Dual Involvement of Caspase-4 in Inflammatory and ER Stress-Induced Apoptotic Responses in Human Retinal Pigment Epithelial Cells

Zong-Mei Bian, Susan G. Elner, and Victor M. Elner

**PURPOSE.** To investigate the functional involvement of caspase-4 in human retinal pigment epithelial (hRPE) cells.

**METHODS.** Expression and activation of caspase-4 in hRPE cells were measured after stimulation with proinflammatory agents IL-1β (2 ng/mL), TNF-α (20 ng/mL), lipopolysaccharide (1000 ng/mL), interferon-γ (500 U/mL), or monocyte coculture in the absence or presence of immunomodulating agent cyclosporin (3 or 30 ng/mL), dexamethasone (10 μM), or IL-10 (100 U/mL) and endoplasmic reticulum (ER) stress inducer thapsigargin (25 nM) or tunicamycin (3 or 10 μM). The onset of ER stress was determined by expression of GRP78. The involvement of caspase-4 in inflammation and apoptosis was further examined by treating the cells with caspase-4 inhibitor Z-LEVd-fmk, caspase-1 and -4 inhibitor Z-YVAD-fmk, and pan-caspase inhibitor Z-VDAD-fmk.

**RESULTS.** Caspase-4 mRNA expression and protein activation were induced by all the proinflammatory agents and ER stress inducers tested in this study. Caspase-4 activation was blocked or reduced by dexamethasone and IL-10. Elevated ER stress by proinflammatory agents and ER stress inducers was shown by increased expression of the ER stress marker GRP78. The induced caspase-4 and caspase-5 activities by tunicamycin and the stimulated IL-8 protein expression by IL-1β were markedly reduced by caspase-4 inhibitor Z-LEVd-fmk. Although caspase-4 inhibitor Z-LEVd-fmk and caspase-1 and -4 inhibitor Z-YVAD-fmk reduced tunicamycin-induced hRPE apoptotic cell death by 59% and 86%, respectively, pan-caspase inhibitor Z-VDAD-fmk completely abolished the induced apoptosis.

**CONCLUSIONS.** Caspase-4 is dually involved in hRPE proinflammatory and proapoptotic responses. Various proinflammatory stimuli and ER stress induce hRPE caspase-4 mRNA synthesis and protein activation. ER stress-induced hRPE cell death is caspase and, in part, caspase-4 dependent. (Invest Ophthalmol Vis Sci. 2009;50:6006–6014) DOI:10.1167/iovs.09-3628

Caspases are a family of cystolic, aspartate-specific, cysteine proteases involved in apoptosis, inflammation, proliferation, and differentiation.1-4 At least 17 members of the caspase family have been identified, of which 13 are found in humans.5 Human caspase-4 was cloned independently in three laboratories and designated as ICH2,6 ICEcII,7 and TX.8 The caspase-4 gene is expressed ubiquitously in various tissues with the exception of brain.6,7 Although human caspase-4 has no corresponding mouse orthologue,1 human caspase-4 and -5 are possibly the orthologues of mouse caspase-11.9 Caspase-4 cDNA exhibits 68% sequence homology with human caspase-1.7 As with caspase-1, caspase-4 is composed of a large prodomain (p22) and two small domains (p20 and p10) that are cleaved on activation.7 Transient expression of the cloned caspase-4 gene causes apoptotic cell death in fibroblasts,7 S9 insect cells,6 and COS cells.8 Subsequent studies have confirmed the apoptotic role of caspase-4 in endoplasmic reticulum (ER) stress-induced cell death.9-12

The ER is responsible for folding, maturation, and storage of membrane and secreted proteins. ER is also the major organelle that stores second-messenger calcium ions, which sense and respond to changes in cellular homeostasis. ER stress occurs when the cellular demand for ER function exceeds its capacity. Overloading of unfolded protein aggregates triggers a signaling cascade of events, called unfolded protein response (UPR). Excess UPR leads to irreversible commitment to cell death. There is accumulating evidence to suggest the involvement of caspase-4 in ER stress-induced apoptosis. First, caspase-4 is localized mainly to the ER.9 Second, caspase-4 is closely associated with many essential proteins in ER stress-induced cell death pathways, including GRP78, a well-known marker of ER stress10; CARD-only protein (Cop or pseudoICE), a regulator of procaspase-111; Apf1, a protein involved in death protease-mediated cell death12; and TRAF6, a member of the TNF receptor-associated factor.13 Third, caspase-4 inhibitor Z-LEVd-FMK (z-LEVd-fmk) selectively and effectively blocks ER stress-induced apoptosis in many cancer cells, such as neuroblastoma cells,14 lung and esophageal cancer cells,15 Jurkat cells,16 and melanoma cells.17 Fourth, knocking down caspase-4 expression by siRNA in multiple myeloma cells,18 leukemia cells,19 glioma cell lines,20 and neuroblastoma cells,9 introducing caspase-4 antisense oligonucleotides to lymphoblastoid AIH-1 cells,21 expressing catalytically inactive caspase-4, and microinjecting anti-caspase-4 antibodies into HeLa cells22 abolishes ER stress-induced cell death. Conversely, overexpression of caspase-4 in COS-7 cells induces activation of caspase-3 and -9, the two well-known death proteases.23

Chromosomal mapping reveals that the human caspase-4 gene is colocalized within a cluster of functionally related genes caspase-1, -5, and -12 and caspase-1 pseudogenes ICEBERG, COP, and INCA in human chromosome 11q22-23.24 The chromosomal colocalization of caspase-4 with inflammatory caspases implies that these caspases are derived from a common ancestor through gene multiplication and that they share common functions in innate immunity and inflammation, despite the common acceptance that caspase-4 is a member of the inflammatory caspase family. Most previous functional studies have focused on the role of caspase-4 in apoptosis. Thus far, only one study has shown, by having demonstrated its role in lipopolysaccharide (LPS)-induced inflammatory responses, that caspase-4 is involved in inflammation.15

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6006
In this study we investigated the functional involvement of caspase-4 in hRPE cells. Our data showed that caspase-4 is involved in both inflammation and apoptosis in hRPE cells.

**Materials and Methods**

**Materials**

Recombinant human IL-1β, TNF-α, interferon-γ (IFN-γ), and IL-10 were purchased from R&D Systems (Minneapolis, MN). Dexamethasone, cyclosporine, and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO). The cell-permeable general caspase inhibitor ZVAD (OMe)ValAlaAsp (OMe)-fmk and the caspase-4 inhibitor z-LEVD-fmk were from R&D Systems and BioVision (Mountain View, CA), respectively. The mouse monoclonal antibody (4B9) against caspase-4 was from Abcam (Cambridge, MA). The goat polyclonal antibody against GRP-78 was from Santa Cruz Biotechnology (Santa Cruz, CA). A shredding system (QIAshredder) and a purification kit (RNeasy Mini Kit) were purchased from Qiagen (Venlagen, Valencia, CA). Reverse transcription system was obtained from Invitrogen (Carlsbad, CA). RQ1 RNase-free DNase was purchased from Promega (Madison, WI). Detection kits (Cell Death Detection ELISA and In Situ Cell Death Detection) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). A visualization kit (EnVision G/2 System/AP (Permanent Red)) was obtained from DAKO (Carpinteria, CA). All other reagents were obtained from Sigma-Aldrich.

**Cell Isolation and Culture**

The hRPE cells were isolated within 24 hours of death from the donor eyes, as previously described.25-28 In brief, the sensory retina tissue was separated gently from the hRPE monolayer, and the hRPE cells were removed from Bruch’s membrane with papain (5 U/mL). The hRPE cells were cultured in Dulbecco’s modified Eagle’s Ham’s F12 nutrient mixture medium, containing 15% fetal bovine serum, penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (0.25 μg/mL) in culture plates (Falcon Primaria; Becton Dickinson, Mountain View, CA), respectively. The mouse monoclonal antibody (4B9) against caspase-4 was from Abcam (Cambridge, MA). The goat polyclonal antibody against GRP-78 was from Santa Cruz Biotechnology (Santa Cruz, CA). A shredding system (QIAshredder) and a purification kit (RNeasy Mini Kit) were purchased from Qiagen (Venlagen, Valencia, CA). Reverse transcription system was obtained from Invitrogen (Carlsbad, CA). RQ1 RNase-free DNase was purchased from Promega (Madison, WI). Detection kits (Cell Death Detection ELISA and In Situ Cell Death Detection) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). A visualization kit (EnVision G/2 System/AP (Permanent Red)) was obtained from DAKO (Carpinteria, CA). All other reagents were obtained from Sigma-Aldrich.

**Monocyte Isolation and hRPE-Monocyte Coculture**

Human monocytes were freshly isolated from the peripheral blood of healthy volunteers, as described previously.29 In brief, the peripheral blood was drawn into a heparinized syringe and 1:1 diluted in 0.9% saline. Mononuclear cells were separated by density gradient centrifugation. The cells were washed and then layered onto density gradient saline. Mononuclear cells were separated by density gradient centrifugation. The cells were stained with TdT-mediated dUTP nick end labeling (TUNEL) according to the manufacturer’s protocol. Briefly, hRPE cells were seeded and grown in 96-well plates until cells were close to confluence. After treating the cells with or without various of inducers or inhibitors for 24, 48, or 72 hours, apoptosis was quantified. Cultures were lysed with the lysis buffer of the ELISA kit (Cell Death Detection kit; Roche Molecular Biochemicals), and then the cytoplasmic fraction was transferred to the wells coated by streptavidin in the microplate modules for further analysis. Next, the immunoreagent was added to each well, which contained anti-histone-biotin and anti-DNA-peroxidase. The immunoreagents bonded to or reacted with the histone and DNA part of the mononucleosomes and oligonucleosomes that were out of the cytoplasm of cells during apoptosis. After removal of the unbound components with wash, the substrate 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] was added to determine the amount of peroxidase by reading absorbance difference between A_{505nm} and A_{505nm} in an ELISA reader as a measure of apoptosis.

**TUNEL Staining**

The cells were stained with DiT-mediated dUTP nick end labeling (TUNEL) according to the manufacturer’s protocol. Briefly, hRPE cells were fixed and incubated with TUNEL mixtures for 1 hour at 37°C. The incorporated fluorescein was detected by sheep anti-fluorescein antibody conjugated with horseradish peroxidase using the substrate diaminobenzidine. hRPE cells were distinguished by subsequent labeling with anti-β-actin antibody, alkaline phosphatase-labeled polymer, and permanent red substrate (EnVision G/2 system; DAKO). The stained cells were analyzed by light microscopy. Apoptotic cells in the cultures were quantified by counting the number of TUNEL-positive cells in five random microscope fields.

**Caspase-3 Activity**

Caspase-3 activity was assayed using a cellular caspase-3 activity assay kit (Biomol, Plymouth Meeting, PA), according to the manufacturer’s protocol. Briefly, cell extracts were added to the microtiter wells, and the reaction was initiated by adding 200 μM Ac-DEVD-pNA substrate. In parallel, the samples were reacted with this substrate in the presence of 0.1 μM Ac-DEVD-CHO, a specific caspase-3 inhibitor, to measure the nonspecific hydrolysis of the substrate. Absorbance was read at 405 nm in a microtiter plate reader at the indicated time intervals.
RESULTS

Expression and Activation of Caspase-4 in Response to Proinflammatory Stimulation

To determine the involvement of caspase-4 in proinflammatory response by hRPE cells, a group of known proinflammatory agents was selected for this study, including IL-1β, TNF-α, LPS, IFN-γ, and monocyte coculture. The concentrations of IL-1β (2 ng/mL), TNF-α (20 ng/mL), LPS (1000 ng/mL), and IFN-γ (500 U/mL) and the conditions for monocyte-hRPE coculture used in this study have been shown to maximally stimulate proinflammatory responses in hRPE cells.26 –28,30 After hRPE cells were treated with these agents for 6 hours, total cellular mRNA was isolated and subjected to RT-PCR analysis. To compare caspase-4 mRNA levels, expression of the housekeeping gene β-actin was used to monitor gel loading. As shown in Figure 1A, treatment of hRPE cells with IL-1β, TNF-α, LPS, or monocyte coculture increased caspase-4 mRNA synthesis by 0.7-, 0.5-, 0.5-, or 0.6-fold, respectively.

Next, whole lysates from hRPE cells treated under the same conditions, but for 24 hours, were subjected to Western blot analysis. To compare caspase-4 mRNA levels, expression of the housekeeping gene β-actin was used to monitor gel loading. As shown in Figure 1B, a treatment of hRPE cells with IL-1β, TNF-α, LPS, or monocyte coculture increased caspase-4 mRNA synthesis by 0.7-, 0.5-, 0.5-, or 0.6-fold, respectively.

The activation of caspase-4 by proinflammatory agents suggested that caspase-4 may participate in proinflammatory responses in hRPE cells. To prove this is the case, IL-1β-induced IL-8 protein production was examined by Western blot analysis of the whole cell lysates from the hRPE cells treated with 2 ng/mL IL-1β in the presence or absence of caspase-4 inhibitor Z-LEVD-fmk. As we have shown previously,27 IL-1β induced IL-8 protein production significantly above basal levels. As shown in Figure 1C, the presence of caspase-4 inhibitor completely eliminated the induced IL-8 protein production.

Effect of Dexamethasone, IL-10, and Cyclosporine on IL-1β- and IFN-γ-Induced Activation of Caspase-4

Given that expression and activation of caspase-4 were induced by IL-1β and IFN-γ, we next investigated whether anti-inflammatory agents could neutralize this induced caspase-4 activation by these two proinflammatory agents. As expected, treatment with dexamethasone (1 μM) and IL-10 (100 U/mL) reduced IL-1β- and IFN-γ-induced cleavage of procaspase-4 by 60% and 53% and by 15% and 50%, respectively (Fig. 2). On the other hand, cyclosporine (3 ng/mL) inhibited IFN-γ-induced caspase-4 activation by 47% but markedly increased IL-1β-induced caspase-4 activation by more than twofold.

Induction of Caspase-4 mRNA Synthesis and Protein Activation by ER Stress-Inducer Tunicamycin or Thapsigargin

Tunicamycin and thapsigargin are the two well-known ER stress inducers that act by blocking, respectively, N-glycosylation of newly synthesized proteins and Ca²⁺-ATPase, which maintains Ca²⁺ homeostasis in the ER. We used these two agents to study apoptotic involvement of caspase-4. Similar to the results from using proinflammatory agents, as described, tunicamycin (3 μM) and thapsigargin (25 ng/mL) both moderately increased caspase-4 mRNA by 0.3- and 0.4-fold, respectively (Fig. 3A). Tunicamycin (10 μM) induced caspase-4 cleavage that appeared as early as 24 hours after stimulation, and activation remained up to 72 hours (Fig. 3B). The induced caspase-4 activation was reduced by coinoculation with caspase-4 inhibitor Z-LEVD-fmk (2 μM) for 30 minutes and then coincubated with IL-1β for another 24 hours. Proteins from whole hRPE cell lysates were subjected to Western blot analysis by anti-IL-8 antibody.

ER Stress in Response to Apoptotic and Inflammatory Stimuli

Tunicamycin has been widely used to induce ER stress. To confirm the existence of ER stress when caspase-4 was activated by this agent, expression of GRP78, a specific marker of ER stress, was examined. In untreated hRPE cells, GRP78 pro-
tein was barely detectable by Western blot analysis (Fig. 4A). Induction of GRP78 production appeared at 24 hours after hRPE cells were treated with 10^(-9)M tunicamycin. The induced GRP78 expression continued to increase up to 48 hours. Tunicamycin treatment also triggered a significant increase in expression of the antiapoptotic protein Bcl-2 as early as 24 hours after stimulation, and the increase was sustained up to 2.5-fold at 48 hours after treatment (Fig. 4B). On the other hand, tunicamycin modestly (by 0.7-fold) increased the pro-apoptotic protein Bax. As a result, the Bcl-2/Bax ratios were enhanced 1.1-fold by tunicamycin.

Because GRP78 has been shown to have immunosuppressive activity,31,32 we then examined the expression of GRP78 after treating hRPE cells with IL-1β, TNF-α, LPS, or overlaid monocytes. As shown in Figure 5A, these treatments increased hRPE GRP78 expression. In monocyte coculture, for example, the enhanced GRP78 protein levels appeared as early as 2 hours and reached steady state in 8 hours.

Caspase-4 in Inflammatory and Apoptotic Responses by hRPE

The effect of dexamethasone (Dex), cyclosporine (CsA), and IL-10 on caspase-4 activation by IL-1β (A, C) and IFN-γ (B, C) in hRPE cells. The hRPE cells were pretreated with Dex (1 μM), CsA (5 ng/mL), or IL-10 (100 U/mL) for 30 minutes and then cocultivated with IL-1β (2 ng/mL) and IFN-γ (500 U/mL) for an additional 24 hours. Proteins from whole hRPE cell lysates were detected by anti-caspase-4 antibody specific for pro-caspase-4 and cleaved caspase-4. The fold changes of the cleaved caspase-4 were calculated by relative density between treated and untreated samples, as determined by densitometry after normalization with actin protein.

ELISA cell death detection kits were used to measure apoptotic cell death at the same concentrations used for caspase-4 activation. At 72 hours after treatment, tunicamycin induced substantial hRPE apoptotic cell death compared with untreated

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933449/)

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933449/)
control cells, which had undetectable levels of cell death. Arbitrarily taking the ELISA readings under tunicamycin treatment as 100% cell death, the induced hRPE apoptosis was inhibited by caspase-4 inhibitor Z-LEVD (2 μM) and caspase-1 and -4 inhibitor Z-YVAD-fmk (20 μM) by 59% and 86%, respectively (Fig. 6). The pan-caspase inhibitor Z-VAD-fmk (50 μM) completely abolished the induced hRPE cell death.

To validate the tunicamycin-induced apoptosis, TUNEL staining was performed. Normal hRPE cells in the culture exhibited typical hexagonal arrays (Fig. 7A, upper left). First we conducted microscopic analysis of the hRPE cells in the presence (Fig. 7A, lower panels) or absence (Fig. 7A[b], upper right) of 10 μM tunicamycin. As shown in Figure 7A, TUNEL staining is dark brown and hRPE vimentin staining is red. Nuclear condensation and cell shrinkage were evident after hRPE cells were treated with tunicamycin for 24 hours (Fig. 7A, lower panels). Next we determined the relative levels of the tunicamycin-induced apoptotic cell death by TUNEL assays in the presence of caspase-4 inhibitor Z-LEVD or pan-caspase inhibitor Z-VAD for 24 or 48 hours (Fig. 7B). After hRPE cells were treated with tunicamycin (10 μM) for 24 and 48 hours, 13% and 24% of the cells exhibited apoptotic cell death as detected by TUNEL assay. In the presence of the caspase-4 inhibitor Z-LEVD-fmk (2 μM), tunicamycin-induced apoptotic cell death at 24 and 48 hours was reduced by 62% and 53%, respectively. In contrast to Z-LEVD-fmk, pan-caspase inhibitor Z-VAD-fmk almost completely blocked the induced apoptotic cell death. Furthermore, treatment of hRPE cells with IL-1β (IL-1; 2 ng/mL), TNF-α (TNF; 20 ng/mL), and lipopolysaccharide (LPS; 1000 ng/mL) for 24 hours (Fig. 6B, left) or with cyclosporine (CsA; 3 ng/mL), IL-10 (100 U/mL), and dexamethasone (Dex; 1 μM) for 72 hours (Fig. 6B, right) did not result in apparent cell death compared with tunicamycin control.

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**Figure 4.** Time-dependent effects of tunicamycin (Tu) on GRP78 (A), Bcl-2, and Bax (B) protein expression. The hRPE cells were cultured either without or with tunicamycin (10 μM) for 24 and 48 hours. The hRPE whole cell lysates were subjected to Western blot analysis for GRP-78, Bcl-2, and Bax expression. The data shown represent results from a typical experiment. The fold changes were calculated by normalization of band density with actin and assigned control value as 1.

**Figure 5.** Expression of GRP78 protein in hRPE cells that were stimulated by proinflammatory agents (A) or in combination with anti-inflammatory agents (B). The hRPE cells were cultured either without (Ctl, control) or with IL-1β (IL-1, 2 ng/mL), TNF-α (TNF, 20 ng/mL), LPS (1000 ng/mL), IFN-γ (500 U/mL), or overlaid monocytes (RM) for 2 to 24 hours (A, middle and bottom) or 24 hours (A, top; B, C) Cultures were pretreated with or without dexamethasone (Dex, 1 μM), cyclosporine (CsA, 3 or 30 ng/mL), or IL-10 (100 U/mL) for 30 minutes and then were coincubated with IL-1β and IFN-γ for an additional 24 hours. Whole hRPE cell lysates were subjected to Western blot analysis for GRP-78 expression. The fold changes of cleaved caspase-4 were determined by the ratios between treated and untreated samples of the band densities, which were quantified by densitometry and normalized by actin.
Human caspase-12 was originally thought to play distinctive roles in inflammation and apoptosis, corresponding to those of caspase-12 in mice. However, given that most human populations express only the C-terminal truncated form of caspase-12, the role of this caspase in human apoptosis has been challenged.2,33,34 The short form of human caspase-12 is not a null product in hRPE cells; we have recently shown that it may play an immunomodulatory role in these cells.35 Phylogenetic analyses suggest that caspase-4 and -5 may be the functional counterparts of caspase-12 in humans.4 The data from the present study support the notion that human caspase-4 may correspond to murine caspase-12, involved in both inflammation and apoptosis.36

Despite the accumulating evidence supporting an apoptotic role of caspase-4, negative results have been reported in tunicamycin- and thapsigargin-induced apoptosis in human multiple myeloma cell lines,37 homoharringtonine-induced apoptosis in MUTZ-1 cells,58 and Z α-1 antitrypsin-induced apoptosis in HEK293 cells.39 Although apoptosis usually does not require de novo synthesis of caspases, ER stress may increase mRNA expression of certain caspases, including caspase-4.40 Our study demonstrated that proinflammatory stimuli (IL-1β, TNF-α, LPS, IFN-γ, and monocyte coculture) induced both mRNA synthesis and activation of caspase-4 in hRPE cells. IL-1β-induced IL-8 protein production was reduced by the caspase-4 inhibitor Z-LEVD-fmk, suggesting that caspase-4 is involved in the proinflammatory responses of hRPE cells. These results are consistent with a previous study in which LPS-induced IL-8 mRNA synthesis and protein production are reduced by caspase-4 knockdown in human THP1 monocytic cell lines.15 On the other hand, the ER-stress inducer tunicamycin induced expression of GRP78, a specific marker of ER stress. Although tunicamycin increased the expression of proapoptotic Bax, this study also found increased expression of the antiapoptotic Bcl-2 protein, resulting in an increased Bcl-2/Bax ratio. In most cases, the ER stress-induced apoptotic response is regulated by or dependent on the Bcl-2 family of proteins.41 The Bcl-2/Bax ratio has been clinically used as an indicator of the susceptibility of tumor cells to the induction of apoptosis by chemotherapeutic agents.42 However, the involvement of Bcl-2 and Bax in tunicamycin-induced apoptosis has not been well characterized. Increased Bcl-2 protein expression by tunicamycin has been reported in one previous report.43 The increased Bcl-2 expression shown in our study, however, might not have affected caspase-4 activation because a previous study has shown that activation of caspase-4 by...
tunicamycin is only slightly affected by overexpression of Bcl-2. The increased Bcl-2/Bax ratio by tunicamycin may suggest an early protective response of hRPE cells to apoptotic stimuli by enhanced expression of the antiapoptotic protein Bcl-2 to counteract the increase in proapoptotic protein Bax.

Furthermore, in response to ER stress, hRPE cells increased the production and activation of caspase-4. The latter is consistent with a recent report in ARPE19 cells, an immortalized hRPE cell line. These authors showed that treatment of ARPE19 cells with tunicamycin and thapsigargin stimulated the activation of caspase-4, though the effect of tunicamycin on caspase-4 mRNA expression was negligible. In agreement with this recent report, apoptotic hRPE cell death by staurosporine was insensitive to caspase-4 inhibitor; thus, caspase-4 is unlikely to be involved in the staurosporine-induced mitochondrial apoptotic pathway (data not shown). We further demonstrated in this study that tunicamycin-induced caspase-4 activation and apoptotic hRPE cell death were highly sensitive (59%) to inhibition by caspase-4 inhibitor Z-LEVD-fmk, implying that ER stress-induced apoptosis in hRPE occurs in part through the activation of caspase-4. The difference in potency between caspase-4 inhibitor Z-LEVD-fmk (59% inhibition) and pan-caspase inhibitor Z-VAD (98% inhibition) suggests the existence of parallel caspase-4-independent apoptotic pathways that are also induced by ER stress. Although apoptotic cell death can be caspase independent,44 the hRPE apoptotic cell death induced by ER stress was totally caspase dependent because this induction was completely abolished by a pan-caspase inhibitor. The caspase inhibitor Z-YVAD-fmk has been used as a caspase-4 inhibitor,45,46 but it is more frequently used as a caspase-1 inhibitor.47 In fact, Z-YVAD-fmk inhibits both caspase-1 and -4.48 The stronger inhibition (86%) of apoptosis by Z-YVAD-fmk suggests that caspase-1 is likely to be responsible for the ER stress-induced, caspase-4-independent hRPE cell death. Caspase-1 and -4 have been shown to coordinate in TNF-α-induced, but not in tunicamycin-induced, cell death in Cop-transfected HeLa cells.49 Our data also show that caspase-4 inhibitor Z-LEVAD-fmk abolished both tunicamycin-induced activation of caspase-3 and apoptotic hRPE cell death, suggesting that caspase-3, the central effector of apoptosis, acts downstream from caspase-4 in the ER stress-induced RPE apoptotic pathway, as reported in other cell types.10,23,31,46,49 Taken together, our hRPE data support the proposal by Momoi et al.36 that caspase-4 may be a functional surrogate of the truncated human caspase-12.

The proinflammatory agents tested in this study, including IL-1β, TNF-α, and LPS, upregulated the ER stress marker GRP78. However, when treating hRPE cells with these three agents at the concentrations used for other experiments in this study for up to 24 hours, none of these agents caused apoptotic cell death. Uptregulation of GRP78 in response to inflammation suggests its immunosuppressive and protective role.31,32 Moreover, the drugs dexamethasone and cyclosporine and the anti-inflammatory cytokine IL-10, when added together with proinflammatory agent IL-1β, exerted differential consequences on the inductions of GRP78 expression. Although dexamethasone reversed IL-1β-induced increases in hRPE GRP78, cyclosporine at 3 and 50 ng/ml enhanced the already elevated GRP78 production caused by IL-1β. The different impact by dexamethasone and cyclosporine, as indicated by the ER stress marker GRP78 response, suggests potential risks of long-term treatment with some anti-inflammatory drugs, such as cyclosporine. Indeed, when we treated hRPE cells with cyclosporine (30 ng/ml) for 72 hours, all hRPE cells died. In contrast, neither dexamethasone (10 μM) nor IL-10 (100 U/ml) induced noticeable apoptosis under the same conditions. The cyclosporine concentration used in this study (3–30 ng/mL) was well within the drug concentrations used clinically (5–100 ng/ml),50 and 30 ng/mL cyclosporine appeared to be cytotoxic in this study. Indeed, cyclosporine itself has been shown to induce ER stress and GRP78 causing nephrotoxicity and apoptotic cell death,51 whereas dexamethasone has been shown effective in treating acquired glomerular diseases by suppressing ER stress and GRP78.52

The signaling pathway mediating caspase-4 proinflammatory responses remains elusive. Caspase-4 does not appear to act via caspase-1 activation. Activation of caspase-4 requires both dimerization and proteolysis, a feature that combines the requirement for activation of initiator caspases such as caspase-1 (dimerization) and effector caspases such as caspase-3 (interdomain cleavage).53 Caspase-4 does not promote the maturation of caspase-1 substrate pro-IL-1β,54 and no specific substrates have been identified for caspase-4. These observations suggest that the proinflammatory mechanisms involving caspase-4 differ from those involving caspase-1.6,8 It has been proposed that caspase-4 may function mainly via the NF-κB signal pathway in inflammatory responses.15 Our previous studies have shown that the NF-κB pathway is essential for the expression of IL-8 and other cytokines in hRPE cells, and the inhibition of NF-κB activation effectively blocks IL-1β-induced cytokine production in hRPE cells.56 Because knockdown of the caspase-4 gene by siRNA significantly reduces NF-κB activation and nuclear translocation,13 this mechanism for caspase-4 in the IL-8 pathway is likely. Caspase-4 has been associated with activation of signal transducer TRAF6.13 In response to stimulation by proinflammatory cytokines, TRAF6 activates IκB kinase in the NF-κB pathway.54 Thus, TRAF6 may also be involved with ER stress response-induced hRPE IL-8 expression.

This study shows that caspase-4 appears to be a key mediator of apoptosis and inflammation in hRPE cells, underscoring its potential value as a novel therapeutic target. The pathophysiological relevance of this dual role in hRPE responses warrants further investigation. Inflammatory cytokines are essential mediators of the innate immune response. Given that hRPE cells play important roles in ocular functions under normal and diseased conditions,27,55 caspase-4-mediated cytokine expression could be relevant to many noninfectious and infectious retinal diseases, such as proliferative vitreoretinopathy,56 age-related macular degeneration,57 uveitis,58 and endophthalmitis.59 On the other hand, apoptotic cell death is an established response in many ocular diseases, such as age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa, retinal ischemia, photoreceptor degeneration, and glaucoma.60–63 Blockade of caspase-3, the target downstream of caspase-4, may represent a therapeutic strategy in the protection of retinal degeneration.64,65 Therefore, it is of interest to investigate whether caspase-4 is involved under those diseased conditions. Further delineating the signaling pathway, regulation, and functional roles of caspase-4 may suggest novel strategies for developing therapies for ocular disease.

References


5. Eckhart L, Ballaun C, Hermann M, et al. Identification of novel mammalian caspases reveals an important role of gene loss in the signaling pathway mediating caspase-4 proinflammatory responses remains elusive. Caspase-4 does not appear to act via caspase-1 activation. Activation of caspase-4 requires both dimerization and proteolysis, a feature that combines the requirement for activation of initiator caspases such as caspase-1 (dimerization) and effector caspases such as caspase-3 (interdomain cleavage).53 Caspase-4 does not promote the maturation of caspase-1 substrate pro-IL-1β,54 and no specific substrates have been identified for caspase-4. These observations suggest that the proinflammatory mechanisms involving caspase-4 differ from those involving caspase-1.6,8 It has been proposed that caspase-4 may function mainly via the NF-κB signal pathway in inflammatory responses.15 Our previous studies have shown that the NF-κB pathway is essential for the expression of IL-8 and other cytokines in hRPE cells, and the inhibition of NF-κB activation effectively blocks IL-1β-induced cytokine production in hRPE cells.56 Because knockdown of the caspase-4 gene by siRNA significantly reduces NF-κB activation and nuclear translocation,13 this mechanism for caspase-4 in the IL-8 pathway is likely. Caspase-4 has been associated with activation of signal transducer TRAF6.13 In response to stimulation by proinflammatory cytokines, TRAF6 activates IκB kinase in the NF-κB pathway.54 Thus, TRAF6 may also be involved with ER stress response-induced hRPE IL-8 expression.


