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## **Extra-views**

### **Programmed cell death in retinal degeneration**

Targeting apoptosis in photoreceptors as potential therapy for retinal degeneration

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### **KEY WORDS**

Calpain, AIF, Caspase-12, apoptosis, retinitis pigmentosa, photoreceptor

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## **ABSTRACT**

Retinal degenerations are the major cause of incurable blindness characterized by loss of retinal photoreceptor cells. Several genes causing these genetic diseases have been identified, however the molecular characterization of a high percentage of patients affected by retinitis pigmentosa (RP), a common form of retinal degeneration, is still unknown. The high genetic heterogeneity of these diseases hampers the comprehension of the pathogenetic mechanism causing photoreceptor cell death. Therapies are not available yet and for this reason there is a lot of interest in understanding the etiology and the pathogenesis of these disorders at a cellular and molecular level. Some common features have been identified in different forms of RP. Apoptosis was reported to be the final outcome in all RP animal models and patients analyzed so far. We recently identified two apoptotic pathways co-activated in photoreceptors undergoing cell death in the retinal degeneration (*rd1*) mouse model of autosomal recessive RP. Our studies opened new perspectives together with many questions that require deeper analyses in order to take advantage of this knowledge and develop new therapeutic approaches. We believe that minimizing cell demise may represent a promising curing strategy that needs to be exploited for retinal degeneration.

## APOPTOSIS IN RETINAL DEGENERATION

Due to the specialized nature of the eye, a non life-essential organ, mutations in retina specific genes are not lethal and can cause very specific and disabling forms of visual loss. Retinitis pigmentosa (RP), a type of retinal degeneration, represents one of the most prevalent causes of visual handicap and is characterized by loss of photoreceptor cells and invasion of the retina by the retinal pigment epithelium. As rod photoreceptors die, cone viability is also compromised and the disease proceeds toward reduction of peripheral field with tunnel vision and finally loss of sight. It is a progressive disease causing complete blindness in later life. RP is a genetically heterogeneous disease and, up to now, over 50 genes have been identified (<http://www.sph.uth.tmc.edu/Retnet/>). There is a great functional diversity in the types of genes that have been implicated in RP and they can be eye specific (e.g. visual transduction cascade, structural proteins, retinoid cycle, outer segment renewal, transcription factors) or widely expressed (e.g. splicing factors, nucleotide metabolism) <sup>1</sup>. Due to genetic and functional diversity of the involved proteins, the molecular mechanisms underlying the different forms of RP are still not well understood. In all the types of RP analyzed so far apoptosis was reported to be the final and common cause of photoreceptor degeneration but the apoptotic pathway engaged in the process was not defined until recently. Interestingly, the involvement of executioner caspases has been quite controversial. Activation of caspase-3 was detected in transgenic rats with a mutation in the *Rhodopsin* gene <sup>2</sup>. Nonetheless, several data on different models of the disease indicate a caspase-independent mechanism <sup>3-5</sup>. Moreover the impact of caspase-3 was evaluated in caspase-3-deficient mice. Although ablation of caspase-3 provided transient

photoreceptor protection, rod demise proceeded, suggesting that caspase-3 may be involved but is not critical in mediating retinal degeneration *in vivo* <sup>6</sup>.

### **APOPTOSIS IS INITIATED BY CALPAINS IN PHOTORECEPTOR CELLS**

Accumulating evidences imply that mitochondria and the endoplasmic reticulum (ER) are major points of integration of pro-apoptotic intrinsic signaling and, in some of these cases, caspase inhibition does not prevent apoptosis. Notably the cell death pathways elicited by ER stress and following calcium overload differ from classical caspase mediated apoptosis and engage calpains <sup>7</sup>. Calpains are cysteine proteases synthesized as inactive pro-enzymes and activated by increase of intracellular calcium levels that stimulate autoprocessing. Calpains are present in the cell as heterodimers, composed of an 80 kDa catalytic subunit and a 28 kDa regulatory subunit. In the ER the heterodimer is associated with an endogenous calpain inhibitor called calpastatin (Figure 1). Activation of calpains requires calpastatin dissociation and then translocation to the cytosolic side of the ER <sup>8</sup>. The active protease originates by autolytic cleavage of the 80 kDa catalytic subunit after  $Ca^{2+}$  stimulated dissociation from the 28 kDa subunit. Calpain I and II (micro- and milli-calpain) are expressed in the retina <sup>9</sup> and are activated by micromolar and millimolar calcium concentrations respectively. Activation of calpain and cathepsin D has been associated to cell death in RP <sup>10-12</sup>. In addition, a correlation between cell demise and downregulation of calpastatin at the mRNA and protein level has been reported in degenerating photoreceptors <sup>11</sup>. Calpains do not directly cause chromatin condensation but they are proteases activating apoptotic factors, such as caspase-12 and AIF. *In vivo* and *in vitro* studies showed that AIF and caspase-12 are

co-activated and co-localized with fragmented chromatin in apoptotic photoreceptors <sup>12</sup>. Caspase-12 is a murine caspase belonging to the inflammatory caspase subgroup including caspase-1, -4, -5 and -11 localized to the cytoplasmic side of the ER <sup>13</sup>. Caspase-12 can be activated by cleavage by calpain II following elevation of intracellular calcium. The cleaved active form of caspase-12 participates to the apoptotic event by translocation to the nucleus or possibly through other caspases <sup>14, 15</sup> (Figure 1). Apoptosis-inducing factor (AIF) is a flavoprotein localized in the mitochondrial intermembrane space. Upon proper apoptotic stimuli AIF exits the mitochondrion and directly translocate to the nucleus. Cleavage and release of AIF from mitochondria is regulated by calpain I and can occur without cytochrome *c* release <sup>16</sup>. In several neuronal degeneration models, including retinal degeneration, translocation of AIF to the nucleus was observed prior to the mitochondrial release of cytochrome *c* <sup>12, 17, 18</sup>. The activated form of AIF translocates to the nucleus participating to DNA fragmentation that culminates in cell death (Figure 1).

The *rd1* mouse model of recessive RP has elevated levels of cGMP <sup>19, 20</sup> and this results in elevated intracellular calcium <sup>10, 12</sup>. This is explained by lack of PDE activity controlling cGMP concentration in photoreceptor cells and therefore calcium influx through cGMP-gated channels (CNGA, figure 1). Based on these and other data we reasoned that increase of intracellular calcium concentration and concomitant down-regulation of calpastatin <sup>11</sup> may activate calpains and, subsequently, caspase independent death pathways. We showed that increase of intracellular calcium and activation of calpains are the first events observed in *rd1* dying photoreceptors. Activation of calpains is followed by translocation of AIF and caspase-12 to the nucleus

and chromatin fragmentation. *In vitro* treatment of *rd1* mutant photoreceptors with a calcium channel blocker prevented intracellular calcium increase and all the following events. This result confirmed the direct link between intracellular increase of calcium ions, activation of calpains and apoptosis initiation in mutant photoreceptor cells <sup>12</sup>.

## **APOPTOSIS IN DIFFERENT FORMS OF RETINITIS PIGMENTOSA**

One of the main questions that now need to be addressed is whether different genetic lesions trigger the same apoptotic mechanism. This issue will be fundamental for curing this genetically heterogeneous but phenotypically similar group of diseases.

Mutations in the *Rhodopsin (RHO)* gene underlie 25% of dominant RP and, for that reason, are extensively studied. Mutant RHO has been suggested to accumulate in the ER and to form aggresomes <sup>21, 22</sup>. Demonstration of ER stress as a consequence to mutations in RHO has not been provided in vertebrates so far, nonetheless is most likely. In fact, activation of an ER stress response following expression of mutant RHO has been recently reported in *Drosophila* <sup>23</sup>. We have shown in fibroblast cells that co-activation of AIF and caspase-12 occurs as a response to changes in intracellular calcium concentrations as well as to protein misfolding <sup>7</sup>. We therefore expect these same pathways to be activated when mutant RHO aggregates in the ER. We also reported that the relative contribution of the two apoptotic factors appears to be distinctive during the different stressful events. AIF has a predominant role in response to calcium increase as also observed in *rd1* mutant photoreceptors. In fact downregulation of AIF expression in *rd1* photoreceptors completely blocked apoptosis. Conversely, decrease of caspase-12 mRNA and protein had only marginal effects in

dying rods <sup>12</sup>. On the other hand, caspase-12 appeared to play the key function after protein accumulation in the ER of fibroblast cells <sup>7</sup>. Taking into account all these data, we expect co-activation of AIF and caspase-12 in dominant forms of RP caused by toxic effects of an aberrant protein such as mutant RHO (Figure 1). We also suppose that, differently from what we have shown in *rd1* rods, down-regulation of caspase-12 but not AIF will provide the best approach to reduce apoptosis caused by RHO mutation.

A remarkable finding of these studies was the identification of calpains as common proteases that act upstream to AIF and to caspase-12 and are activated in response to calcium increase and protein misfolding stresses <sup>7</sup>. This was also true for the RP affected retina. In fact, inhibition of calpain activity prevented *in vivo* activation of AIF and caspase-12 and consequently apoptosis in the *rd1* degenerating retina <sup>12</sup>. Similarly, we also expect that by using inhibitors of retina expressed calpains we can delay or stop apoptosis caused by *RHO* mutations. These results have important implications for development of therapeutic strategies, yet further studies are needed to translate these preliminary results in effective treatments. The first issue regards the type of molecules and delivery procedures used to block calpain activity in our study. The aldehydes that have been intraocularly injected in the mutant mouse and used to down-regulate calpain activity are non-specific cysteinyl protease inhibitors and are also effective against the proteasome. For this reason, our treatment blocked calpains but possibly also other proteases. Secondly, the inhibitors are small molecules that have the advantage to be cell-permeable, yet they quickly undergo clearance. Thus, in order to define their long time efficacy, repetitive injections should be applied with the risk of retinal damage.

Some preliminary study undertaken with other molecules targeting calpains confirmed that calpains are key regulators of apoptosis in RP, nevertheless development of long-lasting application procedures of specific calpain inhibitors will be necessary to develop proper therapies for RP patients.

## **CASPASE INVOLVEMENT IN RETINITIS PIGMENTOSA**

The participation of executioner caspases (i.e. caspase-3, caspase-6 and caspase-7) to the activation of programmed cell death in photoreceptors cannot be completely excluded. *In vivo* studies showed that, although rods eventually died in the absence of caspase-3, lack of this executioner caspase provided transient photoreceptor protection<sup>6</sup>. We also evaluated the contribution of executioner caspases to photoreceptor apoptosis in the *rd1* mouse model. We observed that treatment with the pancaspase inhibitor zVAD.fmk did not abolish apoptosis but reduced the number of apoptotic cells<sup>12</sup>. The 20% reduction of cells undergoing apoptosis in the presence of zVAD.fmk can be explained by a partial contribution of executioner caspases. How can executioner caspases be activated in this system? As previously mentioned, AIF plays the key role in the activation of apoptosis in *rd1* photoreceptors while down-regulation of caspase-12 does not abolish apoptosis. Nevertheless, caspase-12 probably holds a reinforcing effect because apoptosis is reduced in the absence of caspase-12. It is known that caspase-12 acts directly by translocation to the nucleus but can also activate executioner caspases. We can envisage the scenario in which the boosting effect of caspase-12 is partially mediated by executioner caspases and therefore impaired in the presence of pancaspase inhibitors or siRNA targeting caspase-12.

An alternative explanation for the reduction of apoptotic cells in the presence of pancaspase inhibitor derives from the specificity of zVAD.fmk. High doses of zVAD.fmk, like those applied in our studies, can also affect calpain activity<sup>24</sup>. If so, a reduced calpain protease activity will also influence apoptosis and our results can be justified simply by calpain activation and rule out involvement of executioner caspases. Hence, we cannot exclude that the apoptotic process in the RP retina engages also executioner caspases, nevertheless activated forms of caspase-3 have not been revealed by immunohistochemistry or immunoblotting so far.

### **NON-CELL AUTONOMOUS EFFECTS ON CONE VIABILITY**

RP usually begins with night blindness and decreased peripheral vision due to loss of rod photoreceptors. The most devastating effect comes with subsequent degeneration of cones, which causes loss of color and high acuity vision, ending in total blindness. Rod photoreceptors death is therefore followed by reduction of cone viability and it is unclear whether this is due to loss of trophic factors or to activation of toxic molecules or to both. The fact that cones die in the retina of patients with mutations in rod specific genes suggests the presence of a non-cell autonomous mechanism of death that is also demonstrated by degeneration of genetically normal photoreceptors adjacent to rods that express the mutant protein in chimera mice<sup>25</sup>. It is not known whether the same or different mechanisms mediate rod and cone degeneration. To address this question we counted the number of apoptotic cells in which AIF and caspase-12 were activated inside the nucleus. At all the different time points analyzed during retinal degeneration we always failed to detect activation of these two apoptosis effectors in 20% of dying

cells. We suggested that *in situ* detection of AIF and caspase-12 fails to detect low amounts of these factors in some apoptotic nuclei, but this issue has not been solved yet. What is the identity of these cells? Are these cells undergoing apoptosis through a different mechanism? It is possible that some photoreceptors, such as cones, die through an alternative apoptotic pathway as a consequence of death of neighbouring cells. On the contrary, the idea that different mutant rod photoreceptors activate different apoptotic pathways in response to the same genetic insult appears unlikely. Specific studies aimed at the cellular identification of these dying cells are required to define whether the degenerating retina activate several apoptotic mechanisms that need to be specifically targeted in order to effectively block or at least delay cell demise in this disabling disease.

## **PERSPECTIVES AND CONCLUSIONS**

As previously discussed, now that the apoptotic pathways have been partially unraveled, we need to define whether these same molecular mechanisms are activated during cell demise in RP caused by different genetic lesions. In fact, a limiting aspect in designing a cure for RP is the still inadequate molecular diagnosis for RP patients and the high percentage of isolated instances, occurring in about 40% of RP cases. General and common factors activated by mutations of different genes can be the keystone to the cure. We started with the identification of calpains that are engaged by high calcium and ER stress and are probably upstream to both AIF and caspase-12 activation. Specific experiments aimed at defining whether calpains are really activating all the downstream catastrophic events leading to cell death or whether they cooperate with

other proteases are fundamental to plan effective approaches to stop cell death during retinal degeneration. This is an essential issue important to define whether apoptosis should be exploited as a therapeutic target for this genetically heterogeneous disease.

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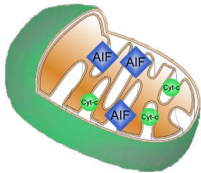
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**Figure 1. Interaction between AIF and caspase-12 apoptotic pathways.**

Increase of intracellular  $\text{Ca}^{2+}$  levels upon opening of CNGA (cyclic nucleotide gated channel alpha) channel activates calpain proteases. Activated calpains act on mitochondrial AIF and ER caspase-12. Cleaved AIF and cleaved caspase-12 translocate to the nucleus and are involved in chromatin fragmentation. siRNA data showed that downregulation of caspase-12 in *rd1* mutant photoreceptors does not block cell death and AIF activation but reduces the number of cells with nuclear AIF and undergoing apoptosis. This suggested that caspase-12 has a reinforcing effect shown here as dashed arrows and a question mark. The question mark refers to the lack of information on the molecular mechanisms involved in the cross-talk between AIF and caspase-12: it is still unclear whether these effects are direct or indirect.

Protein misfolding in the ER causes increased  $\text{Ca}^{2+}$  (arrow inside the ER) that can activate calpains and then AIF and caspase-12.



Calpain

