Inhibition of Retinal Detachment-Induced Apoptosis in Photoreceptors by a Small Peptide Inhibitor of the Fas Receptor

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PURPOSE. To test the effect of a small peptide inhibitor (Met12) of the Fas receptor on the activation of extrinsic and intrinsic apoptosis pathways after retinal detachment.

METHODS. Retinal-RPE separation was created in Brown Norway rats by subretinal injection of 1% hyaluronic acid. Met12, derived from the Fas-binding extracellular domain of the oncoprotein Met, was injected into the subretinal space at the time of separation. A mutant peptide and vehicle administered in a similar fashion acted as inactive controls. The extrinsic apoptotic pathway was induced in 661W cells using a Fas-activating antibody in the presence or absence of Met12. Caspase 3, caspase 8, and caspase 9 activities were measured with calorimetric and luminescent assays in retinal extracts and cell lysates. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was performed in retinal sections 3 days after separation. Histology was performed in retinal sections 2 months after retinal detachment.

RESULTS. Met12 inhibited Fas-induced caspase 8 activation in 661W cells. Similarly, administration of Met12 into the subretinal space inhibited the activation of caspase 3, caspase 8, and caspase 9 after retinal detachment. This corresponded to a decreased level of TUNEL-positive staining of photoreceptors after retinal-RPE separation in animals that received Met12, but not inactive mutant, peptide treatment. After 2 months, the outer nuclear layer was significantly thicker, and the photoreceptor count was higher in animals treated with subretinal Met12.

CONCLUSIONS. The small peptide Met12 may serve as a photoreceptor-protective agent in the setting of retinal-RPE separation. (*Invest Ophthalmol Vis Sci.* 2010;51:2177–2184) DOI:10.1167/iovs.09-4439

Photoreceptor cell death is the primary mechanism of vision loss after retinal detachment. The retinal pigment epithelium (RPE) provides the major metabolic and nutritional support for the photoreceptors.^{1,2} Numerous retinal diseases cause the separation of photoreceptors from the RPE, and this leads to the disruption of normal photoreceptor homeosta-

sis.^{3–6} Interruption of this close relationship activates cell death in photoreceptors. Initially, a subpopulation of photoreceptors undergoes rapid cell death.^{7,8} This is followed by a slower, chronic loss of photoreceptors. Studies have demonstrated that photoreceptors die by apoptosis after separation from the RPE.^{9–12} Two major cell death pathways play critical role in activating apoptosis in photoreceptor cells, the Fas-mediated extrinsic death pathway and the mitochondrial intrinsic death pathway. Our previous studies have shown that photoreceptors activate both the Fas proapoptotic pathway and the intrinsic death pathway in a time-dependent fashion after retinal detachment.^{13,14} Fas pathway activation occurs when the Fas ligand (FasL) binds to the Fas receptor (Fas). This pathway is upstream of the intrinsic death pathway. Inhibition of Fas signaling with a neutralizing antibody or a small inhibitory RNA against the Fas transcript prevents the activation of the intrinsic cell death pathway and the separation-induced death of photoreceptors.¹⁴ Identifying novel means of inhibiting retinal Fas signaling may lead to the discovery of small molecules or biologicals that may be developed into new therapeutic agents.

Recent studies have identified Met as a novel inhibitor of the Fas pathway. Met is an oncogene that encodes the tyrosine kinase receptor for hepatocyte growth factor.¹⁵ Met is a disulfide-linked heterodimer composed of an extracellular 50-kDa α chain and a transmembrane 145-kDa β chain. In normal mouse liver tissue, Met associates directly with Fas and prevents Fas activation by its sequestration.¹⁶ Studies have demonstrated that only the extracellular α chain domain of Met is required to form the Fas-Met complex.¹⁷ The extracellular α chain contains an N-terminal sequence motif, TyrLeuGlyAla (YLGA), which shows high similarity to the normal FasL.¹⁷ In cell cultures, α -Met or YLGA-containing peptides protect Jurkat cells from FasL-induced apoptosis.¹⁷ This inhibition is secondary to the sequestration of the Fas and decreasing the number of Fas molecules available to interact with FasL.

We investigated the in vitro and in vivo effects of a small peptide containing the YLGA motif and the surrounding amino acids on the Fas proapoptotic pathway and its downstream targets in the 661W photoreceptor cell line and in a rodent model of retinal detachment. Treatment of 661W cells with a Fas-activating antibody induced caspase 8 activation, which was competitively blocked by the Met YLGA 12-mer (Met12). Similarly, the administration of Met12 during retinal detachment in rat eyes prevented Fas-induced extrinsic cell death pathway activation. Inhibition of Fas signaling by Met12 prevented activation of the intrinsic cell death pathway in detached photoreceptors. Finally, inhibition of the Fas signaling pathway by Met12 in detached rodent retinas reduced Fas-mediated apoptosis and increased photoreceptor survival.

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Supported by grants from the Michigan Eye Bank (DNZ), the International Retinal Research Foundation, Inc. (DNZ), and Departmental Core Center for Vision Research Grant EY-07003.

Submitted for publication August 5, 2009; revised August 29 and September 23, 2009; accepted September 25, 2009.

Disclosure: C.G. Besirli, None; N.D. Chinskey, None; Q.-D. Zheng, None; D.N. Zacks, None

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METHODS

Experimental Model of Retinal Detachment

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. Detachments were created in adult male Brown-Norway rats (weight range, 300–400 g) (Charles River Laboratories, Wilmington, MA), as described previously.¹² Briefly, rodents 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 20-gauge microvitreoretinal blade (Walcott Scientific, Marmora, NJ) was used to create a sclerotomy 2 mm posterior to the limbus, carefully avoiding lens damage. Under direct visualization through an operating microscope, a subretinal injector (Glaser, 32-gauge tip; BD Ophthalmic Systems, Sarasota, FL) was introduced through the sclerotomy into the vitreous cavity and then through a peripheral retinotomy into the subretinal space. Sodium hyaluronate (10 mg/mL) (Pharmacia and Upjohn Co., Kalamazoo, MI) was slowly injected to detach the neurosensory retina from the underlying retinal pigment epithelium. In all experiments, approximately one-third to one-half of the superonasal neurosensory retina was detached. In all animals, detachments were created in the same location to allow for direct comparison of retinal cell counts. Detachments were created in the left eye, leaving the right eye as the control. In some eyes, a wild-type Met YLGA 12-mer (HHIYLGA VNNYIY, Met12, 50 μ g), a mutant Met 12-mer (HHGSDHERNNYIY, mMet, 50 μ g), or vehicle (dimethyl sulfoxide [DMSO]) was injected into the subretinal space in the area of the detachment in a 10- μ L volume using a Hamilton syringe (Hamilton Company, Reno, NV) immediately after the creation of the detachment.

Cell Culture

The 661W photoreceptor cell line was generously provided by Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK). The 661W cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 300 mg/L glutamine, 32 mg/L putrescine, 40 μ L/L β -mercaptoethanol, 40 μ g/L hydrocortisone 21-hemisuccinate, and 40 μ g/L progesterone. The media also contained penicillin (90 U/mL) and streptomycin (0.09 mg/mL). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Caspase Activity Assays

Caspase 3, caspase 8, and caspase 9 activities were measured with colorimetric tetrapeptide cleavage assay kits, per the manufacturer's instructions (Bio-Vision, Mountain View, CA). Total retinal protein was extracted according to our previously published protocol.¹⁴ One hundred micrograms of total retinal protein from either attached or detached retinas was incubated with caspase 3 (DEVD-pNA), caspase 8 (IETD-pNA), or caspase 9 substrates (LEHD-pNA) at 200 μ M final concentration for 60 minutes. Absorbance was measured at 405 nm in a microplate reader (Spectra-MAX 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 3, caspase 8, or caspase 9 was incubated with the tetrapeptide alone. The caspase activity in the detached retina was normalized against the caspase activity in attached retina at the same time point. For each group, the data represented the average caspase activity levels of four or five independent samples, each sample consisting of protein from five eyes.

In cell culture experiments, caspase 8 activity was measured by a commercially available luminescent tetrapeptide cleavage assay kit (Promega, Madison, WI). The 661W cells were seeded in 96-well plates (Nunc, Rochester, NY) at 1500 cells/well for 24 hours before treatment. Cells were pretreated with various concentrations of Met12,

mMet, or vehicle for 1 hour before treatment with 500 ng/mL Fas-agonistic Jo2 monoclonal antibody (BD Biosciences, San Jose, CA). Caspase 8 activity was measured at various time points by incubating the cells with the proluminescent substrate in 96-well plates according to the manufacturer's instructions. Controls included untreated cells and wells with no cells. Luminescence was measured in a plate reader luminometer (Turner Biosystems, Sunnyvale, CA).

Western Blot Analysis

Retinas from experimental eyes with detachments and control eyes without detachments were dissected from the RPE-choroid, homogenized, and lysed in buffer containing 10 mM HEPES (pH 7.6), 0.5% IgEPal, 42 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 5 mM MgCl₂ and with 1 tablet of protease inhibitors per 10 mL buffer (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were incubated on ice and centrifuged at 22,000g at 4°C for 60 minutes. The protein concentration of the supernatant was then determined (DC Protein Assay kit; Bio-Rad Laboratories, Hercules, CA). The protein samples were loaded and run on SDS-polyacrylamide gels (Tris-HCl Ready Gels; Bio-Rad Laboratories). After electrophoretic separation, the proteins were transferred onto polyvinylidene fluoride membranes (Immobilon-P; Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized with Ponceau S staining, and the lanes were assessed for equal loading by densitometry of a nonspecific band present across all lanes. Membranes were then immunoblotted for cleaved caspase 3, cleaved caspase 8, and cleaved caspase 9 (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions.

TUNEL Staining and Histology

At varying intervals after creation of the detachment, the animals were euthanatized, and the eyes were enucleated. For TUNEL staining, whole eyes were fixed overnight at 4°C in phosphate-buffered saline with 4% paraformaldehyde (pH 7.4). The specimens were embedded in paraffin and sectioned at a thickness of 5 to 6 μ m. TUNEL staining was performed on the sections with a detection kit (ApopTag Fluorescein *In Situ* Apoptosis Detection Kit; Millipore, Billerica, MA) according to the manufacturer's instructions. For light microscopic analysis, paraffin sections were stained with 0.5% toluidine blue in 0.1% borate buffer.

Cell Counts and Retinal Thickness Measurements

Photoreceptor cell apoptosis was quantified as the percentage of total cells in the outer nuclear layer (ONL) that were TUNEL positive. Three nonoverlapping high-power fields (40 $\times$) at the maximal height of the retinal detachment were selected per section and were averaged unless there were less than three non-overlapping high-power fields, in which case fewer fields were used. One representative section was used per eye. The total number of cells in the ONL was measured in a similar fashion. The total thickness of the retina (measured from the outer edge of the ONL to the inner limiting membrane) was measured in three places in each of three nonoverlapping high-power fields (40 $\times$) at the maximal height of the retinal detachment per section and averaged for each eye. Photoreceptor inner and outer segments were not included in the total retinal thickness measurement, given the variable retraction of these elements after detachment of the neurosensory retina, which does not necessarily correlate with viability of the photoreceptors after reattachment.^{18,19} For toluidine blue-stained specimens, normalization of ONL cell count to the total retinal thickness of each section (i.e., ONL cell count divided by total retinal thickness) was performed to account for possible differences in angles of sectioning and to allow for intersample comparison. ONL cell counts and total retinal thicknesses in each group of the rat experiments were also normalized to corresponding values of attached retinas in that group to allow inter-sample comparison. For each experi-

mental group, measurements were made on three sections of 4 to 11 eyes; each eye was from a separate animal.

Statistical Analysis

Statistical analysis comparing groups was performed using two-tailed Student's *t*-tests without assuming equal variance. Differences were considered significant at $P \leq 0.05$.

RESULTS

In Vitro Analysis of Met12

The 661W cell line is a photoreceptor line that has been immortalized by the expression of SV40-T antigen under control of the human interphotoreceptor retinol-binding protein promoter.²⁰ 661W cells express cone photoreceptor markers, including blue and green cone pigments, transducin, and cone arrestin,²¹ and can undergo caspase-mediated cell death.²² Our previous studies have shown that Fas signaling plays a critical role in caspase 8 activation and photoreceptor apoptosis in vivo. To determine whether activation of the Fas-signaling pathway would lead to caspase activation in an in vitro model of photoreceptors, we treated 661W cells with a Fas-activating antibody (Fas-Ab). Addition of the Fas-Ab resulted in cell death. Activity of caspase 8 measured in 661W cell lysates increased with increasing concentrations of Fas-Ab, peaking with the 500-ng/mL dose (Fig. 1A). To determine the time course of caspase 8 activation, we treated the 661W cells with 500 ng/mL Fas-Ab and measured activity levels at various time points. Caspase 8 activity was significantly increased at 48 hours in 661W cells exposed to Fas-Ab (Fig. 1B; $P < 0.001$).

Met has recently been shown to be a novel inhibitor of the Fas pathway. A small 12-mer peptide, Met12, containing the amino acids surrounding the Fas-binding YLGA motif of Met has been shown to protect Jurkat cells from FasL-induced apoptosis.¹⁷ To determine whether the same peptide could block Fas death receptor signaling in photoreceptors, we treated 661W cells with Fas-Ab in the presence of Met12 or the inactive mutant peptide mMet, in which the central six amino acids containing the YLGA motif were randomly changed. Caspase 8 activity was determined 48 hours after treatment as a measure of Fas receptor pathway activation. Our results showed that Fas-Ab-induced caspase 8 activation was inhibited by Met12 treatment in a dose-dependent manner (Fig. 1C). In contrast, treatment of cells with the mMet peptide or vehicle alone had no effect on Fas-mediated caspase 8 activation. These results demonstrated that 661W cells have an intact Fas death receptor pathway and that Met12 peptide can inhibit Fas signaling in an in vitro photoreceptor model.

In Vivo Effect of Met12

Inhibition of the Fas signaling by Met12 in 661W cells suggested that this peptide may also block photoreceptor Fas signaling in vivo. We have previously shown that retinal detachment leads to the activation of the Fas/FasL pathway and caspase 8 cleavage.^{13,14} To determine whether Met12 could inhibit retinal detachment-induced caspase 8 activation, we examined the effect of Met12 on photoreceptor caspase 8 activation 24 hours after retinal detachment. Rat retinas were detached in the presence of Met12 (50 μ g), mMet (50 μ g), or vehicle. Caspase 8 activity was significantly increased in detached retinas injected with vehicle or mMet, compared with attached retinas (Fig. 2). Subretinal injection of the Met12 peptide at the time of retinal detachment reduced caspase 8 activity by approximately 50% ($P = 0.028$). These results demonstrated that, similar to 661W cells, treatment of detached

FIGURE 1. Fas-induced caspase 8 activation is blocked by Met12 in 661W cells. (A) 661W cells were treated with various concentrations of Fas-activating antibody (Fas-Ab). Caspase 8 activity was measured at 48 hours. Compared with no treatment, the increase in caspase 8 activity was statistically significant for all concentrations of Fas-Ab ($P < 0.05$ for each treatment group). Mean $\pm$ SE; $n = 8$. (B) 661W cells were treated with 500 ng/mL Fas-Ab, and caspase 8 activity was measured at various time points. Data were normalized to untreated controls at each time point. * $P < 0.001$. Mean $\pm$ SE; $n = 16$. (C) 661W cells were treated with 500 ng/mL Fas-Ab in the presence of Met12, mMet, or vehicle (DMSO). The difference between the DMSO-treated eyes and the Met12 (10.0 μ g/mL)-treated eyes was significant ($P = 0.01$). Control group did not have Fas-Ab treatment. Caspase 8 activity was measured at 48 hours. Mean $\pm$ SE; $n = 8$.

retinas with Met12 inhibits Fas-mediated caspase 8 activation in photoreceptors.

During rodent photoreceptor apoptosis, Fas/FasL signaling acts as an upstream regulator of the intrinsic death pathway. This is demonstrated by the reduction of caspase-9 activity after the injection of neutralizing antibodies against either Fas or FasL into the subretinal space of the detached retina.¹⁴ To determine whether Met12 inhibits activation of the intrinsic death pathway, we measured caspase 3 and caspase 9 activity levels 24 hours after retinal detachment in the presence of Met12 (50 μ g), mMet (50 μ g), or vehicle. Subretinal Met12 injection reduced caspase 3 activity by approximately 50% after 24 hours (Fig. 2; $P = 0.017$). Similarly, caspase 9 activity was also reduced by approximately 50% in detached retinas injected with Met12 (Fig. 2; $P = 0.037$). In contrast, subretinal injection of the mMet did not affect the activity level of either of these caspases. The conversion of procaspase 8 to cleaved caspase 8 was confirmed on Western blot analysis (Fig. 2), as was the cleavage of caspases 3 and 9 (data not shown). These results demonstrated that Met12-mediated inhibition of the Fas receptor leads to reduced activation of the intrinsic cell death pathway in detached photoreceptors.

In rodent eyes, the peak of TUNEL staining occurs at 3 days after retina/RPE separation, with a rapid decline in TUNEL-positive cells to near preseparation levels by day 7.⁸ We tested whether Met12 inhibition of Fas pathway signaling would prevent photoreceptors from entering the apoptotic cascade, as measured by TUNEL staining. Rat retinas were detached in the presence of Met12, mMet, or vehicle. At 3 days after retinal detachment, TUNEL-positive cells were confined to the ONL of photoreceptors (Fig. 3A), consistent with previous studies of experimental retinal detachment. Approximately 5% of ONL cells displayed TUNEL-positive staining at day 3 after separation (Fig. 3B). Injection of Met12 into the subretinal space resulted in approximately 77% fewer TUNEL-positive photoreceptors than separated retinas injected with mMet (Fig. 3B; $P = 0.025$). No gross histologic change could be detected because of injection of the vehicle alone.

We next sought to test whether the decrease in caspase activation and TUNEL-staining corresponded to increased survival of photoreceptors after retina-RPE separation of longer duration. Rat retinas were detached as described, and Met12, mMet, or vehicle was injected in the subretinal space at the time of detachment. After 2 months, detached retinas showed significant reductions in ONL thickness compared with attached retinas (Fig. 4A). Rat retinas injected with Met12 showed a 37% increase in ONL cell counts (Fig. 4B; $P = 0.007$) and a 27% increase in ONL thickness measurements (Fig. 4C; $P = 0.017$) compared with mMet-injected retinas after 2 months of retinal detachment. These results showed that inhibition of Fas signaling and caspase activation by Met12 increases the survival of photoreceptors after prolonged retina/RPE separation.

DISCUSSION

In this study, we report a novel method of inhibiting Fas signaling and preventing photoreceptor apoptosis after retinal

detachment. Our results demonstrate that a small peptide inhibitor of the Fas death receptor blocks caspase activation and increases photoreceptor survival after retina-RPE separation. We initially tested this small peptide, Met12, in an in vitro model of cone photoreceptors and confirmed that it prevents the Fas-dependent activation of caspase 8. We extended this study to show that Met12 significantly reduced Fas signaling and photoreceptor apoptosis in our in vivo model of experimental retinal detachment.

Previous studies from our laboratory have shown that retinal detachment activates the Fas receptor^{13,14} and that this event controls activation of the intrinsic cell death pathway in photoreceptors. We have previously been able to prevent photoreceptor apoptosis by using large molecules, such as neutralizing antibodies, to inhibit the Fas receptor or by preventing the detachment-induced increase in the Fas receptor transcript with inhibitory RNA.¹³ We now show that we can achieve this same significant level of photoreceptor preservation using a small peptide, Met12.

Our laboratory uses a rodent model to investigate molecular mechanisms regulating photoreceptor death after retinal detachment. Using animal models to study diseases is labor intensive and has substantial cost. The 661W cells are immortalized photoreceptors that express cone photoreceptor markers, including blue and green cone pigments, transducin, and cone arrestin.²¹ When transfected into the 661W cells, Fas-associated death domain, FADD, was a potent inducer of cell death.²³ To determine whether 661W would be a good in vitro model of retinal detachment-induced photoreceptor apoptosis, we tested the effect of Fas activating antibody on caspase activation. We showed that Fas-Ab treatment activates caspase 8 in 661W cells in a dose- and time-dependent manner, indicating that the Fas receptor signaling pathway is intact in these cells. Our results indicate that the 661W cell line is a functional in vitro model of photoreceptor apoptosis mediated by Fas signaling. Further studies are needed to determine whether other apoptotic signals, such as components of the intrinsic pathway, are also activated in 661W cells. A cell line model of photoreceptor death will allow us to investigate apoptotic pathways using in vitro assays and to identify therapeutic candidates that warrant further testing in our in vivo model.

Although significant levels of photoreceptors survived after Met12-mediated Fas inhibition, some were still lost after chronic retinal detachment. Several underlying mechanisms may explain this partial survival effect. First, the dose of Met12 that was injected in the subretinal space at the time of retinal detachment may be suboptimal for complete protection. Second, subretinal Met12 may not adequately reach detached photoreceptors that are apoptotic. Consistent with either of these hypotheses, caspase activation was only partially blocked by Met12 administration in the in vivo model. This is in contrast to 661W cells, in which Met12 treatment resulted in full inhibition of the caspase 8 activation. A third possibility is reduced Met12 levels and Fas inhibition in retinas with prolonged detachments. We injected Met12 subretinally only during the creation of retina/RPE separation. The amount of Met12 available in the subretinal space most likely decreases with time, thus increasing the number of Fas receptors available for

FIGURE 2. Met12 inhibits activation of caspases. (A) Injection of the Met12 into the subretinal space of detached retinas reduces caspase 8 activity. Rat retinas were detached in the presence of Met12 (50 μ g), mMet (50 μ g), or vehicle (DMSO). Caspase 8 activation was measured in harvested retinas after 24 hours of detachment, as described. Data are presented as fold increase in caspase activity in detached retinas compared with attached retinas. Mean $\pm$ SE; $n = 4-5$. Western blot shows decreased caspase 8 cleavage in the presence of Met12 (*inset*). Assay controls including no lysate samples (blank) and recombinant caspase 8 are shown. (B) Caspase 3 activity was significantly reduced by Met12 treatment during retinal detachment. Mean $\pm$ SE; $n = 4-5$. (C) Caspase 9 activity was also significantly reduced by Met12. Mean $\pm$ SE; $n = 4-5$. Data are presented as fold increase in caspase activity in detached retinas compared with attached retinas. Assay controls including no lysate samples (blank) and recombinant caspase 8 or caspase 9 are shown

A

B

FIGURE 3. Met12-mediated inhibition of Fas pathway signaling prevents photoreceptors from entering the apoptotic cascade. Rat retinas were detached in the presence of Met12 (50 μ g), mMet (50 μ g), or vehicle (DMSO). Eyes were enucleated after 72 hours, and retinas were sectioned for TUNEL staining. **(A)** Representative photomicrographs of TUNEL-stained photoreceptors after 72 hours of retinal detachment. Nuclei of retinal cells are stained with propidium iodide (PI). INL, inner nuclear layer; ONL, outer nuclear layer. **(B)** Quantification of TUNEL-positive cells in the ONL. Mean $\pm$ SE; $n = 3-6$. There were no TUNEL-stained cells in the ONL of attached retinas (data not shown).

FasL binding. This is similar to what occurs with another neuroprotectant, IL-6. We have recently reported that exogenous IL-6 increases the survival of detached photoreceptors.²⁴ When IL-6 was injected in the subretinal space at the time of detachment, its protective effect on detached photoreceptors lasted for 1 month. However, reinjection of exogenous IL-6 at the 1-month time point extended the duration of photoreceptor survival after retina/RPE separation. The half-life of Met12 in the subretinal space is unknown, and additional experiments are needed to determine the optimal dosing and frequency to achieve continued preservation of photoreceptors. The optimal treatment strategy and increased prosurvival effect of Met12 would allow for a greater therapeutic “window of opportunity” to achieve the highest photoreceptor survival rate after retinal-RPE reattachment. This might be most useful in conditions of chronic or recurrent episodes of retina-RPE separation, such as in neovascular age-related macular degeneration (AMD) or tractional retinal detachment because of diabetes or proliferative vitreoretinopathy.

The incomplete rescue of detached photoreceptors by Met12 treatment may also indicate that a population of photoreceptors executes apoptosis independently of Fas death receptor signaling. Supporting this hypothesis, we have previ-

ously shown that the separation-induced activation of the mitochondrial apoptotic pathway is only partially controlled by the activation of Fas.¹⁴ Thus, an alternative signaling pathway may be instrumental in stimulating the intrinsic death pathway in photoreceptors. The critical role of intrinsic pathway caspases in retinal detachment-induced photoreceptor apoptosis was recently shown by Zadro-Lamoureux et al.²⁵ Delivery of X-linked inhibitor of apoptosis (XIAP) with a recombinant adenoassociated virus inhibited caspase 3 and caspase 9 activities and protected photoreceptors from detachment-induced apoptosis. In addition to the mitochondrial apoptotic pathway, caspase-independent death pathways may play a role in photoreceptor loss after retinal detachment. Hisatomi et al.^{26,27} reported the activation of the apoptosis-inducing factor (AIF)-dependent death in an experimental rat model of retina-RPE separation, similar to the one used in this study.^{26,27} Retinal detachment-induced photoreceptor death was reduced in mice carrying hypomorphic mutation of the gene encoding AIF.²⁷

In clinical practice, patients seek treatment after detachment has already occurred. The animal models of retina-RPE separation show that Fas pathway activation takes place early and remains elevated throughout the duration of the detach-

FIGURE 4. Decreases in caspase activation and number TUNEL-positive cells correspond to increased long-term survival of photoreceptors. Retinas were detached in the presence of Met12 (50 μ g), mMet (50 μ g), or vehicle (DMSO), injected into the subretinal space at the time of detachment. Eyes were enucleated after 2 months of detachment, and paraffin sections were stained with 0.5% toluidine blue. (A) Representative photomicrographs. INL, inner nuclear layer; ONL, outer nuclear layer. (B) ONL cell counts normalized to retinal thickness. (C) ONL thickness normalized to retinal thickness. Mean $\pm$ SE; $n = 3-7$.

ment.¹⁴ Thus, the usefulness of photoreceptor-protective therapy would be to help prevent further photoreceptor loss until the retina can be surgically reattached and normal retina-RPE homeostasis can be restored. Separation of retina and RPE is also encountered in a broad spectrum of retinal diseases. Thus, the potential clinical relevance of anti-Fas therapy in photoreceptor survival is not limited to retinal detachment. For example, Fas-mediated apoptosis has been suggested to play a role in photoreceptor cell death in AMD.¹¹ AMD is characterized by progressive degeneration of the RPE,²⁸ and it causes outer retinal degeneration and reorganization similar to that which occurs after retinal detachment.²⁹ The neovascular form of AMD also includes the exudation of fluid under the retina, creating an actual separation of this tissue from the underlying RPE.²⁸ Although there have been significant advances in the

treatment of neovascular AMD,³⁰ there can be prolonged periods of retina-RPE separation and Fas pathway activation. The usefulness of anti-Fas treatment would most likely be as an adjunct aimed at extending the survival of photoreceptors while the underlying disorder is treated. Although additional apoptotic pathways may be activated after retina-RPE separation, our data suggest that a significant number of photoreceptors can still be preserved by inhibiting the Fas death receptor. In addition, combining Met12 treatment with another anti-apoptotic (e.g., XIAP) or with a prosurvival molecule (e.g., IL-6) may increase the efficiency of photoreceptor survival. Further experiments are needed to define the therapeutic parameters within which modulation of the Fas pathway activity and supplementation of exogenous prosurvival factors will facilitate increased photoreceptor survival, allowing improved visual outcomes in patients.

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