Caspase-Dependent Apoptosis in Light-Induced Retinal Degeneration

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PURPOSE. To study the apoptotic mechanism involved in our model of light-induced retinal degeneration.

METHODS. Rats were injected intravitreally with PBS, 2% dimethyl sulfoxide (DMSO), caspase inhibitor ZVAD-FMK (1.06 mM), ZYVAD-FMK (0.16 mM), or Z-DEVD-FMK (2 mM) before they were placed in constant light (3400 lux) for 24 hours. Additional controls included rats that were uninjected or were punctured with a dry needle. Electoretinograms were recorded before injection and 1 day after the cessation of exposure to constant light. A group of rats was killed for apoptotic cell detection in the outer nuclear layer. Fifteen days later, the remaining rats were killed for histology, and the outer nuclear layer (ONL) thickness was measured. Caspase-1, caspase-3, and calpain activities were measured before and 1 day after exposure to the damaging light.

RESULTS. ZVAD, YVAD, and DEVD inhibited caspase-1 and -3 activities, but not calpain activity, from the beginning and up to 1 day after light exposure. In untreated, dry needle–punctured, PBS, DMSO, and YVAD groups, light exposure significantly reduced retinal function and ONL thickness and increased by 51-fold the number of apoptotic cells. ZVAD and DEVD preserved retinal function to 86% and 78%, respectively, and reduced by three times the number of apoptotic photoreceptors. ONL thickness was more preserved in ZVAD (to 72%) than in DEVD (to 56%).

CONCLUSIONS. In the authors' model of retinal degeneration, photoreceptor cells die through a caspase-dependent mechanism. However, the molecular events involved during and after light exposure seemed to implicate different proteases. (Invest Ophthalmol Vis Sci. 2007;48:2753-2759) DOI:10.1167/iovs.06-1258

A apoptosis is a common final pathway in retinal degeneration in humans and in animal models. Although biochemical aspects are similar in all apoptotic cells (nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, membrane blebbing, and internucleosomal DNA fragmentation), different molecular events lead the cell to death. Caspases (cysteine aspartate–specific proteases) are the first proteases identified that coordinate and execute the apoptotic process in many apoptotic systems. These proteases are synthesized as inactive zymogens and are activated by proteolytic cleavage to form a tetramer. Two major pathways, leading to the degradation of key survival proteins, have been described for caspase activation: the extrinsic pathway initiated by ligand binding to a death receptor and the intrinsic pathway involving the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. More recently, calpains (calcium-dependent cysteine proteases) have been shown to be involved in the apoptotic process and to be activated by the increase of cellular calcium concentration during apoptosis. The two major isoforms, calpain 1 or μ-calpain and calpain 2 or m-calpain, are synthesized as inactive proenzyme and are activated by micromolar and millimolar Ca++ concentration, respectively. Activated calpains can cleave a variety of key survival proteins and can activate or inactivate caspases. In addition, the leukocyte elastase inhibitor (LEI), a serine protease inhibitor or serpin, was identified in apoptosis during lens cell differentiation and in retinal development. It induces nuclear fragmentation after its transformation into an endonuclease DNase II. LEI activation depends on pH conditions or activation of serine proteases.

In retinal degeneration, caspases, calpains, and (LEI)-DNase II have been shown to be activated during photoreceptor cell death in several models. Caspase-1 and -2 were detected in the retinal outer nuclear layer during degeneration in Royal College of Surgeons rats, and inhibition of caspase-3 preserved the retina of S334ter rats and tubby mice. In rd mice, calpain mediates apoptosis through caspase-3 activation, and caspase-3 inhibitors preserve the retina. However, no activation of caspase-2, -3, -7, -8, or -9 could be observed by Doonan et al. In light-induced retinal degeneration, caspase-1 was activated in Balb/c mice retinas after exposure to white fluorescent light at 1300 lux and was also overexpressed during exposure to green light at 3500 to 3500 lux. A white fluorescent light at 60 lux or 5000 lux had no effect on caspase activity, but exposure at 5000 lux induced hyperactivation of calpains, suggesting that light-induced retinal damage was caspase independent and calpain dependent. In albino Sprague-Dawley rats, caspase-3 was overexpressed and activated 8 hours after blue light exposure at approximately 60 lux, but no change in caspase-3 expression was observed during prolonged white light exposure at 1700 lux. Endonuclease activation was observed in Balb/c mice exposed for 5 days to white fluorescent light at 900 lux. Consequentially, in light-induced retinal degeneration, the molecular events leading to cell death appear to be dependent on the strain and the light intensity used.

In this study, our model of light-induced retinal degeneration consisted of exposing albino rats to white fluorescent light at 3400 lux for 24 hours. To observe the photoreceptor cell death process (caspase dependent or caspase independent) in our model, we injected intravitreally an irreversible, large, broad-spectrum caspase inhibitor. The results suggest that photoreceptor cells died through a caspase-dependent mechanism. We then tested more specific caspase inhibitors to identify which caspases were involved.

METHODS

Animals

Albino Wistar rats were raised in dim-cyclic light (12 hours dark/12 hours light; less than 10 lux). They were fed ad libitum and had free

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Submitted for publication October 19, 2006; revised February 9, 2007; accepted April 16, 2007.

Disclosure: O. Perche; None; M. Doly; None; I. Ranchon-Cole; None.

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access to water. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and with the permission of the regional animal care committee.

**Damaging Light**

Rats were dark adapted 18 to 19 hours before exposure for 24 hours in the light box. The light box had white reflecting surfaces and was equipped with three fluorescent tubes (cool white fluorescent light, 18 W) fixed on the superior side. The illuminance at the position of the rat’s eyes was measured at 3400 lux (Photomètre S350; United Detection Technologies, Hawthorne, CA). During exposure, the rats had free access to food and water. After exposure to the damaging light, they were placed in darkness for 1 day and then returned to the dim cyclic light conditions.

**Treatments**

Rats were anesthetized by a mixture of ketamine (Imalgén; Merial, Rhône Mérieux, France) and xylazine (Sigma Aldrich, St. Quentin Fallavier, France) at 150 mg/kg and 6 mg/kg, respectively. A drop of antiseptic solution (Betadine 4%; Viatris, Merignac, France) was applied on the cornea, and intravitreal injections were performed under a microscope with a 30-gauge needle mounted on a Hamilton syringe (VWR, Strasbourg, France). Rats were injected intravitreally (2 μL) with a solution of PBS, 2% dimethyl sulfoxide (DMSO), caspase inhibitor Z-VAD-FMK (1.06 mM in 2% DMSO), caspase inhibitor YVAD or DEVD (0.16 mM in 2% DMSO), or DEVD-FMK (2 mM in 2% DMSO). The caspase inhibitors were from Calbiochem (Strasbourg, France). In parallel, control rats were punctured intravitreally with a dry needle to investigate any potential effect of the needle puncture alone (Stung group). A drop of antibiotic (Tobrex; Alcon, Rueil-Malmaison, France) was applied on the cornea after injection, and rats were placed in the dark for 18 to 19 hours. Both eyes of each rat received the same treatment.

**Electroretinography**

Electroretinograms (ERGs) were recorded as described previously with 10-μs flashes and through Ag/AgCl electrodes. The b-wave sensitivity curves were fitted with a software program (Microsoft Origin 6.0; Microcal Software, Northampton, MA) to calculate the saturated b-wave amplitude (Bmax).

**Histology**

Eyes were embedded in paraffin, as described previously. Sections measuring 3 μm were cut along the meridian through the optic nerve. Outer nuclear layer (ONL) thickness was measured every 0.36 mm from the optic nerve to the inferior and to the superior ora serrata. Area under the curve (AUC) was integrated with the use of software (Origin 6.0 program; Microcal Software).

**Apoptotic Cell Detection**

Rats were killed and eyes were enucleated, placed in fixative (4% paraformaldehyde in PBS) at 4°C for 4 hours, and embedded in paraffin. Sections of 5 μm were cut along the meridian through the optic nerve. An apoptosis detection kit (Apoptag S7101; Qiogen, Ilkirch, France) was used in accordance with the manufacturer’s instructions. Positive cells were counted under a microscope 1.2 mm from the optic nerve in the superior part of the retina on a 0.2-mm section length.

**Caspase-1 and -3 Colorimetric Activity Assay**

Retinal caspase-1 and caspase-3 activities were measured with the use of specific colorimetric kits (caspase-1 colorimetric kit [AbCys, Paris, France]; caspase-3 colorimetric kit [R&D Systems, Lille, France]) according to the manufacturer’s instructions. Briefly, two retinas of one rat were homogenized in the kit’s lysis buffer. Total protein content was determined by the BCA method (Pierce, France). Proteins (150 μg) were incubated at 37°C for 1.5 hours with caspase-specific substrates (WEHD-pNA for caspase-1 and DEVD-pNA for caspase-3) in the kit’s reaction buffer. The absorbance of each sample was read at 405 nm. Caspase activity level was directly proportional to the color reaction. Results are expressed as fold increase in caspase activity.

**Calpain Activity**

Total retinal calpain (m-calpain and μ-calpain) activity was measured with a fluorometric kit (calpain fluorometric kit; VWR). Briefly, two retinas of one rat were homogenized in the kit’s lysis buffer, and total protein concentration was determined by the BCA method (Pierce, France). Proteins (150 μg) were incubated at 37°C for 1 hour with calpain-specific substrate (Suc-Leu-Leu-Val-Tyr-AMC) in reaction buffer. The fluorometric substrate (AMC) is released on cleavage by calpain and is measured fluorometrically at an excitation wavelength of 360 to 380 nm and an emission wavelength of 440 to 460 nm. Results are expressed as fold increase in calpain activity. We also tested the inhibitory effect of ZVAD-FMK on calpain activity in vitro by adding 2 μL ZVAD-FMK at 100 μM to human calpain 1 provided in the kit.

**Experimental Paradigm**

Control ERGs were recorded on both eyes of each rat. The rats were then injected with PBS, DMSO, YVAD, DEVD, ZVAD, or Stung (dry needle) before they were placed in the dark. An additional control group included uninjected rats. Eighteen hours later, they were exposed to the damaging light. At the end of light exposure, they were placed in the dark for 1 day (D1). After ERGs were recorded on each eye, a group of the rats was killed for apoptotic nuclei detection. Remaining rats were returned to dim cyclic light for 15 days and then were killed for histology. Unexposed animals were processed in parallel. Therefore, we had 12 groups: Exposed-Uninjected, Exposed-PBS, Exposed-Stung, Exposed-DMSO, Exposed-ZVAD, Exposed-YVAD, Exposed-DEVD, Unexposed-Uninjected, Unexposed-DMSO, Unexposed-ZVAD, Unexposed-YVAD, and Unexposed-DEVD.

In another set of experiments, caspase and calpain activities were measured in untreated or treated retinas just before exposure to the damaging light (0 hours [18 hours after treatment]) and 1 day (D1) after exposure to the damaging light.

**Statistical Analysis**

Analysis of variance (ANOVA) was performed on the electroretinographic and morphometric parameters, apoptotic cell number, or caspase and calpain activities. If ANOVA was significant, multiple comparisons were made to determine which pairs of mean values were different. Significant differences between groups were assessed with the post hoc Newman-Keuls test; the significance level was set at P = 0.05. Significant differences between groups are noted by *, †, and ‡. One symbol for P < 0.05, two symbols for P < 0.01, three symbols for P < 0.0001, and four symbols for P < 0.0001.

**RESULTS**

**Electroretinography**

ERGs were recorded before treatment or exposure to damaging light on both eyes of each rat and were averaged to obtain a control b-wave sensitivity curve (Figs. 1A, 1B, black continuous line) and a control Bmax (1287 ± 199 μV, CTRL; Fig. 1C).

The b-wave sensitivity curves of Unexposed-DMSO, -ZVAD, -YVAD, and -DEVD were similar to those of the control (Fig. 1A). Corresponding Bmax values (1233 ± 175 μV; 1345 ± 71 μV; 1464 ± 61 μV; 1428 ± 131 μV, respectively [data not shown]) were not significantly different from those of the control. Therefore, DMSO, ZVAD, YVAD, and DEVD had no toxic effect on retinal function.

In the Exposed-Uninjected group, the damaging light induced a collapse of the sensitivity curve (Fig. 1B) and a significant (P < 0.0001) reduction of Bmax (251 ± 298 μV) com-
ZVAD (Z-VAD-FMK) and Z-DEVD-FMK results show that the caspase inhibitor ZVAD had a protective effect on retinal function against light-induced damage. Significant difference compared with *control (CTRL), †Exposed-Untreated group, and ‡Exposed-DMSO group.

In the Exposed-ZVAD group, the b-wave sensitivity curve was not affected by the damaging light, but still significantly lower \((P < 0.0005)\) than those of the control group (Fig. 1C). These results show that intravitreal dry needle puncture or injection of PBS, DMSO, or YVAD had no protective effect on retinal function. Therefore, dry needle puncture or injection of PBS, DMSO, or YVAD had no protective effect on retinal structure after light damage. In the Exposed-ZVAD group, ONL thickness on the superior side was affected by the damaging light but less so than in the control group (Fig. 2B). A maximal degenerative zone was observed around 1.2 mm from the optic nerve. ONL areas \((42 ± 4 \mu m^2, 28 ± 6 \mu m^2, 40 ± 1 \mu m^2,\) and \(39 ± 15 \mu m^2,\) respectively) were not significantly different from those of the Exposed-Un treated group. Therefore, dry needle puncture or injection of PBS, DMSO, or YVAD had no protective effect on retinal structure after light damage.

In the Exposed-ZVAD group, ONL thickness on the superior side of the retina (Fig. 2B) was less affected by damaging light than it was in the Exposed-DMSO group. ONL area \((97 ± 15 \mu m^2)\) was significantly \((P < 0.0001)\) greater than in the Exposed-DMSO group but still significantly \((P < 0.0001)\) lower than in the control group (Fig. 2C).

In the Exposed-DEV group, ONL thickness on the superior side of the retina was affected by the damaging light but less so than in the Exposed-DMSO group (Fig. 2B). ONL area of Exposed-DEV group \((75 ± 15 \mu m^2)\) was significantly lower than in the control \((P < 0.0001)\) and Exposed-ZVAD \((P < 0.004)\) but was significantly \((P < 0.0002)\) higher than in the Exposed-DEV group (Fig. 2C).

In the Exposed-DEV group, ONL thickness on the superior side of the retina was affected by the damaging light but less so than in the Exposed-DMSO group (Fig. 2B). ONL area of Exposed-DEV group \((75 ± 15 \mu m^2)\) was significantly lower than in the control \((P < 0.0001)\) and Exposed-ZVAD \((P < 0.004)\) but was significantly \((P < 0.0002)\) higher than in the Exposed-DMSO group (Fig. 2C).

Histology
ONL thickness in the inferior and superior parts of the retinas of Unexposed-DMSO, -ZVAD, -YVAD, and -DEVD groups was not significantly different from that of the Unexposed Untreated group, indicating that these treatments had no significant effect on retinal structure (Fig. 2A).

On the inferior side of the retina, ONL thickness of Exposed groups appeared thinner (Fig. 2B) than that of the Unexposed Untreated group, but there was no significant difference between the ONL area of any exposed group and that of the Unexposed Untreated group (Fig. 2C).

On the superior side, ONL thickness of the Exposed Untreated group was highly affected by the damaging light. AUC \((29 ± 8 \mu m^2)\) was significantly \((P < 0.0001)\) lower than that of the control \((135 ± 4 \mu m^2);\) Fig. 2C). A maximal degenerative zone was observed around 1.2 mm from the optic nerve. Exposed-Stung, -PBS, -DMSO, and -YVAD groups had similar ONL thicknesses. ONL areas \((42 ± 4 \mu m^2, 28 ± 6 \mu m^2, 40 ± 1 \mu m^2,\) and \(39 ± 15 \mu m^2,\) respectively) were not significantly different from those of the Exposed-Untreated group. Therefore, dry needle puncture or injection of PBS, DMSO, or YVAD had no protective effect on retinal structure after light damage.

In the Exposed-ZVAD group, ONL thickness on the superior side of the retina (Fig. 2B) was less affected by damaging light than it was in the Exposed-DMSO group. ONL area \((97 ± 15 \mu m^2)\) was significantly \((P < 0.0001)\) greater than in the Exposed-DMSO group but still significantly \((P < 0.0001)\) lower than in the control group (Fig. 2C).

In the Exposed-DEV group, ONL thickness on the superior side of the retina was affected by the damaging light but less so than in the Exposed-DMSO group (Fig. 2B). ONL area of Exposed-DEV group \((75 ± 15 \mu m^2)\) was significantly lower than in the control \((P < 0.0001)\) and Exposed-ZVAD \((P < 0.004)\) but was significantly \((P < 0.0002)\) higher than in the Exposed-DMSO group (Fig. 2C).

Therefore, DEV had a protective effect on retinal structure, though it was less than that of ZVAD. YVAD had no protective effect on retinal structure. Apoptotic Cell Detection Apoptotic nuclei were counted in the ONL at 1.2 mm from the optic nerve in the superior part of the retina, which was within the maximal degenerative area (Fig. 2B). No apoptotic nuclei were detected in Unexposed retinas, untreated retinas (0 hours; Fig. 3), or retinas injected with DMSO, -YVAD, -ZVAD, or -DEVD (data not shown).
After light exposure, Untreated retinas (24 hours; Fig. 3) contained 34 apoptotic nuclei. This number continued to increase after the light was turned off and doubled (54 apoptotic nuclei) by D1.

At D1, the number of apoptotic nuclei in Exposed-Stung (43 apoptotic nuclei), Exposed-PBS (57 apoptotic nuclei), and Exposed-DMSO (44 apoptotic nuclei) groups were not significantly different from that in the Exposed-Untreated group (Fig. 3). Therefore, dry needle puncture or injection of PBS or DMSO had no effect on the number of apoptotic nuclei.

Compared with the Exposed-DMSO group, the Exposed-ZVAD and Exposed-DEVD groups had a significantly (P < 0.007) lower number of apoptotic nuclei (20 and 10 apoptotic nuclei, respectively), and the Exposed-YVAD group had as many apoptotic nuclei (58 apoptotic nuclei).

Therefore, the caspase inhibitors ZVAD and DEVD, but not YVAD, reduced apoptotic nuclei in the ONL of rats exposed to damaging light.

### Caspase Activities

Immediately before exposure to damaging light (Fig. 4A), no significant variation of caspase-1 and caspase-3 activities was observed in DMSO-treated retinas compared with the Untreated retinas (100%). ZVAD significantly reduced caspase-1 (P < 0.004) and caspase-3 activities (P < 0.039) to 63% and 61%, respectively. YVAD significantly reduced (P < 0.004) caspase-1 activity to 51% but had no effect on caspase-3 activity (98%). DEVD significantly reduced (P < 0.032) caspase-3 activity to 45% but had no effect on caspase-1 activity (94%). These results show that the caspase activities were significantly reduced by the caspase inhibitors, with ZVAD and DEVD being more effective than YVAD.

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**FIGURE 2.** ONL at D15. ONL thickness was measured every 0.36 mm from the optic nerve (ON) to the inferior and superior ora serrata. Rats were untreated, punctured intravitreally (Stung), or injected with PBS, DMSO, ZVAD, YVAD, or DEVD. They were (A) unexposed (n = 4 per groups) or (B) exposed (n = 5 per groups) to the damaging light. The black continuous line represents the ONL of the Unexposed-Utreated group. (C) Areas under the ONL curve in the inferior and superior parts of the retina of the Unexposed-Utreated and Exposed groups. Results are presented as mean ± SD (μm²). Injection of Z-VAD-FMK or Z-DEVD-FMK had a protective effect on the retinal structure against light-induced damage. Significant difference compared with the †Unexposed-Utreated group, ‡Exposed-Utreated group, †Exposed-DMSO group.

**FIGURE 3.** Apoptotic cell number. Apoptotic cells were counted in the ONL at 1.2 mm from the optic nerve on the superior side of the retina on 0.2-mm section length: before (0 hours) and at the end (24 hours) of exposure to the damaging light in Untreated retinas and 1 day (D1) after the cessation of light exposure in Untreated, Punctured (Stung), PBS, DMSO, ZVAD, YVAD, and DEVD retinas. Results are presented as the mean ± SD. These results show that the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK significantly reduced apoptotic photoreceptors induced by exposure to the damaging light. Significant difference compared with the †Exposed-Utreated group at D1 and the ‡Exposed-DMSO group at D1.
vehicle (DMSO) had no significant effect on caspase-1 and -3 activities, YVAD and DEVD specifically inhibited their respective targets, and ZVAD, YVAD, and DEVD were efficient at inhibiting their targets when the damaging light was turned on.

At D1 (Fig. 4B), Exposed-Untreated or Exposed-DMSO retinas showed significant activation of caspase-1 to 165% ± 33% (P < 0.03) and 150% ± 9% (P < 0.03), respectively, and activation of caspase-3 activity to 125% ± 12% (P < 0.007) and 129% ± 29% (P < 0.004), respectively, compared with the Unexposed-Untreated group (100%, 0 hours). Exposed-ZVAD and Exposed-YVAD retinas showed caspase-1 activities of 105% ± 18% and 66% ± 33%, respectively. These values were significantly (P < 0.04 and P < 0.004, respectively) lower than in the Exposed-DMSO group. Caspase-3 activities in the Exposed-ZVAD and Exposed-DEVD groups were reduced, respectively, to 102% ± 11% and 88% ± 11%, levels that were significantly (P < 0.04 and P < 0.015, respectively) lower than in the Exposed-DMSO group. YVAD had no effect on caspase-3 activity (160% ± 19%), and DEVD had no effect on caspase-1 activity (121% ± 15%). These results suggest that these three inhibitors were still effective on their respective targets up to 1 day after the damaging light was turned off. ZVAD, YVAD, and DEVD inhibitors were effective at the beginning (0 hours) of exposure to damaging light and up to 1 day (D1) after the light was turned off.

**Calpain Activities**

In vitro (Fig. 5A), ZVAD at 100 μM significantly decreased (P < 0.002) human calpain activity by 50%. In vivo (Fig. 5B) at 0 hours, no significant variation of calpain activities was observed in retinas treated with DMSO, ZVAD, or DEVD compared with the Untreated group; YVAD induced a significant (P < 0.04) activation of calpains to 336% ± 93%. At D1, calpains were upregulated in Exposed-Untreated (217% ± 125%), Exposed-DMSO (243% ± 16%), Exposed-ZVAD (179% ± 3%), Exposed-YVAD (333% ± 94%), and Exposed-DEVD (241% ± 19%) retinas, and no significant differences were observed between these exposed groups. Therefore, though ZVAD inhibited human calpain activity in vitro, it did not affect retinal calpain activity in vivo. DEVD had no effect on retinal calpain activity, but YVAD activated retinal calpains.

**Discussion**

In this report, we used a model of light-induced retinal damage to study apoptosis in photoreceptor cells. This model consisted of exposing Wistar albino rats for 24 hours to white fluorescent light at 3400 lux. This light condition induced a significant, but not complete, loss of retinal function and structure. Because the severity and the nature of the light insult and the animal strain used determine the molecular events of apoptosis, we first assessed whether a caspase-dependent or caspase-independent mechanism was involved in our model. Based on the hypothesis that if a molecule is implicated in the apoptotic process induced by light inhibiting this molecule should protect against retinal light damage, we injected specific inhibitors intravitreally.

Stress before exposure to damaging light has been shown to reduce retinal damage36; therefore, we tested the effect of intravitreal dry needle puncture (Stung group) and injection of a buffered solution (PBS) or the vehicle (2% DMSO). One day after exposure to the damaging light, these treatments had significant and similarly protective effects on retinal function, indicating that a protective effect was induced by a dry needle puncture and that PBS and DMSO had no further effect. This protection was not observed in the histologic analysis 15 days after light exposure, suggesting that it was a transitory effect or that it affected only retinal function.

The first inhibitor tested was Z-VAD-FMK, which is commercially sold as an irreversible and cell-permeable, broad-spectrum caspase inhibitor. We showed that Z-VAD-FMK injected intravitreally inhibited caspase-1 and caspase-3 (two of its targets) from the beginning and up to 1 day after light exposure, protected retinal function and structure against light-induced damage, and reduced apoptotic nuclei in the ONL. In a recent report, Z-VAD-FMK was demonstrated to
were injected intravitreally with 2 day after exposure to the damaging light (D1). Rats were Untreated or observed at 16 hours after blue light exposure, 35 and that 1 day after exposure to damaging light, as had already been our model of light-induced retinal damage, photoreceptors rat retinal calpain activity in vivo. These data showed that in inhibit human calpain 1 in vitro, it did not have any effect on Z-VAD-FMK on calpain activity. Although Z-VAD-FMK could be estimated to have in our experiments, we tested the effect of this concentration was similar to the final concentration we indicated that these two inhibitors were still inhibiting caspase-3. Consequently, between D1 and D15, inhibiting caspases other than caspase-3 and -7 offered better retinal protection. Once the light was turned off, these other caspases were more important than caspase-3 and -7. These data suggest that two apoptotic pathways are involved in light-induced retinal degeneration: a caspase-3- or -7-dependent pathway during exposure to the damaging light and a second pathway involving other caspases once the light was turned off. Caspase-1 seemed to be a good candidate involved in the second apoptotic mechanism because we observed an upregulation of caspase-1 activity after exposure to damaging light that was supported by upregulation of the caspase-1 mRNA level observed by Grimm et al.4 However, inhibiting caspase-1 and -4 by Z-YVAD-FMK had no protective effect against light-induced retinal degeneration, though caspase-1 was still inhibited to 66% the day after light exposure. Therefore, it is possible that caspase-1 and -4 are not main actors in the apoptotic pathway after the light was turned off or that this second apoptotic process was irreversible once it was initiated. Calpain activation after light exposure occurred in accordance with the description by Donovan et al.34 and suggested a role for these proteases in the apoptotic process once the light was turned off. However, it is interesting to note that though intravitreal injection of Z-YVAD-FMK induced calpain activation, it was not toxic for the retina. Indeed, unexposed retinas treated with Z-YVAD-FMK were not different from unexposed ones, and exposed treated retinas were not more sensitive to the damaging light than untreated ones. Therefore, activation of calpain was not sufficient to induce apoptosis in photoreceptor cells. Nevertheless, to test the potential role of calpains in the apoptotic process during or after light exposure, future experiments are planned on calpain inhibitors.

In conclusion, in our model of light-induced retinal degeneration, photoreceptors died through a caspase-dependent apoptotic mechanism. Our results also suggest that the molecular events during and after exposure to damaging light involve different pathways. During exposure to damaging light, caspase-3 and -7 played major roles. After the light was turned off, they were less important. Further experiments are planned to determine caspase-3 and -7 activation during light exposure and to identify which additional caspases or proteases are involved during and after exposure to damaging light.

**Acknowledgments**

The authors thank Christine Cercy and Thomas Boyer for their excellent technical assistance and Michael Elliot (Dean A. McGee Eye Institute, Oklahoma City, OK) for editing the English.

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