Activation of Multiple Pathways during Photoreceptor Apoptosis in the rd Mouse

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PURPOSE. The primary purpose of this study was to characterize photoreceptor apoptosis in the rd mouse. Given that apoptosis is the final common pathway in many cases of retinal degeneration, the ability to retard or even arrest this process may ameliorate retinal disorders such as retinitis pigmentosa (RP). The absence of any recognized therapy emphasizes the fact that a detailed knowledge of the molecular events involved is necessary to identify rational targets for therapeutic intervention.

METHODS. Flow cytometry was used to measure physical and chemical characteristics in the photoreceptor population. Individual cells flow in suspension past one or more lasers, scattering light and emitting fluorescence. Western blot techniques demonstrated cleavage of calpain-specific substrates. Retinal explant cultures were used for inhibitor studies. Postnatal day 10 (P10) rd retinas were cultured without retinal pigment epithelium (RPE) attached up to P17.

RESULTS. This study demonstrated calcium overload in the cytosol and subsequently in mitochondria. Mitochondrial membrane depolarization and reactive oxygen species (ROS) were detected later, during the peak of cell death. Analysis of downstream events indicated early activation of calcium-activated calpains. Treatment of rd retinal explants with the calpain inhibitor N-acetyl-Leu-Leu-Nle-CHO (ALLN) successfully inhibited o-fodrin cleavage, yet it did not protect against photoreceptor degeneration. Finally, the results demonstrate an increase in the levels of both precursor and processed forms of the aspartate protease cathepsin D.

CONCLUSIONS. Excessive calcium influx is an early event that initiates the activation of calcium-activated proteases. However, these proteases are not singularly the cause of death, because their inhibition does not prevent apoptosis. Indeed, the results presented herein suggest that multiple pathways are involved and that each of these components may have to be addressed for cell death to be successfully inhibited. (Invest Ophthalmol Vis Sci. 2005;46:3530–3538) DOI:10.1167/iovs.05-02488

Caspase-independent photoreceptor apoptosis has been described in a number of in vivo models of retinal degeneration including the rd mouse. Indeed, we have shown that the intrinsic pathway is unavailable due to downregulation of key members of this death program in the developing mouse retina. Given that caspases are not essential for retinal degeneration in the rd mouse it is clear that the actual pathways involved require elucidation. Studies in a variety of systems have revealed that several other proteases including calpains, cathepsins, and serine proteases induce apoptosis in the absence of caspase activation.

The cathepsin family consists of cysteine (cathepsin B and L), aspartate (cathepsin D and E), and serine (cathepsin A and G) proteases. Cathepsins are transported to the lysosomal compartment as proenzymes, where they are activated depending on their type, either by autodegradation in acidic pH or by proteolysis by another cathepsin. The main physiological role of cathepsins is protein turnover in the lysosomal compartment. With regard to the retina, cathepsin D plays a key role in lysosomal digestion of photoreceptor rod outer segments. Transgenic mice expressing a mutated form of cathepsin D exhibit photoreceptor degeneration, shortening of rod outer segments, and accumulation of photoreceptor breakdown products in RPE cells, suggesting an essential role for cathepsin D in retinal maintenance.

In recent years, the role of cathepsins has been elevated from disposal of proteins in the lysosomal compartment to include a role in programmed cell death (PCD). Although the mechanisms by which these largely nonspecific proteases achieve this end remain unclear. Gene expression profiling has identified an eightfold induction in cathepsin S expression during retinal degeneration in the rd mouse, but the potential involvement of these proteases in the execution stage of apoptosis during retinal degeneration has yet to be addressed.

Calpains are calcium-responsive cysteine proteases activated by autolytic processing in both apoptosis and necrosis. The two main isoforms, distinguishable by their calcium requirements (in micro- and millimolar), are calpain1/μ-calpain and calpain2/m-calpain. Other tissue-specific isoforms include the calpain-3 splice variants LP82 and LP85 found in the lens and the retina-specific form Rt88. Calpain substrates include cytoskeletal proteins, intracellular enzymes, membrane receptors and transporters, and regulatory proteins. This laboratory has demonstrated activation of calpains in light-induced retinal degeneration in vivo and in 661W photoreceptor cells in response to sodium nitroprusside (SNP). As just mentioned, calpain activation requires an increase in intracellular calcium levels. Indeed, a link between calcium and apoptosis has been firmly established in several systems including photoreceptors. Calcium influences the function of mitochondria, which act as buffers for nontoxic levels of calcium, extruding these through the sodium–calcium exchanger. However, accumulation of mitochondrial calcium can result in mitochondrial dysfunction, energy depletion, free radical generation, and cell death.

In this study, we sought to characterize the events leading to photoreceptor apoptosis in the rd mouse, given that caspases do not play a role and therefore caspase inhibitors would have little therapeutic value. Primarily, we sought to demonstrate an increase in intracellular calcium (Ca2+) given the likelihood that accumulation of cGMP leads to opening of cGMP-gated channels. Subsequently we analyzed the
effects that changes in intracellular calcium might have on mitochondrial membrane potential ($\Delta \psi$), reactive oxygen species (ROS) production, and calpain activation. Finally, work conducted in this laboratory has demonstrated an important role for caspase pathways in photoreceptor cell death in the rd mouse and suggests the need to manipulate effectors other than caspases to slow or prevent retinal degeneration.

**Materials and Methods**

**Animal Treatment**

All experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. C3H/HeN (rd/rd) and C57BL/6 (wild type) mice were obtained from Harlan UK (Bicester, UK) and reared under light conditions of 60 to 100 lux in a 12-hour light–dark cycle.

**Photoreceptor Staining by Immunocytochemistry**

For all flow cytometry studies, mice were killed by decapitation at postnatal day (P$\_0$, P$\_10$, P$\_11$, P$\_12$, or P$\_13$), and retinal dissection was performed as previously described. Tissue dissociation was achieved in a 0.25% trypsin solution (BioSciences Ltd., Dun Laoghaire, Ireland). Retinal cells were washed in PBS and fixed in 1% para-formaldehyde for 30 minutes at $4^\circ$C. Alddehyde groups were quenched and cells permeabilized in 0.01% Triton X-100 for 5 minutes. Retinal cells were incubated with an anti-rhodopsin antibody (Labvision, UK Ltd., Suffolk, UK) at a dilution of 1:50 for 1 hour at 4°C. Incubation with an FITC-conjugated secondary antibody for 1 hour at 4°C was followed by analysis on flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ).

**Intracellular Free Calcium Measurement**

Intracellular calcium levels were determined using the intracellular calcium probe Fluor-3 AM (acetoxyethyl ester; Molecular Probes, Leiden, The Netherlands). Mitochondrial calcium levels were measured using Rhod-2 AM (Molecular Probes). Cells were incubated with Fluor-3 (250 nM) or Rhod-2 (10 μM) for 15 minutes at $37^\circ$C and fluorescence measured in FL-1 (530 nm) and FL-2 (590 nm), respectively, on the flow cytometer with excitation at 488 nm. Mitochondrial membrane depolarization was analyzed using the probe 5,5’6,6’-tetra-chloro-1,1’3,3’-tetraethylbenzimidazolecarbocyanine iodide (JC-1; Molecular Probes). Cells were incubated with JC-1 (5 μg/mL) for 15 minutes at $37^\circ$C, and fluorescence was measured in FL-2, as previously described. To measure superoxide anion production, we incubated the cells 10 μM dihydroethidium (DHE) for 15 minutes at $37^\circ$C. Fluorescence due to ethidium bromide was measured in FL-2 as described.

**Western Blot Analysis**

For Western blot studies, mice were killed by decapitation at P$\_0$, P$\_10$, P$\_11$, P$\_12$, P$\_13$, or P$\_14$, and retinal dissection was performed. Tissue samples were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) containing protease inhibitor cocktail (Roche, Lewes, UK) and AEBSF (0.1 mM). Western blot analysis has been described in detail. Briefly, equivalent amounts of 5 mM HEPEs, 100 mM NaCl, 1 mM dithiothreitol [DTT], and 0.1% NP40 on ice for 10 minutes, followed by sonication for 20 seconds. The supernatant was retained and equivalent amounts of protein were incubated with variations of 5 mM CaCl$_2$, 5 mM EDTA, ZVAD (Bachem, Heidelberg, Germany), MDL28170, and ALLN (Calbiochem, Nottingham, UK; 10–50 μM) at $37^\circ$C for 90 minutes. Proteins were resolved by SDS-PAGE, as previously described.

**Retinal Explant Culture**

Retinal organ culture was performed according to the protocol of Caffé et al. Briefly, C57BL/6 (wild-type) and C3H/HeN (rd/rd) mice were decapitated at P$\_10$ and the eyes removed. Cleaning with 70% ethanol was followed by incubation in basal medium supplemented with protease K (Sigma-Aldrich) at $37^\circ$C for 15 minutes. The anterior segment, vitreous body, and sclera were removed and the retina mounted on nitrocellulose inserts (Millipore, Millipore, Billerica, MA) photoreceptor side down. Explants were cultured without RPE in 1.2 mL of R16 medium without FCS. Retinal explants were treated with 1 μM ALLN or 1 μM ALLN plus 25 μM peptide A (Sigma-Aldrich) in

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**Figure 1.** (A) Western blot analysis to verify the specificity of the rhodopsin antibody. Rhodopsin was detected in wild-type retinal lysates, but not in lysates prepared from rho$^{-/-}$ mice or 32D cells. GAPDH demonstrated protein loading in each of the lanes. (B) Flow cytometric immunofluorescence was used to detect photoreceptor cells in a mixed retinal population. Rhodopsin binding was indicated by a shift from the lower right quadrant to the upper right quadrant. This photoreceptor population was gated for all subsequent studies ($n = 3$).
dimethyl sulfoxide (DMSO) or DMSO alone, as a control. After treatment, one half of each retina was lysed in RIPA buffer as just described, whereas the other half was retained for TUNEL analysis. Explants were then included or excluded from the study, based on TUNEL staining and morphology. Explants of poor quality exhibited intense TUNEL staining visible in all layers and were disorganized structurally.

Terminal dUTP Nick End Labeling

Briefly, retinal explants were fixed in 10% neutral-buffered formalin for 2 to 4 hours, followed by cryoprotection in 25% sucrose overnight at 4°C. Frozen sections (7 μm) were incubated in 50 μL of reaction buffer containing terminal deoxynucleotidyl transferase (TdT; Promega, Southampton, UK) and fluorescein-12-dUTP (Roche) according to the manufacturer's instructions. Sections were incubated at 37°C for 1 hour in a humidified chamber. After several washes in phosphate-buffered saline (PBS), the sections were mounted in mowiol (Calbiochem) and viewed under a fluorescence microscope (Eclipse E600; Nikon, Micron Optical Co. Ltd., Wexford, Ireland) using a fluorescein isothiocyanate (FITC) filter.

Immunohistochemistry

Retinal explants were fixed in 10% neutral-buffered formalin for 2 to 4 hours, followed by cryoprotection in 25% sucrose overnight at 4°C. After antigen retrieval and quenching of endogenous peroxidase activity, the frozen sections (7 μm) were incubated with anti-active caspase-3 (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Washes in PBS/T were followed by incubation with secondary antibody for 1 hour at room temperature. Antibody detection was achieved with a kit (Vectastain Elite avidin biotin complex [ABC] Kit) and diaminobenzidine (DAB) reagent (Vector Laboratories, Peterborough, UK). Sections were counterstained with hematoxylin to facilitate tissue orientation and mounted (DPx; Sigma-Aldrich).

Subcellular Fractionation

P9 and P14 retinas were dissected in PBS and transferred to a 2-mL homogenizer (Kontes Dounce, AGB Scientific, Dublin, Ireland) containing 120 μL of cell-extraction buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM AEBSF, 10 μg/mL leupeptin, and 2 μg/mL aprotinin). Cells were allowed to swell under hypotonic conditions for 15 minutes on ice and then disrupted with five strokes of the pestle. Centrifugation at 800g removed nuclei and unbroken cells. Further centrifugation at 10,000g removed mitochondria. A lysosome-free cytosol was obtained by centrifugation in an ultracentrifuge (Beckman Coulter, High Wycombe, UK) at 100,000g for 1 hour and then analyzed by Western blot as described.

Statistical Analysis

All results are expressed as the mean ± SEM (n = 4) in all cases unless specified. Counts of TUNEL-positive cells were taken from three fields in each of three independent retinal explants. Fields at the center of the retinal section with a flat outer nuclear layer (ONL) were chosen, because explants flatten toward the periphery. Significant differences across groups were assessed with an unpaired t-test (P < 0.001).

RESULTS

Photoreceptor Identification by Flow Cytometric Immunofluorescence

First, we examined the specificity of the anti-rhodopsin antibody by Western blot. Monomeric rhodopsin was detected in P10 retinal lysates, whereas the dimeric and trimeric forms were detected in adult photoreceptors. Retinal lysates from rhodopsin knockout mice (rho−/−) verified the specificity of the antibody. The hematopoietic 32D cell line also was negative for rhodopsin staining, as expected (Fig. 1A). This anti-

FIGURE 2. (A) Intracellular calcium levels were measured from P9 to P13 with the fluorescent probe Fluo-3. (Aa) The percentages displayed represent the number of cells with increased calcium(i), measured by increased fluorescence in FL-1. (B) Mitochondrial calcium levels were measured from P9 to P13 with the fluorescent probe Rhod-2. (Bb) The percentages displayed represent the number of cells with increased calcium(m), measured by increased fluorescence in FL-2. Data are expressed as the mean ± SEM (**P < 0.001; n = 4).
body was subsequently used to identify photoreceptors in a mixed retinal population. Flow cytometric analysis demonstrated that photoreceptors constituted 70.85% ± 3.89% of the whole retinal population, as assessed by rhodopsin expression (n = 3, Fig. 1B). This percentage is similar to other estimates previously published in the literature.19 The photoreceptor population was gated in the flow cytometry studies published subsequently.

Analysis of Calcium Levels, Mitochondrial Depolarization, and ROS Production

Intracellular calcium levels were monitored before and during the peak of apoptosis, specifically in the photoreceptor population using the fluorescent probe Fluo-3 AM. The level of intracellular calcium was significantly increased in the rd mouse at P10 before the detection of TUNEL-positive cells and remained elevated during the peak of apoptosis (Figs. 2A, 2Aa). Next, we monitored the effect of increased calcium(i) on mitochondria by measuring calcium(m) using the fluorescent probe Rhod-2 AM (Figs. 2B, 2Bb). As expected, levels of calcium(m) increased in rd photoreceptors in parallel with elevated calcium(i), although a slight delay was observed; 18.43% ± 0.59% of the photoreceptor population exhibited an increase in calcium(i) at P10, whereas a shift in calcium(m) of 16.58% ± 0.85% was not detected until P11. These effects were not observed in wild-type photoreceptors over the same period (data not shown).

To determine the possible effect of a sustained increase in calcium on mitochondria, Δψm was monitored from P9 to P13. It was measured by using the lipophilic probe JC-1, which forms aggregates in the presence of intact Δψm. A reduction in fluorescence emission at 590 nm was interpreted as a reduction in Δψm (Figs. 3A, 3Aa). Δψm collapse had occurred in 22.37% ± 2.69% of the rd photoreceptor population by P11, correlating with the increase in calcium(i). Mitochondria are a key site of ROS production, and alterations in Δψm can result in increased ROS production. In rd photoreceptors, measurements of superoxide anion formation were performed using the probe DHE. Increased superoxide levels were not detectable until P13 (34.43% ± 1.38%), significantly later than initiation of apoptosis (Figs. 3B, 3Bb). There was no population shift observed in frequency histograms from wild-type photoreceptors at the same time points (data not shown).

Cleavage of Calpain-Specific Substrates

Subsequently, we investigated whether the sustained increase in intracellular calcium we had observed would initiate activation of calpains. Calpastatin (120–110 kDa), the endogenous inhibitor of μ- and m-calpain, was cleaved in rd retinas at P12, correlating with the rapid induction of apoptosis (Fig. 4A). Calpastatin was not cleaved in wild-type retinas over the same time course. α-Fodrin is a 240–280-kDa protein that is cleaved by calpain yielding fragments of 145 and 150 kDa.20 As a control, we used the 661W photoreceptor cell line, which yielded both fragments on treatment with staurosporine. A band of 145–150 kDa could clearly be seen at P12 in rd retinal lysates, whereas the caspase-3 cleavage product of 120 kDa was absent (Fig. 4B). Neither band was detectable in wt retinas. To confirm further that the proteolysis observed in the rd model was the result of calcium-activated calpains, a retinal cell free extract was prepared and treated with CaCl2, a source of Ca2+ ions. The same 145–150-kDa cleavage product was readily detected by Western blot. This band was absent in samples treated with either a calcium chelator (EDTA) or...
with an antibody specific for cleaved caspase-3 to ensure that this was not a default pathway for photoreceptors under conditions of calpain inhibition. Caspase-3 positive photoreceptors were not detected in either untreated or ALLN treated retinal explants (Fig. 5C). Developmental cell death occurring in the inner nuclear layer (INL) of the retina at P7 provided a positive control for the detection of cleaved caspase-3.

Analysis of Cathepsin D Activation

Inhibition of calpain activity was not sufficient to prevent photoreceptor degeneration, which suggests that another protease may be involved. Data from this laboratory indicate that in addition to caspases and calpains,1,4,22 cathepsin D is activated after treatment of 661W cells with SNP. In the present study, Western blot analysis demonstrated increased processing of procathepsin D to the mature, active 30-kDa form from P10 to P14 in the rd mouse but not in the wild type (Figs. 6A, 6B). SNP treated 661W cells provided a positive control for cathepsin D activation. However, we wanted to confirm that the increase in cathepsin D activity was not associated only with the phagocytic activity of the RPE. Retinas from rd mice at P7 and P14 were isolated, with and without RPE, and lysed in RIPA buffer, as described in the Material and Methods section. Although a significant portion of cathepsin D processing was attributable to the RPE, there was certainly an increase in cathepsin D processing to the active 30-kDa form in RPE-free retina (Fig. 6C). Cellular redistribution of cathepsins from lysosomes to the cytosol appears to play a key role in some systems; therefore, we demonstrated an increase in both precursor and active forms of cathepsin D in rd cytosolic extracts at P14 compared with wild type (Fig. 6D). Finally, retinal explants were treated with both 1 μM ALLN and pepstatin A (1 μM and 10 μM), an inhibitor of cathepsin D activity from P10 to P14. However, treatment with pepstatin A was toxic to retinal cells, particularly photoreceptors, over this concentration range (data not shown).

**DISCUSSION**

The mechanism by which photoreceptor cells execute apoptosis is a matter of continuing debate. There is evidence in the literature to support developmental cell death mediated by components of the intrinsic mitochondrial pathway, such as caspase-3, Bax, and Bak. However, we know that this archetypal pathway is not used in a number of instances of pathologic photoreceptor apoptosis,2–4,24,25 and there is no delay in the rate of degeneration in rd photoreceptor explants treated with the pancaspase inhibitor z-VAD-fmk

(Doonan F., unpublished data, 2004).

The nature of the mutation harbored by the rd mouse leads to a detrimental accumulation of cGMP and potentially excessive calcium influx through cGMP-gated channels.26 Our results confirmed that intracellular calcium levels are amplified at P10 before the onset of apoptosis, and therefore excess calcium seems to provide the initial stimulus. Elevated calcium levels resulting from light damage in mice or developmental lead exposure in rats also gives rise to photoreceptor apoptosis, which potentially identifies a central role for calcium in retinal degenerations.

**Investigation of the Calpain Inhibitor ALLN**

Retinal explants were cultured from P10 up to P17 as described, in the presence or absence of 1 μM ALLN. This concentration was chosen because it consistently prevented α-fodrin cleavage, whereas concentrations of 5 μM and higher were toxic to the whole retina. In the presence of 1 μM ALLN α-fodrin cleavage was reduced to basal levels observed before onset of apoptosis, as determined by Western blot analysis (Fig. 5A). However, TUNEL staining demonstrated that inhibition of calpains did not delay the kinetics of photoreceptor degeneration in the rd mouse at P14, nor did it confer protection up to P17 (Fig. 5B). Counts of TUNEL-positive cells and subsequent statistical analysis indicated that there was not a significant difference in the number of dead cells between untreated and treated explants. TUNEL-positive nuclei were not as condensed and round as in untreated rd explants; instead, they were fragmented and irregular. Retinal sections were also stained

**Figure 4.** (A) Western blot analysis of calpastatin, an endogenous inhibitor of calpain activity. Calpastatin was proteolized at P14 in the rd mouse but not in C57 wild-type mice, clearly correlating with initiation of apoptosis. GAPDH was used as the loading control. (B) Western blot analysis of α-fodrin cleavage. 661W photoreceptor cells treated with 100 nM staurosporine (+) for 6 hours were used to demonstrate α-fodrin processing to both 145/150- and 120-kDa cleavage products indicating caspase-3 activity. The 145/150-kDa calpain-specific cleavage product was detected at P13 in the rd mouse but not in the wild type. (C) Western blot of retinal cell free extracts treated with Ca2+. Wild-type retinas were lysed as described and treated in the following way: -ve: 5 mM Ca2+ + 5 mM EDTA; +ve: 5 mM Ca2+, 145/150 kDa fragment detected; ALLN: 5 mM Ca2+ + 10 mM ALLN, 145/150 kDa fragment absent; MDL28170: 5 mM Ca2+ + 10 mM MDL28170, 145/150 kDa fragment absent; ZVAD: 5 mM Ca2+ + 10 mM ZVAD, 145/150 kDa fragment detected; and rdP12: rd P12 retinal lysate, 145/150 kDa fragment detected. The results are representative of at least three independent experiments.

**Table 2.** Effects of calpain inhibitors (ALLN or MDL28170) but not a caspase inhibitor (ZVAD; Fig. 4C).
The mechanism or mechanisms by which a sustained increase in calcium leads to photoreceptor demise have remained largely unexamined. However, in this study, we provide evidence for activation of calpains. Proteolysis of calpain substrates, such as actin, α/β-fodrin, and vimentin, could induce features of apoptotic morphology by facilitating nuclear

Figure 5. (A) Western blot analysis of α-fodrin cleavage in rd retinal explants treated with 1 μM ALLN. The rd retinas were explanted at P10 and cultured up to P17 in the presence of 1 μM ALLN in DMSO or DMSO alone. This treatment successfully inhibited calpain-mediated α-fodrin cleavage at P12 and P13. (B) TUNEL staining of retinal sections treated with ALLN. TUNEL staining showed that apoptotic nuclei were still detectable under calpain-inhibiting conditions at P13 and P17. TUNEL counts from wild-type and rd explants are represented in graph format, with no significant difference between untreated and treated samples (P > 0.05). (C) Immunohistochemical staining of rd retinal explants with an antibody that specifically recognizes cleaved caspase-3. Slides were counterstained with hematoxylin to facilitate orientation of the sections. The first panel demonstrates caspase-3 cleavage in the INL at P7 during developmental cell death. Active caspase-3 staining was not detected in the photoreceptor layer of either treated or untreated explants.

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condensation and disruption of the cytoskeletal network. Proteolysis of α-fodrin, a protein essential for cytoskeletal structure would certainly contribute to cellular collapse. In addition, we have described cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), generating a 40-kDa fragment, which we attribute to calpain activity. Degradation of PARP in this way disables DNA repair mechanisms in response to stress facilitating cellular disassembly. Calpain isoforms have been implicated in retinal cell death induced by constant light exposure and optic nerve stretch injury in vivo, and in the 661W photoreceptor cell line treated with SNP or calcium ionophore. Furthermore, treatment with the calpain inhibitor SJA6017 protects against retinal ganglion cell loss in response to ischemic injury in vivo. Given the problems encountered when administering drug treatments to early postnatal mice, treatment of rd retinas ex vivo is a more viable option. However, retinas treated with the small peptide calpain inhibitor ALLN at 1 μM from P9 up to P17 died at the same rate as the untreated explants. Under conditions of calpain inhibition, the characteristics of TUNEL-positive nuclei were altered, becoming smaller and less regular. This could result from inhibition of protease-dependent events such as chromatin condensation, whereas other events such as nuclear shrinkage may still take place. Calcium could also contribute to death through activation of a Ca2+/Mg2+-activated endonuclease, leading to DNA fragmentation. Therefore, calpain inhibition may alter some events, whereas others are retained, resulting in different apoptotic morphology.

The absence of photoreceptor rescue after calpain inhibition, together with the involvement of more than one protease in 661W cell death, led us to investigate the activation of other proteases in the rd model. Ordinarily, cathepsins are maintained in the lysosomal compartment; however, leakage can occur after destabilization of the lysosomal membrane. Reports have shown that oxidative stress, TNFα, or the Alzheimer’s-related protein apoE4 can all cause translocation of cathepsins to the cytosol in this manner. Once in the cytosol, there is evidence that many members of the cathepsin family have significant activity above pH 6.5; however, their cytosolic targets remain largely unidentified. Attempts to inhibit cell death with a combination of ALLN and pepstatin A were unsuccessful. In fact, pepstatin A caused an increase in the number of TUNEL-positive photoreceptors, probably because of inhibition of RPE-specific cathepsin D activity that led to accumulation of photoreceptor breakdown products. It cannot be sure that cathepsin D is the only member of the family to be released in response to lysosomal leakage. In this regard, a recent report demonstrating that Hsp70 achieves part of its prosurvival function in tumor cells through stabilization of lysosomal membranes may prove useful as it could block the release of all lysosomal proteases.

It is now clear that multiple destructive pathways are involved in photoreceptor demise in the rd model. Analysis of 661W cell death has provided us with an insight into the apoptotic pathways that may be used by photoreceptors after exposure to different stimuli. We attempted to analyze the effect of pepstatin A in our system but the inhibitor had toxic effects on retinal cells, so at present we cannot exclude a role for cathepsin D or indeed other members of the cathepsin family in photoreceptor apoptosis. An initial calcium insult results in activation of calpains; however, inhibiting these proteases does not curtail molecular events including increased calcium, loss of ΔΨm, and ROS production that can also result in cell death (Fig. 7). Excessive calcium ions entering rod outer segments of the rd mouse through cGMP-gated channels appears to be the apical event; hence, many research groups have tested the efficacy of calcium channel blockers. However the results have been largely inconsistent, with some groups registering survival-promoting effects and others observing none at all.

The belief that caspases are the sole executioners of apoptosis has highlighted the potential to exploit apoptosis as a therapy for retinal degenerations. However, it is becoming apparent that the situation is much more complex and that caspase-dependent or -independent pathways can mediate photoreceptor cell death (Fig. 7). For example, 661W cells have significant activity above pH 6.5; however, their cytosolic targets remain largely unidentified.

**Figure 6.** (A, B) Western blot analysis of cathepsin D. 661W cells treated with SNP for 24 hours were used to demonstrate processing of procathepsin D to its mature, active form. Procathepsin D (42 kDa) expression increased in the rd mouse from P9 to P14. Processing to the mature form (30 kDa) was also detected. The level of both procathepsin D (42 kDa) and the mature, active form (30 kDa) were detected but unchanged from P9 to P14 in wt retinas. GAPDH was used as a loading control. (C) The rd retinas were dissected with (+) and without (−) RPE at P9 and P14 and lysed in RIPA buffer. Cathepsin D processing increased from P9 to P14 in retinas with and without RPE. GAPDH was used as a loading control. (D) Cellular redistribution of cathepsin D to the cytosol during photoreceptor cell death. The lysosomal fraction (L) extracted from rd retinas at P14 confirmed the detection of pro and active cathepsin D by Western blot. Translocation of both pro and active cathepsin D to the cytosolic fraction was detected in rd retinas at P14 but not in wild type. Results are representative of at least three independent experiments.
Pathways of Photoreceptor Apoptosis in the rd Mouse

The pathways involved in photoreceptor apoptosis in the rd mouse. Calcium influx activates calpains, resulting in cleavage of PARP and α-fodrin. Excess calcium is taken up by mitochondria, leading to membrane depolarization and potential mitochondrial dysfunction and energy depletion. Calpain activation and ROS generation are responsible for lysosomal leakage and subsequent cathepsin processing in some systems, but this has yet to be confirmed in the rd model.

References


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provided useful information on the proteases potentially operating during photoreceptor apoptosis, which include caspases, calpains, and cathepsins. However, the pathway used seems to depend on the expression of key constituents and this in turn depends on the stimulus involved and on the age of the animal. Therefore, the ability of photoreceptors to employ multiple death pathways provides an ongoing challenge in the prevention of retinal degeneration.


