FAS-Mediated Apoptosis and Its Relation to Intrinsic Pathway Activation in an Experimental Model of Retinal Detachment

David N. Zacks, Qiong-Duan Zheng, Ying Han, Rita Bakbru, and Joan W. Miller

PURPOSE. To determine whether the FAS-mediated apoptosis pathway becomes activated in the retina after retinal detachment and to investigate the temporal relationship between the activation of the FAS-pathway and the intrinsic apoptosis pathway involving caspase-9 and cytochrome c.

METHODS. Experimental retinal detachments were created in Brown-Norway rats by injecting 10% hyaluronic acid into the subretinal space. Retinal tissue was harvested at 2, 4, 8, 24, 72, and 168 hours after creation of the detachment. Immunoprecipitation was performed to assess for FAS-receptor/FAS-ligand complex formation, and activation of caspase-8 and BID (a member of the Bcl-2 family of proteins) was assessed by Western blot analysis. A caspase-9 activity assay and immunoprecipitation of the caspase-9/cytochrome c complex were performed at these same time points. Specific pathway inhibition was performed with the caspase-9 inhibitor zLEHD.fmk or neutralizing antibodies against either the FAS-receptor or FAS-ligand. Transcription levels of FAS and intrinsic pathway intermediates were assessed as a function of time after retinal detachment by using quantitative real-time polymerase chain reaction.

RESULTS. Retinal detachment resulted in the time-dependent formation of the FAS-receptor/FAS-ligand complex that preceded the peak of caspase-9 activity and caspase-9/cytochrome c complex formation. Cleavage of caspase-8 and truncation of BID were also observed. Injection of zLEHD.fmk into the subretinal space of a detached retina resulted in decreased caspase-9 activity, as did injection of anti-FAS-receptor antibody into either the subretinal space or the vitreous. Retinal detachment resulted in the transcriptional upregulation of the FAS-receptor, FAS-ligand, caspase-8 and BID, but not caspase-9 and cytochrome c.

CONCLUSIONS. The FAS-mediated apoptosis pathway becomes activated and transcriptionally upregulated after retinal detachment. The peak of FAS activity precedes that of the intrinsic pathway, and inhibition of FAS activation can decrease caspase-9 activity. (Invest Ophthalmol Vis Sci. 2004;45:4563–4569) DOI:10.1167/iovs.04-0598

A common feature of many retinal diseases is the physical separation of the neurosensory retina from its normal underlying structures. The detachment of the retina from its source of metabolic support results in apoptotic photoreceptor cell death.1–3 However, the molecular mechanisms of activation, transduction, and execution of the apoptotic cascade in this setting are poorly understood.

In general, apoptosis is activated by one of two main pathways: the receptor-mediated pathway and the intrinsic (mitochondrial) pathway (Fig. 1). Components of the FAS/FAS-ligand system represent the prototypical receptor-mediated apoptosis pathway. Both FAS and FAS-ligand are surface membrane proteins that belong to the tumor necrosis factor-a superfamily of proteins.4 As shown in Figure 1, there is a sequential activation of various intermediary proteins, including caspase-8, BID (a member of the Bcl-2 family of proteins), and caspase-3.

Apoptosis can also become activated through an intrinsic pathway. This pathway does not involve a surface receptor, but rather is formed by the modification of intracellular pools of proteins. Such modulators include BID, as well as other members of the Bcl-2 family. Environmental or intracellular stressors result in post-translation modification of these proteins, which then exert their effect on the mitochondria to release cytochrome c. The released cytochrome c then binds with apoptosis-activating factor-1 and caspase-9 to form a complex known as the apoptosome, which in turn activates more downstream apoptotic reactions. We have previously shown the initiation of photoreceptor cell death by the intrinsic apoptosis pathway.1 Retinal detachment activates the apoptosis initiator caspase-9 and the downstream apoptosis intermediates caspase-3 and -7 and poly-ADP ribose-polymerase (PARP).

In this study, we sought to determine whether only the intrinsic pathway becomes activated during detachment or whether the receptor-mediated pathway also contributes to photoreceptor death. Using our experimental model of retinal detachment, we demonstrated the activation of the FAS/FAS-ligand pathway as a function of time after the creation of the retinal detachment. FAS pathway activation preceded that of the intrinsic pathway, and its inhibition decreased the extent of intrinsic pathway activity. In addition, FAS pathway intermediates were transcriptionally upregulated, whereas intrinsic pathway intermediates were not.

METHODS

Animal Model of Retinal Detachments

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the University Committee on Use and Care of Animals of the University of Michigan. Retinal detachments were created in adult male Brown-Norway rats (300–400 g), as previously described. Briefly, rats were anesthetized with a 50:50 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL), and pupils were dilated with topical phenylephrine (2.5%) and tropicamide (1%). A
Intrinsic Pathway

FAS-mediated Pathway

![Diagram of apoptosis pathways](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933228/)
Caspase-9 Activity Assay and Caspase-9 Inhibition

Caspase-9 activity was measured with a colorimetric tetrapeptide LEHD-pNA cleavage assay kit, per the manufacturer’s instructions (BioVision, Mountain View, CA). In this assay, 100 μg total retinal protein from either attached or detached retinas was incubated with substrate (LEHD-pNA, 200 μM final concentration) at 37°C for 60 minutes. Absorbance was measured at 405 nm in a microplate reader (SpectraMAX 190; Molecular Devices, Sunnyvale, CA). As a negative control, retinal protein was incubated with assay buffer without the tetrapeptide. A second negative control was used in which assay buffer alone was incubated with the tetrapeptide. As a positive control, purified caspase-9 was incubated with the tetrapeptide alone. At each time point, the caspase-9 activity in the detached retina was normalized against the caspase-9 activity in attached retina at the same time point.

The ability of different compounds to inhibit caspase-9 activity in vivo was also measured. The retina was detached with sodium hyaluronate according to the protocol described earlier, followed immediately by the injection of 5 μL of inhibitor. First, the direct inhibitor of caspase-9 –zLEHD.fm Kong was tested. Five microliters of the zLEHD.fm Kong (2-mM solution in dimethyl sulfoxide [DMSO]; BioVision) was injected into the subretinal space of the detached retina with a syringe (Hamilton Syringe; Hamilton Corp., Reno, NV). Five microliters DMSO was injected into the subretinal space of the detached retina as a control for the solvent in which the zLEHD.fm Kong was dissolved. Second, the neutralizing antibody against the FAS-receptor (5 μg in phosphate-buffered saline; clone ZB4; Upstate Biotechnology, Lake Placid, NY) or FAS-ligand (5 μg in phosphate-buffered saline; clone NOK-1; BD Biosciences) was injected into either the subretinal space or the vitreous cavity. In all inhibition experiments, the retinas were harvested at 24 hours after detachment, as this was the peak of caspase-9 activity after detachment (described in the Results section). The caspase-9 activity in the detached retina was normalized against the caspase-9 activity in attached retina at the same time point.

Quantitative Real-Time Polymerase Chain Reaction

Retinal samples were harvested as for the caspase-9 assay. Total RNA was isolated (TRIZol reagent; Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed with 3 μg of total RNA, 200 U reverse transcriptase (Superscript III; Invitrogen), and oligo(dT)20 in a total reaction volume of 20 μL at 42°C for 2 hours. The reaction was terminated by heating. RNaseH was used to remove the template RNA. The reverse transcription products were diluted 1:4 in dH2O.

Real-time PCR was performed by using 1 μL of cDNA, 100 nM of each primer, and PCR master mix (IQ SYBR Green Super Mix; Bio-Rad Laboratories) to make a final volume of 25 μL. Reaction mixtures were incubated in a thermocycler (iCycler; Bio-Rad Laboratories) in the following temperature/time sequences: 95°C for 2 hours; 45 cycles of 95°C for 30 minutes, 57°C for 30 minutes, and 72°C 30 minutes; and a final cycle of 58°C for 10 minutes. Primers specific for the rat hypoxanthine phosphoribosyl transferase (rHPRT) gene were used as an internal control to allow for normalization and direct comparison between multiple samples. All primers (Table 1) were designed to span intron/exon boundaries, to distinguish between transcripts and any contaminating genomic DNA. Samples lacking reverse transcriptase or cDNA template served as the negative control. For each primer set, quantitative (q)RT-PCR was performed on samples derived from three different animals and repeated three times per animal. Quality analysis of the PCR products was performed by running an aliquot of each sample on a 2% Tris-acetate-EDTA (TAE) gel. In addition, melting curves for each PCR reaction were analyzed to ensure that dimerization of PCR primers did not generate a false-positive response. Each reaction was performed three times on three independent samples derived from separate animals. The average % fold change in expression relative to the rHPRT transcript level was calculated and significance analyzed with Student’s t-test.

FAS-Mediated Apoptosis in Retinal Detachment

**TABLE 1.** Primer Sequences Used for Quantitative Real-Time PCR Reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Caspase 3</td>
<td>F—GACAAGACGAAACCTCGGT</td>
</tr>
<tr>
<td></td>
<td>R—GACGGCTATTTCTAGGGCCCA</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>F—TAAAAAGGACCAGCCACAGGAA</td>
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<td></td>
<td>R—ATCCAGAAGCGCTAGGTG</td>
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<tr>
<td>BID</td>
<td>F—TCTTACGTTAGGAATTGTT</td>
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<tr>
<td></td>
<td>R—AGCTTCACAATTCTTCGCGT</td>
</tr>
<tr>
<td>FAS receptor</td>
<td>F—TGAAATGCGAAAGGTGGC</td>
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<tr>
<td></td>
<td>R—CTTGGACGCTTGAGCTGTA</td>
</tr>
<tr>
<td>FAS ligand</td>
<td>F—TCTTCCCTGAGACTGATCGA</td>
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<tr>
<td>Caspase-9</td>
<td>F—CTCAGGCGAGGGTCTTCA</td>
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<tr>
<td></td>
<td>R—ATTCCTCATCAAGAGCGGTGAC</td>
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<tr>
<td>Cytochrome c</td>
<td>F—GAGGCCAAGATGACCTGCGG</td>
</tr>
<tr>
<td></td>
<td>R—GGCTGCCCATTGGCTTCTG</td>
</tr>
</tbody>
</table>

All sequences are listed from the 5′-3′ ends. F, forward primer; R, reverse primer.

**Immunohistochemistry**

Immunohistochemistry was performed on sections obtained from paraffin-embedded retinas. Epitope unmasking was performed by incubating the sections in boiling 10 mM citrate buffer (pH 6.0) for 10 minutes. Slides were then washed with PBS (PBS with 0.3% Tween-20), incubated for 10 minutes in 3% H2O2 at room temperature, and washed again in PBS. The incubation with primary and secondary antibodies was performed with a tyramide signal amplification (TSA)-fluorescence detection kit (Perkin-Elmer, Boston, MA), per the manufacturer’s instructions.

**RESULTS**

Our first objective was to determine whether the FAS pathway would be activated. Figure 2 shows the immunoprecipitation of the FAS-receptor/FAS-ligand complex. Activation of caspase-8 and BID was demonstrated on Western blot analysis by formation of their cleaved forms (Fig. 2), as would be expected by the formation of functional FAS-receptor/FAS-ligand complex. The peak of FAS-receptor/FAS-ligand complex formation occurred at 8 hours after creation of the detachment (Fig. 3). This preceded the peak of caspase-9 activity, which occurred at 24 hours after creation of the detachment (Fig. 4), corresponding to the peak of caspase-9/cytochrome c complex formation (Fig. 4). Normalizing the densitometry readings for any decrease in outer nuclear layer thickness that might result from the retinal detachment did not significantly alter the relative values shown.

Direct inhibition of the intrinsic pathway was possible by the use of a caspase-9 inhibitor or, indirectly, by inhibiting FAS complex formation. Caspase-9 activity levels were used as a measurement of the intrinsic pathway activation. The activity levels were tested 24 hours after the retinal detachment was created and inhibitor applied, as this was the time of peak caspase-9 activity (Fig. 4). Injection of the caspase-9 inhibitor zLEHD.fm Kong into the subretinal space of a detached retina significantly reduced caspase-9 activity to approximately 50% of the control level (P = 0.05; Fig. 5). Injection of neutralizing antibodies against either the FAS-receptor or the FAS-ligand into the subretinal space of the detached retina also resulted in the reduction of caspase-9 activity by approximately 50% (P = 0.05; Fig. 6). The effect of intravitreal injection of these antibodies was less than that of the subretinal injection and did not reach statistical significance. Intravitreally injected anti-FAS-receptor antibody reduced caspase-9 activity by only approximately 30% (P = 0.13). Intravitreal injection of anti-FAS-ligand...
antibody resulted in only a 10% reduction of caspase-9 activity ($P = 0.54$).

We observed a time-dependent increase in the levels of FAS-receptor, FAS-ligand, caspase-8, BID, and caspase-3 on Western blot analysis of protein extracts from detached retinas (data not shown). Quantitative RT-PCR was used to determine whether increased transcription causes the detachment-induced changes in levels of apoptosis intermediates (Table 1, Fig. 7). Members of the intrinsic pathway and receptor-mediated pathways were assessed for upregulation of transcripts. All expression levels were normalized to the expression of HPRT within the same group (detached or attached), to minimize any artificial elevation in intergroup comparisons that might result from a decreased photoreceptor cell count in the detached retinas. A change of onefold represents no change above baseline expression. The transcript levels for FAS-receptor, FAS-ligand, caspase-8, and caspase-3 were increased at both 3 and 7 days after detachment ($P = 0.05$). BID transcript level was also elevated, but not until 7 days after the retina was detached ($P = 0.05$). This is in contrast to the intrinsic pathway intermediates cytochrome $c$ and caspase-9, which had no significant changes in their expression levels. Despite their early activation in retinal detachment, increased transcription of this pathway’s components did not occur. This is consistent with the Western blot results, which did not reveal any time-dependent increase in the level of these two proteins. Immunostaining of the detached retina for caspase-3 shows that the increased transcription occurs primarily at the level of the outer nuclear layer (Fig. 8).

**DISCUSSION**

Retinal detachment results in apoptotic photoreceptor cell death.1–3 We have previously shown the activation of components of the intrinsic apoptosis pathway, particularly caspase-9.1 In this study, we provided the first demonstration that retinal detachment upregulates and activates the FAS/FAS-ligand pathway. This upregulation occurs at the transcription level, as demonstrated by the increased levels of messenger RNA. These components are not just present at increased levels of the pro form, but become activated by the detach-
ment, as evidenced by their cleavage into enzymatically active states.

Our data showing that FAS activation precedes that of the intrinsic pathway, when taken in conjunction with the ability to decrease the latter’s activity by inhibition of the former, suggest a direct linkage of activation between the two. This linkage may be provided by the proapoptosis protein BID (Fig. 1). This protein is known to be activated by the FAS pathway through the activation of caspase-8. BID, through the activation of BAX—another member of the Bcl-2 protein family, can activate the intrinsic pathway by stimulating the release of cytochrome c from the mitochondria. Reducing FAS activation with a neutralizing antibody can thus result in decreased activation of the intrinsic pathway. This finding is consistent with the results of Yang et al.,7 who found decreased photoreceptor cell loss in a similar experimental model of retinal detachment in a Bax-deficient mouse.

**Figure 4.** Kinetics of intrinsic pathway activation as measured by caspase-9 activity levels (top) and caspase-9/cytochrome c complex formation (bottom). For both panels, the units on the ordinate correspond to the normalized densitometry readings from either the activity assay or the immunoprecipitation experiments. Inset: actual immunoprecipitation band for the complex in retinas detached for 24 hours versus control (attached) retinas. Loading across both lanes was equal.

**Figure 5.** Inhibition of caspase-9 activity after 24 hours of detachment, as measured in vitro, by the injection of the caspase-9 inhibitor zLEHD.fmk at the time of detachment. The difference between the two groups was significant at \( P = 0.05 \).

**Figure 6.** Inhibition of caspase-9 activity after 24 hours of detachment, as measured in vitro, by the injection of anti-FAS-receptor (anti-FAS) or anti-FAS-ligand (anti-FASL) neutralizing antibodies at the time of detachment. Subretinal injection of either antibody significantly reduced caspase-9 activity \( (P = 0.05) \). Intravitreal injection was less effective in reducing caspase-9 activity and the difference did not reach statistical significance \( (P = 0.13 \) for the anti-FAS-receptor antibody, and \( P = 0.54 \) for the anti-FAS-ligand antibody).
It is important to note that the inhibition of caspase-9 activity was not complete, either with the direct inhibitor of caspase-9 or with the use of the neutralizing antibody. This could represent a dose effect, or the inability of these agents to access the target cells sufficiently. In addition, the intrinsic pathway may be activated by FAS-independent mechanisms, not susceptible to inhibition by FAS-neutralizing antibodies. Our data do not demonstrate increased survival of photoreceptor cells with these inhibitors, but rather the modulation of the intrinsic pathway activity. Further work is necessary to demonstrate the utility of this strategy in functional preservation of the retina during retinal detachment.

Upregulation of the FAS/FAS-ligand pathway has been well documented in experimental models of brain ischemia. The intermediates of this pathway normally exist at very low levels, and the onset of expression occurs only after the injury. Morphologically, the expression occurs most predominantly in the penumbral region of the ischemic injury, suggesting that receptor-mediated apoptosis plays a role in delayed cell death. In contrast, early or immediate cell death appears to be mediated by the mitochondrial pathway. Cells undergoing early death use pre-existing intermediates, rather than rely on de novo synthesis. Cells surviving this early death may even decrease levels of intrinsic intermediates such as caspase-9.

In our previous description of this experimental model of retinal detachment we described the initial and immediate death of a subpopulation of cells, followed by a slow attrition of photoreceptors when the detachment persisted for an extended period. The data presented herein suggest that the population of cells that do not undergo immediate cell death upregulate the components of the receptor-mediated apoptosis pathway. It is not clear why this upregulation occurs, but it may provide another mechanism for control and regulation of the slow and cumulative loss of photoreceptor cells that occurs in chronic retinal detachments. This delayed apoptosis may occur independently of the intrinsic pathway, which has a peak activity at 24 hours after detachment and is not transcriptionally upregulated after the detachment.

Hisatomi et al. were unable to show inhibition of early cell death in FAS-deficient mice. They showed that caspase-independent apoptosis via the mitochondrial release of apoptosis-initiating factor (AIF) plays an important role in detachment-induced photoreceptor death. AIF is a known mediator of cell death in a variety of systems that exerts its effect by relocating from the mitochondria to the nucleus and promoting DNA degradation. Using a model of retinal detachment similar to the one described herein, they were able to show AIF relocation from the mitochondria of photoreceptors to their nuclei. This relocation occurred early after retinal detachment and temporally coincided with the amount of TUNEL-positive staining in the outer nuclear layer. It is not known what stimulates the release of AIF from the mitochondria nor what its relationship is to the FAS pathway in this model of experimental retinal detachment. Perhaps the AIF-mediated apoptosis observed provides an alternate route for cell death in the absence of FAS activation.

FAS-mediated apoptosis has recently been suggested to play a role in photoreceptor death during macular degeneration. In addition, macular degeneration results in a pattern of outer retinal reorganization and degeneration similar to that which occurs after retinal detachment. The experimental model of retinal detachment may therefore be a suitable model for dissecting the molecular pathways resulting in the photoreceptor loss common to both diseases. Preventing the upregulation or directly inhibiting the FAS/FAS-ligand pathway may provide a

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933228/)

**Figure 7.** Quantitative real-time PCR results for various apoptosis pathway intermediates. A change of onefold represents no difference in expression between detached and attached retina. Each data point represents the average of three individual assays on three separate samples. FAS pathway intermediates: caspase-3, caspase-8, BID, FAS-R (FAS-receptor), and FAS-L (FAS-ligand). Intrinsic pathway components: Caspase-9 and cytochrome c. Error bars, SEM. *Statistical significance in expression over the control rHPRT ($P = 0.05$; Student’s t-test).

![Figure 8](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933228/)

**Figure 8.** Caspase-3 immunostaining of attached retina (left) and a retina detached for 7 days (right). There was a marked increase in caspase-3 staining in the outer nuclear layer of the detached retina (∗).
therapeutic target for preventing photoreceptor cell loss and its associated decrease in vision.

References