Fas Mediates Apoptosis and Oxidant-Induced Cell Death in Cultured hRPE Cells

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PURPOSE. To investigate whether Fas ligand (FasL) and the Fas receptor system mediates apoptosis in cultured human retinal pigment epithelial (hRPE) cells and contributes to oxidant-induced death of hRPE cells.

METHODS. Expression of FasL and Fas in cultured hRPE cells was examined by Western blot analysis and flow cytometry. The susceptibility of hRPE cells to Fas-mediated apoptosis was determined by incubating cells with recombinant soluble Fas ligand (sFasL). Characteristics of apoptosis assessed included chromatin condensation, DNA cleavage, and phosphatidylserine exposure. To investigate the possible involvement of Fas-mediated apoptosis in oxidative killing of hRPE cells, the effects of the oxidant tert-butylhydroperoxide (tBH) on the expression of FasL and Fas were studied. The specificity of effects of oxidant was examined using the antioxidants glutathione and N-acetylcysteine (NAC). The requirement for the Fas pathway in tBH-induced apoptosis was investigated using an antagonistic anti-Fas antibody ZB4 that blocks the interaction between FasL and Fas.

RESULTS. Cultured hRPE cells constitutively expressed FasL and Fas. Ligation of Fas receptor with recombinant sFasL triggered apoptosis in hRPE cells. tBH treatment of hRPE cells resulted in increased expression of FasL and Fas. Glutathione and NAC completely abrogated tBH-induced increase in FasL and Fas expression and apoptosis. Blocking FasL and Fas interaction by ZB4 inhibited tBH-induced apoptosis, but only partially.

CONCLUSIONS. A functional Fas-mediated apoptotic pathway is present in cultured hRPE cells and can be activated with sFasL or by upregulation of FasL and Fas expression with an oxidant. The incomplete inhibition by blocking antibody indicates that the Fas pathway is involved in oxidant-induced apoptosis, but other triggering mechanisms are also important. (Invest Ophthalmol Vis Sci. 2000;41:645–655)

The retinal pigment epithelium (RPE) is a single layer of cuboidal cells situated external to the photoreceptor layer of the retina and internal to Bruch’s membrane.1 The function of the RPE is to maintain and support the photoreceptors by phagocytosis and degradation of debris shed from retinal outer segments. Therefore, injury to the RPE results in degeneration of the sensory retina and may contribute to the pathogenesis of age-related macular degeneration (ARMD), the leading cause of blindness in the developed world.2

The molecular mechanism for the injury to RPE cells is not understood clearly. We have recently shown that the pro-oxidant tert-butylhydroperoxide (tBH) induces apoptosis in cultured hRPE cells.3 Apoptosis is a form of cell death characterized by chromatin condensation, DNA fragmentation, and phosphatidylserine (PS) externalization.4 Besides pro-oxidants, other stimuli such as experimental ischemia, light exposure, and protein kinase inhibitors are also reported to trigger apoptosis in vitro.5–7 In the highly vascularized choroidal neovascular membrane surgically excised from eyes of patients with ARMD, extensive apoptosis in RPE cells has been observed, suggesting that apoptosis of RPE cells is involved in the pathogenesis of ARMD.8

In many systems, apoptosis is mediated by the interaction between Fas ligand (FasL) and Fas.7–10 FasL is a member of the tumor necrosis factor family of cytokines. Its receptor Fas belongs to the tumor necrosis factor receptor superfamily. Binding of FasL, or an agonistic anti-Fas antibody to Fas initiates a signal transduction pathway for apoptosis in susceptible cells. Fasl and Fas are constitutively expressed in a wide range of tissues. The Fas system provides an important mechanism for regulation of immune response and killing of tumor cells. A malfunction of Fas system has been found to contribute to the development of many diseases including cancer, acquired immune deficiency syndrome, hepatitis, and autoimmune diseases.9–10

A substantial expression of Fasl has been found in the corneal epithelium and endothelium, iris, ciliary cells, and retina of eyes and has led to the proposal that Fasl participates in maintenance of the immune privilege of the eye by inducing apoptosis in infiltrating, Fas-bearing, activated lymphocytes.11,12 In the normal eye, Fasl is weakly expressed in the RPE monolayer.13 However, an increased Fasl expression has been found in the RPE monolayer of the choroidal neovascular membranes from patients with ARMD.13 The FasL expressed on RPE cells may be necessary for immune privilege of the post-
rior of the eye. However, if its expression is upregulated or if the expression of Fas in RPE cells is increased, death signals could be activated. Affected cells may undergo apoptosis (suicide) or cause paracrine-induced death of neighboring cells (fratricide). Indeed, in the highly vascularized choroidal neovascular membrane from the eye of patients with ARMD, a correlation between Fas expression and the extent of apoptosis exists, although the association between FasL expression and apoptosis is not found.8 A study by Esser et al.13 has demonstrated that cultured human (h)RPE cells constitutively express Fas but are rather resistant to apoptosis by an agonistic Fas antibody. However, upregulation of Fas expression in hRPE by cytokines can overcome the resistance to the agonistic Fas antibody. However, upregulation of Fas expression in hRPE by cytokines can overcome the resistance to the agonistic Fas antibody.

Cytokine treatment is normally accompanied by oxidative stress, and reactive oxygen intermediates (ROI) are involved in the induction of FasL expression by cytostatic drugs and T-cell activation.14,15 Pro-oxidants are found to upregulate FasL expression and enhance Fas-mediated apoptosis.14,15 In Fas-mediated apoptosis, generation of ROIs is an early signaling event, and inhibiting its production with antioxidants can prevent apoptosis.16,17 All this evidence suggests that ROI is a key regulator of the Fas system. However, the contribution of the Fas pathway to oxidant-induced apoptosis in the RPE cells has not been established. Thus, in the present study, we investigated whether the Fas system mediates apoptosis in cultured hRPE cells and whether it could be modulated by an oxidant and contribute to oxidative injury of RPE.

**MATERIALS AND METHODS**

**hRPE Cell Cultures and Treatments**

hRPE cell cultures were established as previously described.18 Cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified incubator under an atmosphere of 5% CO2 in air. Cells were passaged every 10 days.18 For experiments, cells

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**FIGURE 1.** Expression of FasL and Fas on cultured hRPE cells. (A) Western blot analysis of total FasL and Fas protein. Lysates (50 µg protein) from hRPE cells were probed with mouse IgG to human FasL (250 ng/ml) or Fas (100 ng/ml). Lysates (10 µg protein) from human endothelial cells and human T leukemia Jurkat cells were used as positive controls for FasL and Fas, respectively. Note that the appearance of more FasL detection was due to longer exposure of x-ray film. (B) Flow cytometry analysis of surface FasL and Fas. To detect FasL, hRPE cells were incubated with a rabbit anti-human FasL antibody or with rabbit IgG, respectively, followed by an FITC-conjugated antibody against rabbit IgG. To detect Fas, hRPE cells were incubated with a mouse anti-human Fas antibody or with mouse IgG, respectively, followed by an FITC-conjugated antibody against mouse IgG. Cells were analyzed by flow cytometry. Data are presented as the number of cells plotted as a function of cell fluorescent intensity that is dependent on the amount of FasL or Fas expression in each cell. Representative data of five separate experiments are shown.

**FIGURE 2.** Chromatin condensation in sFasL-treated hRPE cells. hRPE cells were untreated or treated with 500 ng/ml sFasL for 48 hours. Cells were fixed, stained with PI, and visualized by confocal microscopy. Untreated cells had nuclei with diffuse chromatin, whereas sFasL-treated cells showed small nuclei with condensed chromatin (arrows). Data presented are representative of three independent experiments. Bars, 5 µm.
between passages 6 and 10 were taken and plated at the following densities: $2 \times 10^4$ cells/well in 300 μl medium for chambered coverglass (Lab-Tek 136439; Miles, Napierville, IL), $2 \times 10^5$ cells/well in 1.5 ml medium for 6-well plates, and $1 \times 10^6$ cells in 10 ml medium for a 100-mm culture dish. Experiments were begun 2 days after plating. To trigger apoptosis, stock solutions of tBH and recombinant sFasL (Oncogene, Cambridge, MA) were added to the RPE cell culture without changing the growth medium. To examine the effects of antioxidants and antagonistic anti-Fas antibody ZB4 (MBL, Woburn, MA), cells were pretreated with these agents for 1 hour and then incubated with tBH or sFasL for the different times indicated.

### Western Blot Analysis of FasL and Fas Protein

Cells were washed once with phosphate-buffered saline (PBS) and then lysed in a boiling solution containing 10% glycerol, 250 mM Tris (pH 6.8), 4% sodium dodecyl sulfate, and 2% β-mercaptoethanol. The cell lysates were immediately boiled for 5 minutes and centrifuged at 10,000g for 5 minutes at 4°C. The supernatant was collected, and the amount of protein was measured by the Bradford method. Protein extracts (50 μg/lane) were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel, and the separated proteins were blotted to 0.45 μm polyvinylidene difluoride membranes (Hybond; Amersham, Arlington Heights, IL). Nonspecific binding was blocked by incubating the membranes overnight at 4°C in blocking buffer containing 5% nonfat dry milk, 0.1% Tween-20, 10 mM Tris (pH 7.5), and 100 mM NaCl. The membranes were then stained with 1:2500 diluted anti-Fas antibody or 1:1000 diluted anti-FasL antibody (F37720 and F22120; Transduction Laboratories, Lexington, KY) in blocking buffer for 1 hour at room temperature with agitation. After they were washed three times with washing buffer containing 10 mM Tris (pH 7.5), 100
mM NaCl, and 0.1% Tween-20, the membranes were incubated with 1:3000 diluted horseradish peroxidase-coupled anti-mouse antibody in blocking buffer for 1 hour at room temperature. The specific proteins were then visualized by incubating the membranes with reagent (Renaissance Western Blot Chemiluminescence Reagent; NEN, Boston, MA) and exposing the membranes to film (X-OMAT; Eastman Kodak, Rochester, NY). Equal protein loading was verified by ponceau red treatment of membranes.

Flow Cytometric Detection of FasL and Fas on Cell Surface

Cells (2 × 10⁵) were washed twice in washing buffer containing 0.2% bovine serum albumin and 0.02% sodium azide in PBS and incubated with 2.5 µg/ml a rabbit anti-FasL antibody C20 (Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse anti-Fas antibody DX2 (Oncogene) for 30 minutes at 4°C. Cells were then washed, resuspended in 50 µl washing buffer containing 2.5 µg/ml fluorescein isothiocyanate (FITC)–conjugated anti-rabbit IgG or goat anti-mouse IgG, respectively, and incubated for 30 minutes at 4°C. Nonstaining cells, cells stained with the FITC-conjugated secondary antibodies alone, and cells stained with isotypically matched control immunoglobulin were run in parallel as negative controls. After staining, cells were washed twice in washing buffer and resuspended in PBS. Data acquisition and analysis were performed in a flow cytometer (FACScan using the CellQuest software; Becton Dickinson, Mountain View, CA).

Visualization of Apoptotic Nuclei by Confocal Microscopy

hRPE cells grown in chambered coverglass were incubated with sFasL for 48 hours. Cells remaining on the coverglass after treatment were washed twice with PBS and fixed with ice-cold 80% methanol for 30 minutes. Cells were then gently washed three times and stained with 50 µg/ml propidium iodide in PBS for 5 minutes at room temperature. The coverglass were analyzed by laser scanning confocal microscope (model 1024; BioRad, Richmond, CA).

Assessment of Apoptosis by TdT-Mediated dUTP Nick-End Labeling

TdT-mediated dUTP nick-end labeling (TUNEL) was performed using a kit (In Situ Cell Death Detection kit with fluorescein; Boehringer–Mannheim, Mannheim, Germany) according to the standard protocol provided by the manufacturer. Briefly, after treatment, both floating and adherent (released by trypsin) cells were collected and washed twice with PBS. Cells were then fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. To label DNA strand breaks, cells were incubated with 50 µl TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-dUTP in the binding buffer and incubated for 1 hour at 37°C in a humidified atmosphere. Cells were then washed twice with PBS and analyzed by flow cytometry. Alternatively, cells grown on chambered coverglasses were incubated with sFasL for 48 hours. Cells remaining on the coverglass were washed twice with PBS and stained with the TUNEL kit as described. The coverglasses were analyzed by laser scanning confocal microscopy.

Assessment of Apoptosis by Annexin V-FITC Staining of PS

Cells were stained with annexin V-FITC (TACS; Trevigen, Gaithersburg, MD) according to the manufacturer’s instructions. Briefly, after treatment, both floating and adherent cells were collected and washed twice with PBS. Cells were then resuspended in 100 µl binding buffer containing 10 mM HEPES-KOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 1 mg/ml annexin-V–FITC. After 15 minutes of incubation at room temperature in the dark, 400 µl binding buffer was added, and cells were analyzed by flow cytometry.

Total RNA Preparation and Reverse Transcription–Polymerase Chain Reaction for Fas mRNA and FasL mRNA

Total RNA was prepared with a kit (RNeasy, Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Single-stranded DNA was synthesized from RNA in a 15-µl reaction mixture containing 100 ng random hexamer and 200 units of murine MLV reverse transcriptase (Gibco, Grand Island, NY). The reaction mixture was diluted with 20 µl water and 2 µl of which was used for polymerase chain reaction (PCR). The PCR reaction mixture contained 10 picomoles each of forward and reverse primers and 2 units DNA polymerase (Taq, Perkin-Elmer, Branchburg, NJ). Amplification was performed for 30 cycles in a thermal cycler.
Each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 57°C, and 1 minute of extension at 72°C.

The sequences of primers used for analysis were as follows: Fas (forward: 5'-CGGAGGATTGCTCAACAAC-3', reverse: 5'-TTGG-TATTCTGGGTCCG-3'), FasL (forward: 5'-GTTTGCTGGGGCTGGCCTGACT-3', reverse: 5'-GGAAAGAATCCCAAAGTGCTTC-3'), and β-actin (forward: 5'-CGTGGGCCGCCCTAGGCAACCA-3', reverse: 5'-TGGGACCTAGGGTCAAGGGG-3'). The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining. The quantities of the PCR products were determined by densitometric scanning using Lynx software (Applied Imaging, Santa Clara, CA).

**RESULTS**

**Expression of Fasl and Fas in hRPE Cells**

Western blot analysis of total Fasl and Fas showed that both Fasl and Fas protein were detected in cultured hRPE cells (Fig. 1A). Fasl and Fas can be present within cells and on the cell surface, but the latter form is important in the transduction of the death signal. To determine whether Fasl and Fas are expressed on the cell surface of RPE, we treated intact cells with antibodies that react with Fasl and Fas, respectively, and analyzed the cells by flow cytometry (Fig. 1B). With anti-Fasl antibody C20, we were able to detect cell surface Fasl in untreated hRPE cells, although the expression level was low. Using anti-Fas antibody DX2, a relatively high expression of Fas was detected on the surface of hRPE cells.

**Apoptosis in hRPE Cells by Ligation of Fas Receptor**

To investigate whether the Fas expressed in cultured hRPE cells functionally transduces an apoptotic signal, cells were treated with recombinant soluble Fas ligand (sFasl) that binds to Fas and induces apoptosis in sensitive cells. Apoptosis was assessed by three criteria, chromatin condensation, DNA cleav-
Confocal microscopy images of cells stained with the DNA-intercalating dye propidium iodide (PI) illustrate that the nuclei of untreated cells had dispersed chromatin structure and visible nucleoli, whereas the nuclei of cells treated with sFasL became smaller in size and condensed—that is, sFasL-treated RPE cells underwent typical nuclear morphologic changes of apoptosis (Fig. 2).

Extensive DNA cleavage is another characteristic event that occurs in the cells undergoing apoptosis. The cleavage of the DNA yields double-strand breaks that can be detected after labeling the free 3'-OH termini with dUTP-FITC by TUNEL assay. The cells with DNA cleavage can then be analyzed in individual cells by confocal microscopy and in cell populations by flow cytometry. Confocal microscopy revealed only a background level of fluorescence in untreated cells, whereas, cells treated with sFasL had intensely labeled nuclei (Fig. 3A). When these cells were measured by flow cytometry, untreated cells had relatively little fluorescence compared with apoptotic cells. sFasL-induced apoptosis was shown by an increase in the number of cells staining with dUTP-FITC (region R2 in Fig. 3B) after treatment with 250 ng/ml and 500 ng/ml sFasL. Pretreatment of cells with 125 ng/ml ZB4, an antibody that blocks interaction between FasL and Fas, blocked the sFasL-induced increase in dUTP-FITC fluorescence. Thus, the results indicate that sFasL-induced apoptosis is mediated through the Fas receptor.

In cells undergoing apoptosis, the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-kDa, Ca2+-dependent, phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Therefore, staining cells by annexin V in conjugation with vital dye PI allows identification of early apoptotic cells (annexin V-positive and PI-negative). In the present study, untreated hRPE cells showed primarily low fluorescence for both annexin V-FITC and PI, indicating that
they were viable and not undergoing apoptosis (Fig. 4, lower left quadrant). After treatment with sFasL, the portion of cells staining with annexin V but not PI (early apoptotic cells, lower right quadrant) was increased as the concentration of sFasL increased, indicating sFasL treatment induced PS externalization in hRPE cells.

Taken together, these data demonstrate that hRPE cells possess a functional Fas-mediated apoptosis pathway and can be induced to undergo apoptosis by ligation of the Fas receptor.

**Induction of FasL and Fas Expression by tBH and Inhibition by Antioxidants**

Although RPE cells express both FasL and Fas, they may not undergo suicide or fratricide if their expression levels are lower than the threshold for initiating Fas ligation or if the Fas-mediated signaling cascade is inhibited by regulatory components. However, if stimuli can induce FasL or Fas expression in RPE and result in activation in the Fas-mediated signaling pathway, apoptosis may be triggered. Our previous study has shown that the oxidant tBH can induce apoptosis in cultured hRPE cells. Because oxidants upregulate FasL expression in other cell types, we investigated whether the Fas pathway could mediate tBH-induced apoptosis in cultured hRPE cells. We first examined whether tBH induces expression of FasL protein in cultured hRPE. By Western blot, an increase in the expression of total FasL was detected in tBH-treated hRPE cells after 1 hour, and it decreased after 4 hours (Fig. 5A).

When cell surface FasL was measured by flow cytometry, the histogram, plotted as FasL expression against cell number, demonstrated that untreated hRPE cells showed a basal expression of cell surface FasL. After tBH treatment, hRPE revealed a marked upregulation in FasL expression (Fig. 5B). The increase started 1 hour after treatment and continued to increase during incubation up to 6 hours. Dot plot analysis of FasL expression versus forward side scatter (FSC, indicating cell size) showed that the cells undergoing apoptosis after tBH treatment (shown here as cell shrinkage with decreased FSC) had higher FasL expression.

We next examined whether the induction of FasL by tBH was due to enhanced transcriptional activation. hRPE cells were treated with tBH for various times, and mRNA was isolated, reverse transcribed, and amplified using FasL-specific primers. As shown in Fig. 5C, the FasL-specific PCR product was detected at a very low level in untreated cells, whereas tBH treatment resulted in a substantial increase (3.1-fold) in FasL expression after 1 hour. In contrast, expression of β-actin mRNA, which was measured as a control for equal loading and integrity of the RNA, was not affected by tBH treatment.

To obtain further insight into the mechanism of tBH-induced apoptosis, we also analyzed Fas expression on tBH treatment. The Western blot data show that tBH treatment of RPE cells caused an increase in total Fas expression at 3 hours (Fig. 6A). T BH also induced cell surface Fas expression (Fig. 6B). The increase in cell surface Fas began 1 hour after treatment and occurred earlier than the increase in total Fas. RT-PCR data demonstrated that tBH-induced upregulation of Fas was also associated with transcriptional activation of gene expression (1.4-fold increase in Fas mRNA, Fig. 6C). Together, these results suggest that hRPE cells may first translocate the preexisting intracellular Fas to the cell surface and then transcribe and translate more Fas. Compared with tBH-induced FasL expression, the effect of tBH on Fas was less prominent.

The specific effect of tBH as an oxidant dependent activation of the expression of FasL and Fas was then determined using the antioxidants glutathione (GSH) and N-acetylcysteine (NAC). Cultured hRPE cells were first treated with GSH or NAC for 1 hour and then incubated with tBH. As shown in Figure 7A, pretreatment of hRPE with GSH or NAC blocked induction of FasL and Fas by tBH. When apoptosis was determined in these cells by the TUNEL assay, it was found that the antioxidants also blocked tBH-induced apoptosis (Fig. 7B). This protection could also be seen by light microscopy, in which tBH alone caused an increase in cells detaching from the plates, whereas pretreatment with antioxidants protected this change (Fig. 7C).

**Inhibition of tBH-Induced Apoptosis by Blocking FasL and Fas Interaction**

In a previous study, we found that tBH induced a loss of cytochrome c from mitochondria beginning at 2 hours and increasing at 6 and 8 hours. There was a close temporal association with a substantial activation of caspase 3–like activity by 4 hours. A decrease in mitochondria membrane potential was detected as early as 2 hours, indicating that oxidant-induced activation of the permeability transition could directly contribute to activation of the apoptosis pathway. However, because the permeability transition is also inhibited by thiol antioxidants, these data do not allow any distinction between a mitochondria-mediated activation of apoptosis and a Fas-mediated activation of apoptosis.

To further elucidate the involvement of the Fas-mediated death pathway in tBH-induced apoptosis, hRPE cells were preincubated with an antagonistic anti-Fas antibody ZB4 before treatment with tBH. This antibody completely blocked Fas-mediated apoptosis in hRPE cells (Fig. 3B) and human T leukemia Jurkat cells (data not shown). However, pretreatment of hRPE cells with ZB4 only partially inhibited tBH-induced apoptosis in hRPE cells (Fig. 8). This suggests that tBH-induced apoptosis is mediated, at least in part, through a death signal activated by the increased FasL that binds to the increased Fas in RPE cells. However, the data are also consistent with another mechanism’s having a role in activation of apoptosis in hRPE cells, such as the previously documented mitochondrial-activated pathway.

**DISCUSSION**

Histopathologic studies indicate that loss of RPE is a pivotal event in the development of ARMD. Circumstantial evidence suggests that oxidative mechanisms contribute to this process and that supplementation with antioxidants protect against onset or progression of ARMD. We have previously studied the cellular mechanism of oxidant-induced death in human retinal pigment epithelium using human cell lines established from cadaveric donors. The results have shown that cellular and extracellular GSH protect against oxidative stress, apparently by different mechanisms. Our recent research shows that after exposure to the model oxidant tBH, cells undergo apoptosis with a sequence of process involving loss of mitochondrial membrane potential, release of cytochrome c...
and activation of caspase 3-like activity. These results are associated with an activation sequence in which an oxidant-induced mitochondrial permeability transition provides a triggering event, but they do not exclude the involvement of other activating mechanisms.

In the present study, we have examined an alternative mechanism—namely, activation by the plasma membrane Fas receptor. Rapid progress has been achieved during recent years in the elucidation of the signaling pathway of Fas-mediated apoptosis. Fas ligation by FasL or by agonistic anti-Fas antibody can result in oligomerization of receptors in the cell membrane and formation of a death-inducing signal complex that includes the adapter protein Fas-associated death domain (FADD) and leads to the activation of caspase 8. The activated caspase 8 then propagates the apoptotic signal by activating downstream proteins through proteolytic cleavage. Among the proteins activated by this cascade are caspase 3 and BID. BID is a proapoptotic protein that triggers mitochondrial release of cytochrome c, which in turn activates caspases 9 and 3. Thus, in principle, activation of the Fas pathway could contribute to the mitochondrial changes in tBH-treated RPE cells that we have previously reported.

In this study, we show that cultured hRPE cells constitutively express low levels of FasL but relatively high levels of Fas. Under normal culture conditions, these expression levels are insufficient to activate apoptosis because apoptosis occurred at only a low level in untreated cells. However, when oligomerization of Fas was facilitated with a high concentration of recombinant sFasL or the agonistic anti-Fas antibody CH-11 (data not shown), apoptosis was induced. Compared with other Fas-expressing cells, such as human T leukemia Jurkat cells, hRPE cells appeared less sensitive to Fas-mediated apoptosis. Only 20% to 40% of RPE cells underwent apoptosis after 48 hours, whereas more than 80% of Jurkat cells undergo apoptosis under these conditions. This difference may be due to blocking of the Fas pathway in RPE cells by intracellular inhibitors. hRPE cells are found to have a very high level of intracellular Zn$^{2+}$, which is a potent inhibitor of caspase 3 and endonucleases.

Our data show that, besides sFasL, Fas-mediated apoptosis could also be induced in RPE cells by oxidative stress. Treatment of RPE cells with the oxidant tBH resulted in increased FasL and Fas expression; blocking FasL and Fas interaction with an antagonistic antibody inhibited tBH-induced apoptosis. However, unlike the antioxidants that completely inhibited tBH-induced apoptosis, the antagonist antibody only partially inhibited apoptosis. This suggests that the Fas-mediated pathway is involved in tBH-induced apoptosis but serves as only...
one of multiple death-signaling mechanisms triggered by the oxidant. Therefore, mitochondrial damage previously observed in tBH-treated cells may occur in part through a Fas pathway that involves sequential events including activation of caspase 8, cleavage of BID, and damage of mitochondria and also by an alteration of mitochondria components directly by ROIs or by a shift in intracellular or intramitochondrial redox status.34

The present findings could have significant clinical importance. Although sensitivity to oxidant-induced apoptosis by a mitochondrial mechanism could increase because of life-long accumulation of genome damage in the mitochondrial DNA, this sensitivity may not be readily reversible by antioxidant treatments.34 However, the Fas-mediated pathway appears to be potentiated by increased Fasl and Fas expression, and this could be suppressed by antioxidants. In vitro studies have demonstrated that many factors can induce Fasl expression and stimulate release of sFasL. These include T-cell activation, phagocytosis, viral infection, metalloproteinase activation, and oxidative stress.14,15,35–38 A number of studies suggest that oxidative damage in the RPE cells may contribute to the pathology of ARMD.18,24–27 Therefore, a mechanism for oxidative injury of RPE cells in ARMD may involve upregulation of FasL in T cells or other cell types. When cells with increased FasL infiltrate to the subretinal space, they may facilitate oligomerization of Fas receptors on RPE cells and kill them. In healthy, noninflamed eyes, Bruch’s membrane may protect against cells infiltrating from the choriocapillaris.1 However, Bruch’s membrane function may be impaired in inflamed eyes. Moreover, T cells and monocyte/macrophages can also enter the subretinal space adjacent to the RPE cells through retinal capillaries. Indeed, the presence of T cells in the subretinal space has been found in the rat with experimental autoimmune uveitis.39 In exudative ARMD, a choroidal neovascular membrane grows under or through the retinal pigment epithelium through breaks in Bruch’s membrane.2 The endothelial cells of this neovascular net lack tight junctions, and therefore fluid and blood leak into the subpigment epithelial layer of the retina.40 This could attract more cells that express high Fasl, such as activated T cells and activated macrophages, allow access to Fasl-bearing endothelial cells, or exposure to circulating sFasL and thereby cause death of RPE cells. Thus, if Fas-mediated apoptosis contributes to pathogenesis of ARMD,
it may be involved both in the initiation and progression of the disease.

Although there are no data showing an increase of sFasL in the sera of ARMD patients, an elevation of sFasL has been observed in several diseases including large granular lymphocytic leukemia, natural killer cell lymphoma, hemophagocytic syndrome, Diamond–Blackfan anemia, and hepatitis.11–13 Therefore, besides the infiltrating cells that express high FasL, a high level of serum sFasL could also facilitate Fas ligation in Fas-expressing RPE cells and cause death of the RPE cells.

Our present study shows that oxidative stress also causes increases in the expression levels of FasL and Fas in RPE cells themselves, and pretreatment of RPE cells with antioxidants GSH and NAC can inhibit oxidant-induced FasL and Fas expression and prevent apoptosis. Therefore, oxidative stress, in addition to its possible role in elevating FasL expression in T cells or macrophages or serum sFasL level, also directly up-regulates FasL and Fas expression in RPE cells and triggers autocrine and paracrine death of RPE cells. Because we did not perform the measurement of sFasL in the culture medium of tBH-treated cells, we do not know whether RPE-derived sFasL is involved in oxidant-induced apoptosis in our system. However, the inhibition of sFasL-induced apoptosis in RPE cells by antioxidants (S. Jiang, M-W. H. Wu, P. Sternberg, and D. P. Jones, unpublished data, October 1998) indicates that oxidants have an additional role—that is, they act as an activator of the Fas-mediated signaling pathway.

The roles of FasL and Fas in RPE cells may not be restricted to causing death of RPE cells. A recent study by Jørgensen et al.14 has shown that hRPE cells can induce apoptosis in several T cell lines and human peripheral T cells through FasL and Fas interaction. It suggests the FasL expressed in RPE cells may have an important role in maintaining immune privilege of the posterior of the eye. In healthy eyes, when the resting T cells that do not express FasL or activated T cells that express low FasL infiltrate the subretinal space, the overall interaction of FasL and Fas between T cells and RPE cells may activate a signal that travels from the FasL of RPE cells to the Fas of T cells and cause the death of the T cells. This is quite possible, because T cells are much more sensitive than RPE cells to Fas-mediated apoptosis. Therefore, although the FasL expressed in RPE is insufficient to cause suicide or fratricide, it is sufficient to trigger apoptosis in infiltrating T cells and thus to ensure immune privilege of the eye. However, in inflamed eyes, the T cells that infiltrate to the subretinal space are activated and have very high FasL expression. Under this situation, the communication between T cells and RPE may then trigger a death signal that travels from the FasL of T cells to the Fas of RPE cells, before a counterattack signal from RPE cells to T cells can be activated.

In conclusion, the present study demonstrates that Fas mediates apoptosis in RPE cells and that this mechanism may contribute to oxidant-induced apoptosis. Oxidants can induce expression of FasL and Fas in RPE cells, and antioxidant thiols can block this increased expression and associated apoptosis in RPE. Although incomplete inhibition by a blocking antibody indicates that other triggering mechanism(s) also occur, the data suggest that Fas-mediated apoptosis may be relevant to the death of RPE cells such as occurs in the pathogenesis of ARMD.

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