Fas Ligand-Fas Signaling Participates in Light-Induced Apoptotic Death in Photoreceptor Cells

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PURPOSE. We investigated the function of Fas in photoreceptors.

METHODS. Postmortem human eyes and mouse-derived photoreceptor cells (661W) were examined for Fas expression by in situ hybridization and immunofluorescence. 661W cells were treated with FasL or Fas agonistic antibody, or exposed to light with/without pharmacological manipulation of Fas signaling, followed by apoptosis detection by TUNEL, immunofluorescence and fluorescence activated cell scanning (FACS). Fractionated cellular extracts were used to detect protein expression or protein phosphorylation after immunoprecipitation by Western blot.

RESULTS. Fas was expressed in the photoreceptor layer of human retina. Fas and a cleaved form of FasL were found on the cell surface of 661W cells. Treatment with FasL or Fas agonistic antibody induced apoptosis in 661W cells. Blocking the activity of FasL or administration of caspase-8 inhibitor z-IETD inhibited light-induced apoptosis. However, it simultaneously caused induction of necroptosis, which could be blocked by the receptor-interacting protein 1 (RIP1) inhibitor, necrostatin-1. Light exposure in the presence of z-IETD caused hyper-phosphorylation of RIP1. Light exposure did not elevate the expression of Fas, FasL, or the Fas-associated death domain adaptor protein (FADD). Cells or conditioned medium after light exposure induced apoptosis in dark-adapted cells, which could be attenuated by blockade of Fas.

CONCLUSIONS. Fas has a pro-apoptotic role in photoreceptors. Under light stress, soluble and membrane-bound FasL can bind to Fas, inducing apoptosis via a paracrine mechanism. Although blocking Fas signaling inhibits apoptosis, it does not improve the overall photoreceptor survival due to a compensatory activation of necroptosis. Hence, prevention of photoreceptor loss from retinal photo-oxidative stress should target Fas and RIP1. (*Invest Ophthalmol Vis Sci.* 2012;53:3703–3716) DOI:10.1167/iovs.11-8928

Death of photoreceptors is the major pathological endpoint in retinal diseases such as retinitis pigmentosa (RP), age-related macular degeneration and retinal detachment.^{1–3} Photoreceptor loss in these diverse disease conditions involves programmed cell death by apoptosis.^{4–6}

Apoptosis of photoreceptors can result from the environmental changes in the retina rather than a direct cell-autonomous mechanism.⁷ For example, in retinitis pigmentosa, a common inherited retinal dystrophy in humans, the majority of disease-causing mutations are identified in rods, but not in cones. However, cones also can die following the loss of rods, presumably responding to a change in the retinal micro-environment caused by the loss of rods.⁸ This secondary cone death is recapitulated in the transgenic T17M rhodopsin mouse model of RP under bright white light-induced retinal damage.⁹ Interestingly, cones do not always die after loss of rods. For example, photopic exposure-induced increase of rod death in P23H rhodopsin transgenic rats, a model of autosomal dominant RP, is not followed by any detectable cone death.¹⁰ Therefore, it appears that specific rhodopsin mutations, intensity, and duration of damaging light to the retina, and condition of rods all may affect the susceptibility and/or timeline of cones to commit cell death.

The intracellular signals and mediators for apoptotic death in photoreceptors have been studied extensively.^{11–13} However, the extrinsic mediators that activate the apoptotic death signal remain unknown, as is the relevant mechanism by which dying photoreceptors affect the survival and function of their healthy neighbors. Death of photoreceptors caused by genetic mutations and/or environmental stress, such as hypoxia and excess light in the retina, results in an increased oxygen level in the retina,^{14–16} due to decreased oxygen consumption by the viable photoreceptors and the inability of the choroidal vessels, which nourish the outer retina to auto-regulate. Oxygen is the primary precursor, which, through electron donation, generates various reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, and reactive hydroxyl radicals, all of which can induce irreversible damage to DNA, oxidize cellular proteins, and can cause enzymes to alter their functions and activities, and fatty acids to generate secondary toxic by-products through lipid peroxidation.

Light has a precipitating role in the damage and death of photoreceptors, and long has been used as a relevant model to study apoptosis of photoreceptors that occurs in human retinal dystrophies.^{11,17} Photo-oxidative damage following ROS attack under prolonged and intense light is the primary cause of death in photoreceptors. In non-neuronal cells, ROS can act not only as signaling molecules to activate stress response pathways, but also as mediators for cell death through modulation of death receptors. For example, production of ROS by the DNA damaging agent, cisplatin, in leukemia cells can promote Fas aggregation to stimulate apoptosis.¹⁸ Other studies have shown that induction of apoptosis by FasL is executed through generation of ROS.¹⁹

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Apoptotic death through the death receptor Fas has been well characterized in the immune system. Expression of FasL and Fas is ubiquitous in a variety of tissues including the central nervous system (CNS), and the function of Fas has been shown to be pleiotropic. Beyond its canonical pro-apoptotic role, Fas has been implicated in embryonic growth, inflammation, differentiation, and proliferation.^{20–22} Interestingly, functional regulation of Fas in the CNS is complex, and Fas can be either neuroprotective or pro-apoptotic depending on the neuronal context and disease condition.^{23–26} Upregulation of FasL and Fas has been found in various neurological diseases,²⁷ suggesting that there is an alteration of the underlying Fas signaling pathway that may have a role in the neurodegenerative process.

In the visual system, Fas-mediated apoptosis has been demonstrated in experimental models of retinal detachment.²⁸ Inhibition of Fas promotes photoreceptor survival,²⁹ whereas agonistic activation of Fas in cultures of photoreceptors induces activation of downstream caspase-8.³⁰ Previous studies applying serial analysis of gene expression (SAGE) have shown that FasL is upregulated differentially in the retina of *CRX*^{−/−} knockout mice, which develop photoreceptor degeneration at an early postnatal age.³¹ Although the identity of the specific retinal cells responsible for the upregulation of FasL is unknown, these studies suggest that Fas might be activated to initiate an extrinsic apoptotic death signal in photoreceptors.

We sought to investigate the function of the Fas pathway in modulating extrinsic photoreceptor apoptosis using a cellular phototoxicity model.

MATERIALS AND METHODS

Cell Culture, Growth Conditions, and Human Eye Tissue Sections

Mouse-derived immortalized photoreceptor cells (661W) were provided by Muayyad R. Al-Ubaidi and cultured routinely as described previously³² with a minor modification. Instead of Dulbecco's modified Eagle's medium (DMEM), DMEM+GlutaMAX-1 (Invitrogen, Carlsbad, CA) was used as the base medium. Postmortem human eye globes within 6 hours of subject death were acquired from Midwest Eye Banks (Ann Arbor, MI). Subjects had no previous history of retinal disease. Vertical paraffin sections (5 μ) at post-fixation were prepared on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). For all light stress experiments, 661W cells between 35 and 40 passages were used. Cells at 90% confluence were grown on 6-well plates or 60 mm culture dishes with CellBIND Surface (Corning, Tewksbury, MA). Cells were pre-treated with 10 μ M 9-*cis* retinal (Sigma, St. Louis, MO) and left overnight in darkness in medium containing 10% or 1% fetal bovine serum (FBS). The use of different FBS concentrations in the medium was to assess whether there was any influence of FBS that could affect cellular uptake of 9-*cis* retinal. The following day, the medium was exchanged with serum-free DMEM+GlutaMAX-1. The cells were subject to fluorescent white light (FWL) exposure using a custom-made light box (Aristo Lighting Technologies, Roslyn, NY) with a ventilation device at an emitting intensity of 5500 Lux for 2 to 4 hours. The position of the cultures on the light box was 1 cm above the surface and the surrounding temperature was 32°C. Control cells were maintained in darkness at an identical ambient temperature.

Fluorescent In Situ Hybridization (FISH) of Fas in Human Retina

Three independent DNA probes (5'-TAG TAA TAC TCT CCT GCT CAA A-3', 5'-ACA CAA TCT ACA TCT TCT GCA T-3', and 5'-ATT GTC ATT CTT GAT CTC ATC T-3') complementary to different sequences of

human Fas cDNA (NCBI Accession: NM_000043.4) were designed by an algorithm program (<http://www.exiqon.com>; Exiqon, Vedbaek, Denmark). The synthesized probes were 5'-end labeled with ATP γ S (Vector Laboratories, Burlingame, CA) to transfer a thiophosphate to the 5' hydroxyl group. The thiol moiety then was coupled chemically with the biotin-maleimide. After this point, the biotin-labeled probes finally were purified with phenol extraction. For FISH, paraffin-embedded vertical sections of human retinal samples from donor eyes without retinal diseases were used. Essentially, the retinal samples were soaked in xylene to remove paraffin, rehydrated in serially diluted ethanol, digested with proteinase K (250 μ g/mL in PBS), washed with PBS and finally de-hydrated in ethanol. Hybridization was done at 37°C in a humidified chamber overnight at a probe concentration of 100 ng/mL either singly or in combinations. Adjacent retinal sections without probes or with scrambled sequences of the probes were used as negative controls. After hybridization, samples were washed with PBS containing deionized formamide, incubated sequentially with blocking solution, fluorescein-avidin DCS, and biotinylated anti-avidin for signal amplification (Vector Laboratories), and finally washed with PBS-0.1% Tween 20 (PBST) before mounting with DAPI to identify layers of neural retina. The green fluorescent filter was set for detection of fluorescein avidin DCS.

Immunofluorescent Detection of Fas in Human Retina

Paraffin-embedded retinal sections were de-paraffinized and hydrated in serial dilutions of ethanol. Slides were treated with trypsin-based antigen retrieval solution (Abcam, Cambridge, MA) at 37°C for 20 minutes, blocked with normal goat and/or donkey serum (depending on the experimental goals for single or co-labeling) at room temperature for 1 hour, followed by incubation with the primary rabbit polyclonal anti-Fas antibody (A-20, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 hours in an antibody dilution buffer containing PBS, 1% BSA and 0.3% Triton X-100. Secondary antibody was goat anti-rabbit Texas-Red or FITC conjugated IgG (1:250; Santa Cruz Biotechnology). For co-labeling experiments, Alexa Fluor 488-Lectin PNA (Invitrogen) at 1:100 μ g/mL in PBST was used as a cone identification marker in conjunction with the secondary antibody. Similar experiments were performed using goat polyclonal anti-blue opsin (OPN1SW, N-20, 1:200; Santa Cruz Biotechnology) antibody and donkey anti-goat Texas-Red conjugated IgG as the secondary antibody. To detect expression of Fas in 661W cells, fixed cells at 50% confluence in chamber slides were blocked with goat and/or donkey serum, rinsed, and incubated with the same anti-Fas (1:100) primary antibody used in human retinal labeling at 4°C overnight. Anti-rabbit FITC conjugated IgG was used as the secondary antibody. In co-labeling experiments, goat anti-Na⁺/K⁺ ATPase α 3 antibody (C-16, 1:100; Santa Cruz Biotechnology) was used as a cell surface marker. In all studies, slides without primary or secondary antibody were negative controls. All slides were counterstained with DAPI (Vector Laboratories) at mounting and examined with a Zeiss inverted wide-field fluorescent microscope. We examined 10–17 non-overlapping visual fields in each condition to count TUNEL-positive cells for quantitative comparison.

Recombinant FasL, Fas Agonist, and Apoptosis Detection

Nearly confluent 661W cells grown in chamber slides were treated with enhancer-independent human recombinant SuperFasL (Enzo Life Sciences, Plymouth Meeting, PA) that binds crossly with mouse Fas at different doses from 15 ng to 3.3 μ g/mL in serial dilutions for 6–72 hours. In parallel experiments, cells also were treated with Fas agonist Jo2 monoclonal antibody (2–20 μ g/mL; BD Biosciences, San Jose, CA) or an equivalent concentration of isotype IgG as negative control in the presence of Protein G (1 μ g/mL) as an enhancer for 2–6 hours. In neutralizing experiments, mouse monoclonal anti-human FasL antibody

(NOK-2, 10 μ g/mL; BD Biosciences) was pre-incubated with human recombinant SuperFasL on ice for 30 minutes before the addition of the antibody-ligand complex into the culture medium. In independent experiments, cells were pre-treated with NOK-2 for 1 hour before the addition of the ligand. Cells treated with NOK-2 only were used as a control to assess endotoxicity. At post-treatment, apoptosis was examined by the DeadEnd fluorometric TUNEL system (Promega, Madison, WI) that used fluorescein12-dUTP incorporated fluorescently into the nicked DNA. Cells showing fluorescent DNA staining over a background fluorescence of non-treated control cells at identical fluorescent filter settings were considered TUNEL-positive cells, and recorded by double blind counting for statistical analysis using Student's *t*-test. Significance was determined at $P < 0.05$. In independent experiments, the activity of caspase-3 in the presence or absence of pre-treatment with caspase-8 inhibitor z-IETD-FMK (10 μ M; R&D Systems, Minneapolis, MN) was examined after SuperFasL treatment using Alexa Fluor 488-conjugated primary anti-caspase-3 antibody (Asp175, 1:200; Cell Signaling Technology, Danvers, MA), which only detects the activated form of caspase-3.

Immunoprecipitation and Western Blotting

For immunoprecipitation of receptor-interacting protein 1 (RIP1), cellular lysates from 661W cells were prepared using the ProteoJET mammalian cell lysis reagent (Fermentas, Glen Burnie, MD) plus EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Each lysate sample was mixed with a 50 μ L Dynabeads protein G suspension (Invitrogen) that was pre-bound with the mouse monoclonal anti-RIP1 antibody (Santa Cruz Biotechnology) at room temperature for 10 minutes. An equal aliquot volume of lysates was saved at the same time to be used as an input control. After incubation, the supernatants were removed after separation from the beads with a magnetic device. The beads-antibody-antigen complex was washed 3 times to remove any non-specific binding and the antibody-antigen complex was eluted finally at a final 20 μ L volume. To examine the expression of soluble FasL, confluent 661W cells were serum starved under dark for 24 hours, followed either by light exposure or continuing darkness for an identical duration. Conditioned medium was collected and concentrated using size-exclusion concentrators (9kD cutoff; Pierce, Rockford, IL). Using the same pools of cells for collection of conditioned medium, total membrane proteins, plasma membrane proteins, and cytosolic proteins were extracted sequentially using the protein extraction kit (Abcam). Extracted membrane proteins were dissolved in 0.5% Triton X-100 in PBS. The protein concentration of samples was determined using the microBCA protein assay (Thermo Scientific, Pittsburgh, PA). Heat-denatured protein samples were separated by SDS-PAGE under reduced condition using 10% Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA), followed by transferring to a polyvinylidene fluoride (PVDF) membrane for Western blotting. The following additional primary antibodies were used: mouse monoclonal anti-RIP1, goat polyclonal anti-FasL (Q-20, 1:2000; Santa Cruz Biotechnology), polyclonal HRP-conjugated anti-phosphoserine (Enzo Life Sciences), rabbit polyclonal anti-rat FasL and mouse monoclonal anti-Fas-associated death domain adaptor protein (FADD, 1F7, 1:2000; Millipore, Billerica, MA), and mouse monoclonal anti- β -actin (Cell Signaling Technology). Secondary antibodies were anti-rabbit, anti-mouse, or anti-goat IgG-HRP. Signals were developed using the ECL advance Western blotting detection kit (GE Healthcare, Piscataway, NJ). Densitometry analysis was conducted on digital images of the blots using the Kodak Molecular Imaging software.

Fluorescence Activated Cell Scanning (FACS) Analysis of Apoptosis and Necrosis

To detect the activity of activated caspase-3, 661W cells were pre-treated with z-IETD-FMK, or left untreated for 1 hour in darkness, followed by treatment with recombinant SuperFasL as described in the previous section. At 48 hours post-treatment, the cells (1×10^6) from

each sample were harvested, washed in PBS and incubated with 1 μ L FITC-DEVD-FMK inhibitor (EMD Chemicals, Inc., Gibbstown, NJ) in 300 μ L cell suspension in PBS at 37°C for 30 minutes. The cells were washed twice in a wash buffer supplied by the manufacturer, and resuspended in the same wash buffer for quantitative detection of activated caspase-3. To detect light-induced apoptosis and necrosis, 661W cells were treated alone with necrostatin-1 (Nec-1, 10 μ M; Santa Cruz Biotechnology), or in combination with z-IETD-FMK (10 μ M), pan-caspase inhibitor z-VAD-FMK (10 μ M; Promega), 10 μ g/mL of functional grade mouse anti-FasL monoclonal antibody MFL3 or the Armenian hamster IgG as a corresponding negative control (10 μ g/mL for each; eBioscience, San Diego, CA). Cells (2×10^6) were harvested after light exposure, washed with ice-cold PBS, and divided into two separate sample pools. One was labeled with FITC-DEVD-FMK to detect activity of activated caspase-3 and the other one was labeled with Alexa Fluor 488 annexin V/propidium iodide (PI) using a Dead Cell Apoptosis Kit (Invitrogen) following the manufacturer's protocol. Labeled cell samples were analyzed by a Beckman Coulter Cyan ADP flow cytometer. Apoptotic cells were identified when showing positive labeling for Alexa Fluor 488 annexin V only. Early necrotic cells were identified that were positive for annexin V and PI labeling. Dead cells were identified as positive labeling for PI. Cells that showed negative labeling for annexin V and PI labeling were identified as viable cells. Non-stained cells, or cells stained only with annexin V or PI under continuous darkness were used to set gates for background fluorescence and calibrate the cytometer. Fluorescent emission detection was measured at 530 nm for FITC and Alexa Fluor 488, and at 575 nm for PI using a 25 mW solid-state laser. Summit v. 4.3 software was used for data acquisition and analysis.

Live Cell Fluorescent Labeling

661W cells were seeded on 6-well plates at a density of $\sim 2 \times 10^5$ in regular growth medium with 10% FBS. The next day the cells were washed with pre-warmed serum-free basal DMEM+GlutaMAX-1 medium, replenished with the same medium containing 10 μ M CellTracker Blue fluorescent dye (Invitrogen), and incubated at 37°C for 1 hour. The dye did not affect the viability of cells and could be retained in cells with strong fluorescent signal for at least 72 hours. Dye-labeled cells were washed finally at the end of labeling, followed by incubation for 17 hours at 37°C with the conditioned medium or suspension of cells from light-exposed cells in the presence or absence of co-treatment with the functional grade mouse anti-FasL monoclonal antibody MFL3. Controls were dye-labeled and dye-free cells incubated with either the conditioned medium or the suspension of cells that had remained in continuous darkness. FACS was performed using a 405 nm 50 mW solid-state laser to excite the dye. Two sub-groups of dye-labeled cells were sorted that either had dye labeling indicative of viable cells, or had dye and Alexa Fluor 488 annexin V labeling indicative of apoptotic cells.

Statistical Analysis

P value was calculated from the paired *t*-test on the presumption that there was no prediction of the results on any two groups of samples. Difference with significance was defined when $P < 0.05$. Data points were expressed as value $\pm$ SD as needed.

RESULTS

Fas Is Expressed in Photoreceptors

To detect endogenous expression of Fas in human retina, FISH was carried out in human retinal samples from donor eyes (Fig. 1). Using biotin-labeled DNA probes complementary to human Fas cDNA, strong fluorescent staining for Fas was found in the outer nuclear layer, suggesting that Fas is a transcribed gene in photoreceptors. Very faint and spotted staining also was found

FIGURE 1. In situ hybridization of Fas in human retina. Postmortem human eye vertical sections were hybridized with biotin-labeled anti-Fas DNA probe (*top panel*) or without probe as negative control (*bottom panel*). DAPI was used to stain nucleus and identify layers of neural retina. Two cycles of staining with fluorescein avidin DCS and biotinylated anti-avidin were used to increase the detection sensitivity (see merged picture with *arrow* to show Fas expression). RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer.

in some nuclear bodies in the inner nuclear layer. No apparent staining was found in the remaining retinal layers. To corroborate further the FISH results at the protein levels, immunofluorescent labeling experiments were done in human retinas as well as in the mouse-derived photoreceptor cell line (661W, Fig. 2). Consistent with the FISH results, Fas expression was found in the outer segments of photoreceptor layer (Fig. 2A). Co-labeling experiments with the fluorophore-conjugated general cone identification marker, Alexa Fluor 488-PNA, showed co-localization with the Fas signal (Fig. 2A, merged picture). This result was evaluated further and confirmed in another independent co-labeling experiment using the antibody for blue opsin (Fig. 2B). In fixed 661W cells, strong expression of Fas was found on the cell surface (Fig. 2C). Co-labeling experiments with the ion channel protein Na^+/K^+ ATPase $\alpha 3$,³³ a cell surface marker, further confirmed the localization of Fas at the plasma membrane (Fig. 2C, merged picture).

Recombinant FasL or Fas Agonistic Antibody Treatment Induces Apoptosis

Given the expression of Fas in photoreceptors, we investigated whether the Fas pathway can be activated to induce apoptotic cell death in response to recombinant FasL. The morphology of FasL-treated 661W cells at 48 hours exhibited phenotypic signs of apoptosis that included clumping, shrinkage, loss of cellular adhesion, and cytoplasmic condensation (data not shown). Apoptosis was confirmed further by TUNEL assay (Fig. 3A). To assess the possibility of non-specific endotoxic effects from FasL, cells were pre-treated with NOK-2, an anti-FasL neutralizing antibody followed by ligand treatment (Fig. 3B). In this case, the number of TUNEL-positive cells was reduced substantially. Untreated cells or cells that were treated only with NOK-2 did not show any increase in TUNEL-positive cells (Figs. 3C, 3D). In independent experiments, 661W cells also were treated with the anti-Fas monoclonal antibody Jo2, which acts as a Fas agonist. Similar to the FasL treatment, a significant number of TUNEL-positive cells were observed (Fig. 3E). Virtually no TUNEL-positive cells were observed in the isotype IgG-treated cells at an equivalent dose (Fig. 3F). Quantitative

analysis indicated that there was a correlation between the dosage of FasL (up to 1 $\mu\text{g}/\text{mL}$) and the number of TUNEL-positive cells. The maximum percentage of TUNEL-positive cells was nearly 65% (Fig. 3G). FasL treatment at higher dosage (up to 3 $\mu\text{g}/\text{mL}$) did not increase further the number of TUNEL-positive cells, indicating a saturation effect of apoptosis induction by the ligand. In the Fas agonist Jo2-treated cells at 2–5 $\mu\text{g}/\text{mL}$, 40% of cells were TUNEL-positive. Higher dosages of Jo2 were cytotoxic because the isotype IgG2 serum at equivalent dose increased cell death. These results from *in vitro* treatments indicate that Fas is functionally active in photoreceptors and has a pro-apoptotic role after engagement with the ligand.

FasL-Induced Apoptotic Cell Death Involves Caspase-8 Dependent Caspase-3 Activation

To determine whether caspase-8 is required in FasL-induced apoptotic death, 661W cells were pre-treated with the caspase-8 inhibitor z-IETD-FMK for 1 hour before stimulation by the recombinant FasL. At 48 hours post-treatment, activation of the executioner caspase-3 was detected by immunofluorescence using an antibody that only detects the activated form of caspase-3 (Fig. 4). Non-treated cells showed very low fluorescent signal for this protease (Fig. 4A). In sharp contrast, ligand-treated cells showed intense and patchy fluorescent labeling (Fig. 4B). In the presence of z-IETD-FMK, the distribution and intensity of the fluorescent signal was reduced greatly (Fig. 4C). Only random and weak fluorescent signal was observed in the multiple non-overlapping visual fields. To corroborate this result quantitatively, the cells under identical treatments were labeled with the cell permeable FITC-conjugated caspase-3 inhibitor, DEVD-FMK, followed by FACS analysis. At baseline control, non-treated cells with FITC-DEVD-FMK binding were approximately 17% of counted cells (R4, Fig. 4D). Ligand-treated cells showed a significant increase of fluorescence to approximately 35% in the same numbers of counted cells (Fig. 4E). Cells pre-treated with z-IETD showed substantial reduction of FITC-DEVD-FMK binding to approximately 22% of counted cells (Fig. 4F). Together, results from immunofluorescent labeling and FACS analysis indicate strong-

FIGURE 2. Expression of Fas in human retina. (A) Postmortem human eye vertical sections were labeled immunofluorescently for Fas (red). Labeling indicated Fas was present mostly in the photoreceptor outer segment layer. Alexa Fluor 488-conjugated PNA (green) was used to identify cone photoreceptors (A). (B) A sub-type of cones was identified to express Fas (green) in adjacent sections by co-labeling with the anti-blue opsin antibody (red) and Fas (green). (C) Fixed 661W cells were labeled immunofluorescently for Fas (green) and Na⁺/K⁺ ATPase α3 (red) as a plasma membrane marker. DAPI was used in all labeling to reveal the nucleus at mounting. OS, outer segment; IS, inner segment; GCL, ganglion cell layer.

ly that Fas-mediated apoptotic death in photoreceptors follows a caspase-8-dependent process involving activation of downstream executioner caspase-3.

FWL Induces Apoptosis and Necrosis in 661W Cells

The 661W cells were subjected to FWL exposure with the goal of understanding whether Fas signaling participates in phototoxicity-mediated cell death. FWL induced apoptosis and necrosis in 661W cells. The kinetics of the two death programs, apoptosis and necrosis, were examined by FACS in these cells after light exposure. Alexa Fluor 488-annexin V labeling was used to detect early apoptosis, and PI was used to identify necrotic/dead cells. Light exposure for as early as 2 hours induced apoptosis in approximately 3% of cells and apoptosis approached 16% of cells by 4 hours (data not shown). Prolonged light exposure beyond 4 hours did not enhance further the proportion of cells undergoing apoptosis, but instead induced massive cell lysis. Light exposure also induced necrotic cell death, which could be detected in approximately 7% of cells from 2 hours of light exposure. The proportion of necrotic death increased to nearly 14% by 4 hours. To keep the apoptotic and necrotic death within a dynamic range, light exposure was restricted to 2–4 hours. Compared to light exposure alone, co-treatment of cells with

the caspase-8 inhibitor z-IETD-FMK, pan-caspase inhibitor z-VAD-FMK, or anti-FasL neutralizing antibody MFL3 significantly reduced light-induced annexin V labeling (PI-) as indicative of apoptosis (Fig. 5, compare B–E, and quantitative comparison in G). Intriguingly, under all three treatment conditions, there was a simultaneous fluorescent increase of PI (Fig. 5, compare cells in the top half of the left bottom quadrant in C–E with B), indicating the occurrence of a permeabilized membrane. This result suggests that, while prevention of FasL engagement with Fas or inhibition of caspase-8 decreases apoptosis, it simultaneously induces the activation of a secondary cell death pathway.

RIP1 Is Involved in Necrotic Death under Light Stress

Evidence has suggested that necrosis can be a form of regulated cell death (necroptosis).³⁴ RIP1, which acts downstream of the death receptors, is a key regulator of necroptosis.³⁵ Pharmacological inhibition of RIP1 to block its kinase activity by the specific inhibitor Nec-1 can prevent necroptosis.³⁶ To investigate whether the secondary death phenotype observed after inhibition of FasL-Fas engagement, or after inhibition of caspase-8 under light involves RIP1-mediated necroptosis, 661W cells were co-treated with Nec-1

FIGURE 3. TUNEL assay from recombinant FasL or Fas agonist Jo2-treated 661W cells. Nearly confluent 661W cells grown in chamber slides were treated with recombinant soluble SuperFas ligand (1 $\mu\text{g}/\text{mL}$) alone (A) or together with the FasL neutralizing antibody NOK-2 (B) for 48 hours. Non-treated cells (C) or cells treated only with NOK-2 (D) were controls. In separate experiments, cells were treated with 5 $\mu\text{g}/\text{mL}$ Fas agonist Jo2 (E) or with an identical dose of isotype IgG2 as control (F) for 6 hours. All samples were examined under multiple non-overlapping visual fields. DAPI staining under each treatment condition was shown for comparison. (G) Results for quantitative analysis of TUNEL-positive cells were shown ($^*P < 0.05$).

FIGURE 4. Inhibition of caspase-8 prevented FasL-induced activation of caspase 3. Near confluent 661W cells grown in chamber slides were untreated (**A**) or pretreated with the caspase-8 inhibitor z-IETD-FMK (10 μ M) for 1 hour (**B**) before the addition of recombinant soluble SuperFas ligand (875 ng/mL). Cells were fixed at 48 hours post-treatment and labeled with the Alexa Fluor 488-conjugated caspase-3 antibody, which only detects the cleaved (active) form of this protease (*green*). In separate pools of cells, activation of caspase-3 was analyzed quantitatively by FACS after incubation with FITC-DEVD-FMK in un-treated cells (**D**), or cells treated with FasL alone (**E**) or together with z-IETD FMK (**F**).

in the presence of MFL3 and/or z-IETD-FMK. We expected that co-treatment with MFL3 or z-IETD-FMK to inhibit activation of Fas and caspase-8 would result in enhanced cell death when compared to either inhibition alone. FACS analysis revealed that the sample groups of cells under light exposure alone, or when co-treated with MFL3 and/or z-IETD-FMK underwent significant re-distribution of annexin V and PI labeling. (Fig. 6, compare B-E). In particular, light alone induced a mixture of labeled cells that either were viable (annexin V-/PI-), apoptotic (annexin V+/PI-), or early necrotic (annexin V+/PI+). When co-treated with MFL3 and/or z-IETD-FMK, there was a substantial increase of PI+ dead cells without significant loss of apoptotic protection. This death phenotype was more prominent under co-treatment with MFL3 and z-IETD (Fig. 6E), in which the majority of cells (94%) were PI positive. In sharp contrast, co-treatment with Nec-1 dramatically diminished PI-positive labeling, and shifted the majority of cells (89%) to a viable state (Annexin V-/PI-) and a small portion of cells (6%) to apoptosis (Annexin V+/PI-, Fig. 6F), indicating that the predominant PI-positive labeling for dead cells under inhibition of Fas/caspase-8 involves necroptotic cell death mediated by RIP1. A quantitative comparison of the flow cytometry results (PI+) is presented in Figure 6G. Nec-1 treatment alone resulted in partial prevention of primary necrotic cell death by light as demonstrated by reduced labeling for annexin V and PI (data not shown), suggesting that cell death through necroptosis

also is involved in photoreceptors under light stress. In the control experiment, a Nec-1 inactive analog did not change significantly the overall viability or death profiles of cells compared to cells under light alone (data not shown), further reinforcing the specific ability of Nec-1 to prevent necroptosis and preserve cell viability, when used in combination with MFL3 and z-IETD-FMK.

To investigate further whether RIP1 is subject to regulation by light, we assessed its expression and phosphorylation status in 661W cells. By Western blot analysis after normalization with the expression level of β -actin, expression of RIP1 was similar under conditions of continuous darkness or light exposure alone, or in the presence of co-treatment with the MFL3 antibody or z-IETD-FMK (data not shown). To examine the phosphorylation status, RIP1 was immunoprecipitated and then probed with the anti-phosphoserine antibody. Light stress did not induce any change in serine phosphorylation in this adaptor protein (Fig. 6H, top panel, lane 2). However, under treatment with the z-IETD-FMK, RIP1 was hyper-phosphorylated (Fig. 6H, top panel, lane 7). This finding corroborates the results from FACS analysis and indicates that hyper-phosphorylation of RIP1 contributes to the induction of necroptosis when caspase-8 is inhibited. Interestingly, inhibition of Fas activation by the anti-FasL antibody MFL3 did not alter significantly the phosphorylation of RIP1 (Fig. 6H, top panel, lane 6), suggesting that anti-Fas

FIGURE 5. FACS analysis of early apoptosis and necrosis. 661W cells in serum-free medium were treated in darkness (A), or exposed to light (B), light + 10 μ M caspase-8 inhibitor z-IETD-FMK (C), light + 10 μ M pan-caspase inhibitor Z-VAD-FMK (D), light + anti-FasL monoclonal antibody MFL3 (E), or light + Armenian hamster IgG as control for MFL3 (F) for 3 hours. Cells were harvested and labeled with Alexa Fluor 488 annexin V and PI to detect early apoptosis and necrosis, respectively, by FACS. In each histogram, the left bottom gate is defined as viable, non-apoptotic cells that were negative for labeling of annexin V (*x-axis*) and PI (*y-axis*). Early apoptotic cells show positive labeling for annexin V only (*bottom right quadrant*), and early necrotic cells show positive labeling for both annexin V and PI (*top right quadrant*). (G) Quantitative comparison of apoptotic cells from the FACS results was shown. D, dark; L, light.

FIGURE 6. Necrostatin-1 prevented secondary death by co-treatment with anti-FasL antibody (MFL3) and caspase-8 inhibitor z-IETD under light. 661W cells were treated in darkness (A), or exposed to light (B), light + MFL3 (C), light + z-IETD (D), light + MFL3 + z-IETD (E), or together with Nec-1 (F), followed by FACS analysis. (E) Cells showing PI+ labeling were compared quantitatively ($P < 0.05$). (F) Phosphorylation of RIP1 was detected by immunoprecipitation (IP) of RIP1, followed by blotting with anti-phosphoserine antibody. Aliquots of cell extracts before IP were used as input control to normalize expression of RIP1. The following experimental conditions were applied: Dark (lane 1), light (lane 2), light + Nec-1 (lane 3), light + Nec-1 + MFL3 (lane 4), light + Nec-1 + z-IETD (lane 5), light + MFL3 (lane 6), and light + z-IETD (lane 7). The densitometry ratio of P-serine (RIP1)/RIP1 in each treatment was shown underneath the signal band.

induced secondary necroptosis is independent on the kinase activity of RIP1.

A Cleaved FasL Is Anchored on the Cell Surface

We assessed the expression of the key component proteins in the Fas pathway. Conditioned medium from 661W cells was harvested and concentrated after light stress or under

continuous darkness to detect the expression of the soluble FasL (sFasL) by Western blot (Fig. 7). No sFasL was detected. However, membrane-bound (m)FasL at the expected molecular weight of 40 kDa and sFasL (27 kDa) were detected in the cytosolic fraction. No mFasL was detected in the prepared plasma membrane fraction. However, a 27kD processed protein similar in molecular weight to the reported membrane-inserted sFasL³⁷ was observed repeatedly, suggesting

FIGURE 7. Light stress-induced proteolytic degradation of FasL, Fas, and FADD. Cytosolic (A) and membrane (B) fractions of 661W cells were prepared under continuous dark, after light exposure for 3 hours (L3) or 6 hours (L6) to detect expression of Fas, FasL, and FADD by Western blotting. Expression of β -actin was used as loading control.

that the cytosolic FasL is cleaved proteolytically but remains membrane bound. No significant difference in expression for FasL was found between light stress within 3 hours and continuous darkness. Fas expression was found in the cytosolic and membrane fractions. Prolonged light stress significantly reduced expression of FasL, Fas, and FADD as well as β -actin, suggesting the activation of general proteolysis.

The Cleaved FasL Contributes to Fas-Mediated Apoptotic Death through a Paracrine Mechanism

To understand whether the processed FasL identified on the membrane is able to induce Fas-mediated apoptosis, a live cell labeling experiment was performed. Cells were labeled with the fluorescent cell tracker blue dye, followed by incubation with light-exposed cells or their conditioned medium for apoptotic evaluation by FACS. Dye-labeled cells co-incubated with the medium or cells that had been left in darkness showed

very few apoptotic cells, 0.34% and 1.9%, respectively (Figs. 8A, 8B, in top right quadrant). However, dye-labeled cells co-incubated with conditioned medium or cells that were exposed to light showed a significant elevation in numbers of apoptotic cells to 59% and 39%, respectively (Figs. 8C, 8D). This apoptotic response was attenuated partially with the anti-FasL antibody MFL3 (Figs. 8E, 8F). These findings suggest that a soluble form of FasL is present in the medium and that, under light stress, this soluble form of FasL and the cleaved FasL on the cell surface are active in engagement with Fas to initiate apoptotic death through a paracrine mechanism.

DISCUSSION

Phototoxicity can be used as a model to study apoptotic death of photoreceptors that occurs in inherited retinal dystrophies in humans. Over-activation of the visual cycle pathway under light stress is a prerequisite to initiate the apoptotic death

FIGURE 8. A paracrine mechanism of Fas-mediated apoptosis. CellTracker blue dye-labeled live 661W cells were incubated with conditioned medium or harvested cells that had been left in continuous darkness (A, B), light exposure (C, D), or light exposure + anti-FasL antibody MFL3 (E, F). The blue dye-labeled cells then were co-labeled with Alexa Fluor 488 annexin V to detect apoptosis by FACS. Apoptotic cells were counted if the cells retained the dye (*y-axis*) and were positive for Alexa Fluor 488 annexin V labeling (*x-axis*). Med, medium.

signal.³⁸ Several mediators and the intrinsic signaling events to execute the apoptotic cascade through either caspases or caspase-independent pathways have been identified.^{39–42} However, it remains unknown whether light-induced apoptosis in photoreceptors also can be mediated by an extrinsic death pathway through the conventional death receptors. The FasL-Fas pathway is an intriguing candidate to investigate this possibility because of its established role in execution of an extrinsic apoptotic death signal.

In our study, we first showed by *in situ* hybridization and immunofluorescent studies that Fas is expressed predominantly in normal human photoreceptors. In 661W photoreceptor cell cultures, as in other CNS neurons,⁴³ Fas is a cell surface receptor. The neuronal-like morphology of 661W cells, and the expression of green and blue opsins⁴⁴ make this cell line a good cell culture model to investigate the biology of this prototype death receptor in photoreceptors. 661W cells have been well validated and the findings can be related to *in vivo* photoreceptor death in light-induced retinal degeneration or RP. Examples include studies to characterize the protective role of NF- κ B signaling against apoptosis,⁴⁵ test efficacies of pharmacological compounds to preserve photoreceptor sur-

vival,^{46,47} and assess cGMP-gated calcium channel in oxidative stress response.⁴⁸ In our investigations, we pursued *in vitro* and *in vivo* studies. We demonstrated the expression of Fas in the photoreceptor layer of human retina and as a cell surface protein in 661W cells. *In vitro*, 661W cells treated with recombinant FasL or Fas agonistic antibody induced apoptosis. We thoroughly evaluated and validated the results with a set of independent experiments, confirming the pro-death role of Fas. We also showed that inhibition of caspase-8 significantly prevented ligand-induced activation of caspase-3, demonstrating that Fas-mediated apoptosis in photoreceptors is a caspase-dependent process (Fig. 4). The FACS analysis revealed that there were fewer cells with activated caspase-3 after FasL treatment than the total number of TUNEL-positive cells. This may be due to Fas-mediated activation of other caspases⁴⁹ or activation of caspase-8-independent pathways.⁵⁰

In the light stress experiment, we showed that inhibition of ligand-dependent Fas activation or of caspase-8 substantially prevented 661W cells from undergoing apoptosis, suggesting that Fas activated apoptosis through the canonical death pathway. However, this inhibition of apoptosis was associated with a simultaneous induction of necroptosis, which could be

FIGURE 9. Proposed model of Fas-mediated death of photoreceptors under light stress. Intense and prolonged light causes damage of photoreceptors by photo-oxidative stress via generation of ROS. The toxic all-*trans* retinal (ATR) and retinal derivatives generated during photoisomerization of the chromophore 11-*cis* retinal also render photoreceptors vulnerable to death. Early damaged photoreceptors can have disorganized outer segments, followed by their reduction and loss. Eventual death of photoreceptors can be executed by apoptosis through the “intrinsic” pathway as shown. Necrosis as demonstrated in this study and activation of the scavenging pathway by autophagy also are involved. We also demonstrate that apoptosis in photoreceptors under light stress occurs through the “extrinsic” FasL/Fas pathway involving activation of downstream caspases. While inhibition of caspases or Fas can prevent apoptosis by light, it simultaneously induces secondary cell death by necroptosis.

prevented by treating the cells with the RIP1 inhibitor, Nec-1. Consistent with this finding, inhibition of caspase-8 concomitantly triggered hyper-phosphorylation of RIP1. Since phosphorylation of RIP1 activates its pro-necrotic kinase activity,⁵¹ hyper-phosphorylated RIP1 under conditions of caspase-8 blockade triggered a switch from apoptosis inhibition to necroptosis induction. Interestingly, we found that phosphorylation of RIP1 was unchanged in cells treated with the anti-

FasL neutralizing antibody. There are two possibilities to interpret the RIP1 phosphorylation-independent induction of necroptosis. One is that another RIP kinase(s) might be involved that induces necroptosis when Fas is inhibited. Another possibility is that the necrotic activity of RIP1 can be regulated through interaction with other death signaling proteins. Similar to our finding, a recent study has shown that administration of Nec-1 can prevent necrotic death of photoreceptors in retinal detachment.⁵² Therefore, it appears that RIP1 can regulate necroptotic death in photoreceptors under different photoreceptor pathologies.

We propose two models that relate the Fas pathway to the apoptotic death of photoreceptors under light stress (Fig. 9 and legend). The first model is ligand (FasL)-dependent, by which Fas is activated to initiate apoptosis through caspases upon binding with the sFasL released into the medium by proteolytic processing of the full-length FasL. Alternatively, in this model, the cleaved form of FasL anchored on the cell surface also may activate Fas through a juxtacrine manner.⁵³ The second model is ligand-independent, in which Fas is subjected to redox-based structural modifications through ROS in photoreceptors under photo-oxidative stress by light.^{54,55} Although ROS can activate independently the caspase cascade to induce apoptosis,^{56,57} studies by Janssen-Heininger et al. have shown that reactive free radicals, such as nitrogen dioxide, a potent oxidant similar to ROS, can oxidize Fas directly, which facilitates the recruitment of FADD leading to ligand-independent cell death.⁵⁸ Their further studies have shown that S-glutathionylation of specific cysteine residues in Fas promotes activation of caspases and formation of the death-inducing signaling complex (DISC).⁵⁹ Other studies have shown that administration of ROS scavengers can inhibit Fas-stimulated formation of the apoptosome in Jurkat cells.⁶⁰ Hence, future studies investigating redox regulation of Fas may provide additional insights into its role in photoreceptor death associated with oxidative stress in different retinal degenerative diseases. Our study highlights the importance of combined inhibition of apoptosis and necroptosis in prevention of the death of photoreceptors under light stress.

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