at least one of the BH3-only–containing proteins is almost certainly necessary to activate BAX and/or to sequester the antiapoptotic BAX antagonists present, such as BCL-X. Other BH3-only proteins may also be activated in the ganglion cells before BAX activation; alternatively, some BH3-only proteins may become activated as other downstream events in the cell (such as the generation of elevated reactive oxygen species, increases in cytosolic Ca^{2+}, or extensive DNA damage) come into play.

We have focused on two pre-BAX events that appear to be connected by early changes in chromatin structure. Using *Bax*$^{-/-}$ mice as a tool to help define the pre-BAX events, both these events occur in ganglion cells in the same time frame in both knockout and wild-type mice. The first of these events is the silencing of normal gene expression. This phenomenon was first described for the archetypical ganglion cell marker gene *Thy1*. Studies in which the loss of *Thy1* transcript abundance was examined in crush, acute NMDA toxicity, and experimental glaucoma models all indicated that mRNA abundance dropped several days before actual cell loss occurred.[12] Since this initial observation, several other laboratories have documented similar decreases in a variety of ganglion cell marker genes[13–18] in several models of optic nerve damage. More recently, kinetic analysis of several marker genes has suggested that transcript abundance for most selective genes that are normally expressed in ganglion cells decays with exponential curve kinetics.[19]

A second atrophic event appears to be cell shrinkage, particularly nuclear shrinkage. The process of cell shrinkage has attracted a great deal of interest in the glaucoma community, principally because it challenges an established dogma that magnocellular ganglion cells are more susceptible to damage than are smaller parvocellular cells. This initial concept (that larger cells are more susceptible) originated from seminal morphometric studies that measured the sizes of cells in Nissl-stained retinal wholemounts of patients with glaucoma or monkeys with experimental glaucoma.[20–22] The common finding from these measurements was that glaucomatous retinas contained a greater number of smaller neurons than did their control counterparts. The idea that magnocellular cells are more susceptible is important, because it has generated a flurry of research to develop electrophysiological and psychophysical tests, which are biased to magnocellular visual pathways and, it is hoped, will predict glaucomatous damage earlier.[23–25] This hypothesis is not without controversy, however. Modern and more precise anatomic studies support a more uniform degeneration for both magnocellular and parvocellular neuronal circuits,[26–28] and individual labeling of cells in both human and nonhuman primates with glaucoma indicate that all subtypes of ganglion cells are subject to early-onset atrophy.[29–31] Thus, a caveat regarding the magnocellular susceptibility hypothesis is that the original study did not account for cell atrophy such that magnocellular cells may have shrunk to a size typical for parvocellular cells.

In recent studies in our laboratory, we have used *Bax*$^{-/-}$ mice to distinguish cell shrinkage, and predominantly nuclear atrophy, as a pre-BAX event in damaged ganglion cells (Janssen K, et al., manuscript submitted). Like gene silencing, nuclear atrophy is linked to the activity of histone deacetylases (HDACs), which are necessary to deacetylate histones in euchromatic regions of the nucleus and convert them to heterochromatin. Heterochromatin enables tighter packing of the nuclear material, which is an essential element of nuclear condensation. At this point, other molecular events associated with nuclear shrinkage, such as degradation or modification of nuclear laminin scaffolding, have not been evaluated in dying neurons.

Function of the *Bcl2* Family Proteins in Ganglion Cell Soma Death

As mentioned, there are both anti- and proapoptotic members of the *Bcl2* gene family. The principal antiapoptosis family member expressed in retinal ganglion cells is *BclX*,[32] although survival of these cells can be enhanced by ectopic expression or exogenous *Bcl2* in transgenic mice. On the flip side, the principal proapoptosis family member expressed in ganglion cells is *Bax*. The importance of *Bax* in the process of retinal ganglion cell death has been extensively studied in *Bax*-knockout mice. *Bax*$^{-/-}$ ganglion cell somas are completely resistant to normal programmed cell death pruning of superfluous ganglion cells[35] and to damaging stimuli of the optic nerve, including acute optic nerve crush and chronic glaucoma in DBA/2J inbred mice.[36,37] Ganglion cells require a specific threshold of *Bax* expression to execute the apoptosis program, which is exemplified by mice expressing different alleles of a wild-type *Bax* gene. These alleles differ by a single-nucleotide polymorphism in the promoter that affects the rate of gene transcription.[38] Mice with two wild-type alleles, regardless of the promoter polymorphism, produce enough BAX to completely execute the ganglion cell death program. If one wild-type *Bax* allele is deleted, however, *Bax*$^{+/-}$ mice can exhibit long-term resistance to optic nerve damage if they normally express the low-transcription allele. As a consequence, depending on the steady state level of latent *Bax* transcripts and protein, simply reducing concentration by 50% can have a profound impact on ganglion cell soma susceptibility.

In addition to the major anti- and proapoptotic members, ganglion cells have been reported to express several of the BH3-only members of this family. These proteins include BIM,[39,40] BAD,[41] BID,[42] and BBC3 (PUMA).[6] All these proteins are activated by independent events that have been reported in ganglion cells. *Bim* expression is thought to be activated by signaling pathways that are activated by neurotrophin deprivation. BAD protein activation is often the consequence of elevated levels of intracellular calcium, whereas BID activation is most commonly associated with the extrinsic apoptosis pathway. In the case of this last protein, cytokines, usually from the TNF family of ligands, bind to death receptors on cells and directly activate caspase 8 and the rest of the caspase cascade. Thus, the extrinsic pathway can kill cells without the involvement of BAX and mitochondrial dysfunction. Possibly in a mechanism to accelerate apoptosis, however, caspase 8 targets BID and cleaves it into an active BH3-only protein called tBID. This newly activated protein can then recruit the intrinsic pathway by facilitating BAX activation.

It is possible that the process of ganglion cell apoptosis is mediated by the serial activation of BH3 proteins, suggesting that they act as sentinels of where in the apoptosis program the dying cell is found (Fig. 1). In general, this hypothetical serial activation would ensure that cells undergoing the cell death process would produce enough BH3-only proteins to completely inactivate all the antiapoptotic proteins they have accumulated as healthy cells. In a model of ganglion cell apoptosis, a potential series of BH3-only activation could involve primary activation of BIM from neurotrophin deprivation and BBC3 from subacute DNA damage, subsequent activation of BAD as early events in the death program lead to release of calcium from intracellular stores (possible from endoplasmic reticulum stress), and finally activation of tBID from the exter-

nal influence of activated macro- and microglia (see below). A more comprehensive analysis of these events is reviewed by Nickells et al.[7]

A caveat regarding this model of serial activation is that there is no clear evidence that all BH3-only proteins become active in the same dying ganglion cell. An equally plausible model is that different ganglion cells are activated to die by independent stimuli. This hypothesis implies a much more complicated level of interaction between the initial activating event (stress at the optic nerve head) and the sum of cellular responses in the affected retina. It has been clearly exemplified by studies comparing the dependence of BH3-only proteins on programmed retinal ganglion cell death during development and pathologic ganglion cell death. Deletion of *Bbc3* in mice completely abrogates programmed cell death, whereas it has only a modest and transient protective effect after axonal damage.[6]

Evidence of Secondary Degeneration: Glaucoma as an Axogenic and Somatic Neurodegenerative Disease

The prevailing consensus among glaucoma researchers and clinicians is that the initial site of damage in glaucoma is the optic nerve.[3] This knowledge has lead to the classification of glaucoma as an axogenic disease, since axons are the first compartment of the ganglion cells to become damaged.[1] Damage to the axon becomes manifest as a degenerative signal to the cell body and dendritic arbor sometime thereafter, possibly through the mechanism described in the neurotrophin-deprivation hypothesis, as indicated earlier.

There is a growing body of supporting evidence, however, that ganglion cells initially damaged at the level of the optic nerve head produce a chain of events that leads to damage to the surrounding, normally healthy ganglion cells. This effect has been termed secondary degeneration and means that the complete pathology of ganglion cell death and optic nerve degeneration involves both an axogenic component (leading to primary degeneration) and a somatic component. The most compelling evidence of secondary degeneration comes from studies using acute models of optic nerve damage, such as axotomy or crush. Initially, the effect was measured in crushed rats, in which an initial wave of cell death, which was resistant to the NMDA channel blocker MK801, was followed by a later period of death that could be blocked by MK801.[43] Since MK801 can block glutamate toxicity, it was speculated at the time that secondary degeneration was a function of glutamate release from already dead ganglion cells. This concept was supported by now questionable studies showing glutamate elevation in the vitreous of monkeys with experimental glaucoma.[44] Further evidence of secondary degeneration was also provided by detailed measurements of axonal and ganglion cell body loss in monkey optic nerves and retinas, after a partial lesion to only the superior region of the optic nerve. In these experiments, the corresponding inferior retina showed substantial cell loss. These investigators also noted significant loss of ganglion cell bodies and axons in the inferior nerve and superior retina.[45] Because they had not experimentally induced damage to this region of the optic tract, they interpreted their results as evidence supporting a distant damaging influence of early dying ganglion cells. A similar model has been attempted in rats,[46] but data from these animals are highly variable, possibly because axons in the rat do not stay in highly organized bundles throughout the nerve. It is thus likely that ganglion cells in both hemispheres of the retina could be affected by a microlesion to the superior optic nerve.

The mechanism of secondary degeneration is under great debate. In addition, whether secondary degeneration even occurs in glaucoma or is simply an artifact of the more acute models is controversial. The favored mechanism is that dying retinal ganglion cells (primary) affect other cell types in the retina, principally macroglia and microglia. The microglia are especially compelling, since they represent the resident sentinel cells for activation of the innate immune response.[47] Alternatively, Lebrun-Julien et al.[48] demonstrated that ganglion cell toxicity in vivo was mediated by the activation of Müller cells.

A likely mechanism is that glial cells respond to primary ganglion cell death by synthesizing and releasing cytokines. TNFα has been strongly implicated as the critical cytokine mediating the secondary degenerative response,[48,49] although it has also been implicated in the degenerative changes that may occur in optic nerve head astrocytes in response to ocular hypertension. Importantly, however, retinal ganglion cells express the R1 receptor for TNFα,[50] and activation of these receptors leads to direct activation of the extrinsic apoptosis program. Injection of TNFα into the vitreous leads to ganglion cell loss.[51,52] Consistent with the model of secondary degeneration, *Tnfr1*$^{-/-}$ mice exhibit an early phase of degeneration, but no secondary wave of cell loss, suggesting a mechanism in which secretion of TNFα by activated glia directly activates the apoptosis program in bystander ganglion cells. If this model is correct, then ganglion cell death should not be blocked in *Bax*$^{-/-}$ animals, which are still susceptible to stimuli that do not require activation of intrinsic apoptosis.[36] Since there is no cell loss in *Bax*$^{-/-}$ mice, then either the TNFα pathway is not a major contributor to cell death, or there has to be complete execution of apoptosis of ganglion cells affected by the primary insult. Last, the new-generation antibiotic minocycline is being used to help dissect the role of the innate immune response in the process of secondary degeneration. This antibiotic has been found to suppress inflammatory responses in the central nervous system,[53] including preventing the activation of microglia. Minocycline retarded ganglion cell death in an axotomy model[54] and appears to affect retinal changes associated with secondary degeneration.[55]

Does secondary degeneration occur in glaucoma? This is now a question of considerable debate. The partial optic nerve transection experiments in monkeys suggest that if it does occur, the secondary effect is quite distant, able to damage cells many millimeters away from regions of primary injury. Arguing against this effect is the striking regional degenerative patterns observed in many forms of glaucoma. Perhaps the most dramatic is the wedge pattern of ganglion cell soma loss exhibited in DBA/2J mice and some rat models.[2,56,57] It is difficult to rationalize a diffusible toxic effect that obeys boundaries as strict as those in these models. Nevertheless, TNFα mRNA and protein are upregulated in animal models of glaucoma,[50] and deletion of the *Tnfr2* gene in mice attenuates both optic nerve and ganglion cell soma damage[52] after experimental induction of ocular hypertension. Similarly, minocycline has been shown to attenuate ganglion cell damage in models of glaucoma.[58,59] At this time, it is not possible to rule out that the effect of this cytokine, and the innate immune response, contributes directly to the pathology of the optic nerve head or secondarily to retinal ganglion cell somas in the retina.

Summary: Key Needs and Opportunities

A dominant metric in the evaluation of neuroprotective agents is the simple counting of retinal ganglion cell somas compared with those in control conditions. As *Bax*$^{-/-}$ mice show, complete soma protection can be achieved in glaucoma, but these cells still experience both axonal degeneration and atrophic

events such as shrinkage and loss of normal gene expression. A better defined set of criteria is needed to set the bar for more comprehensive neuroprotection studies, including morphologic, molecular, and functional criteria.

A better appreciation is needed of the self-destruction pathways in different cell compartments and their interrelationship in the pathology of glaucoma. A ganglion cell comprises multiple compartments, including the synapse, axon, dendritic arbor, and soma. Each of these can be independently activated to degenerate, and we must have an understanding of how degeneration of one compartment affects another. In addition, we should understand the sequence of compartmental degeneration, which will provide a more comprehensive understanding of the initiating pathology of this disease.

We can now focus on molecular changes occurring early in the degeneration of the ganglion cell soma: Early degenerative events are linked with cell atrophy. For example, dendritic arbors contract along with the shrinkage and collapse of the soma and cell nucleus, respectively. Early changes in the soma appear to be linked to epigenetic changes occurring in the nucleus, principally the activation of histone deacetylases and the global deacetylation of nuclear histones. These early changes lead to the activation of BAX, which is the committed step in the apoptosis of ganglion cells. There is a need to carefully define the activation events of the BAX-dependent intrinsic apoptosis pathway, specifically by BH3-only proteins. Many different varieties of these proteins have been implicated in glaucomatous ganglion cell apoptosis. Since each different BH3 protein is an initiator from distinct and overlapping signaling events, we must better understand the sequence of their activation and recruitment in the cell death process. For example, are all the BH3-only proteins active in a single ganglion cell, but recruited at different times? Or are different ganglion cells activated to die because of the independent actions of single BH3-only proteins? The answers to these questions will have a significant bearing on whether ganglion cells receive one or many different signals prompting them to die.

Is this mechanism of secondary degeneration an active component of the pathology of glaucoma? If so, what is the chain of events leading to it, and what additional cell types are involved in transmitting the death of cells by primary degeneration to a death-signaling event in secondary degeneration? If secondary degeneration is a real phenomenon, then it provides an important opportunity to inhibit this process and preserve ganglion cells that still have intact axons and may be coaxed back into a functional state.

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