

Regulated Expression of Caspase-12 Gene in Human Retinal Pigment Epithelial Cells Suggests Its Immunomodulating Role

Zong-Mei Bian,¹ Susan G. Elner,¹ and Victor M. Elner^{1,2}

PURPOSE. To investigate the expression and regulation of the short form of caspase-12, caspase-12S, in human retinal pigment epithelial (hRPE) cells.

METHODS. hRPE cells were stimulated by the proinflammatory agents IL-1 β (2 ng/mL) and TNF- α (20 ng/mL); LPS (1000 ng/mL); coculture with monocytes; the immunomodulating agent cyclosporine (Cys; 30 ng/mL); the anti-inflammatory cytokine IL-10 (100 U/mL); and the endoplasmic reticulum (ER) stress inducers tunicamycin (3 or 10 μ M) and thapsigargin (25 or 100 nM) for 6 hours or longer. The total RNAs were isolated and subjected to semiquantitative and quantitative real-time RT-PCR analysis. Effects of tunicamycin and thapsigargin on IL-1 β - and TNF- α -stimulated MCP-1 mRNA expression and protein production were further examined by RT-PCR and ELISA, respectively.

RESULTS. RT-PCR results showed that caspase-12S is the predominant form of caspase-12 in the examined hRPE cells of this study, with expression at levels as high as those in many other human tissues such as pancreas, prostate, small intestine, lung, spleen, and kidney. Treatment with IL-1 β and TNF- α , as well as LPS and coculture with monocytes reduced hRPE caspase-12S mRNA expression within 6 hours. In contrast, hRPE exposure to cyclosporine (Cys) and the cytokine IL-10 for 6 hours increased caspase-12S mRNA expression. Compared to Cys and IL-10, the ER stress activators tunicamycin and thapsigargin were even more potent enhancers of hRPE caspase-12S gene expression. They also caused corresponding reductions in IL-1 β - and TNF- α -induced MCP-1 mRNA expression and protein production.

CONCLUSIONS. hRPE cells express a high level of caspase-12S. The regulated expression of caspase-12S suggests that this caspase recruitment domain (CARD)-only protein may be an endogenous dominant negative regulator that modulates inflammatory responses in hRPE cells. (*Invest Ophthalmol Vis Sci.* 2008;49:5593–5601) DOI:10.1167/iovs.08-2116

Caspases are a family of cysteinyl aspartate-specific proteases that play crucial roles in modulating cellular signaling pathways involved in apoptosis and inflammation.¹ Typically, caspase proteins consist of a prodomain and large and

small domains that are cleaved on activation. One class of prodomain is called caspase recruitment domain (CARD), common in caspase-1, -2, -4, -5, -9, -11, and -12, and some caspase-associated adapter proteins.^{2,3} In response to extracellular and intracellular stimulation, two types of protein complexes, apoptosome and inflammasome, are formed, initiating apoptotic and inflammatory signaling pathways, respectively.⁴ Based on their functional and phylogenetic relationship, caspases are grouped into inflammatory (caspase-1, -4, and -5) and apoptotic caspases (caspase-2, -3, -6, -7, -8, and -10).⁵ Depending on the species, caspase-12 appears to function in apoptosis and/or inflammation.^{1,6-9}

Caspase-12 was first cloned in mice in 1997.¹⁰ Based on corresponding sequences of murine caspase-12 gene and the human genome, a human orthologue has been identified and cloned.¹¹ The human caspase-12 cDNA exhibits 68% and 57% identity to mouse caspase-12 and human caspase-4, respectively. Human caspase-12 has nine splice variants¹¹ and, in most cases, contains a premature stop codon. Thus, the translated short form of caspase-12 protein, caspase-12S, has only the CARD prodomain. By searching the single nucleotide polymorphism (SNP) database, a human caspase-12 allele has also been identified to encode the full length of caspase-12, suggesting that polymorphisms of the human caspase-12 gene exist.⁷ The caspase-12 gene is colocalized in a cluster of functionally related genes, caspase-1, -4, and -5 as well as the caspase-1 pseudogenes, *ICEBERG*, *COP*, and *INCA* in human chromosome 11q22-23, or with caspase-1 and -11 in mouse chromosome 9A. The chromosomal colocalization of caspase-12 with inflammatory caspases implies that these caspases are derived from a common ancestor through gene multiplication and may function in concert during inflammation.⁵

Most studies of caspase-12 function have focused on the roles of murine caspase-12 in endoplasmic reticulum (ER) stress-induced apoptosis.^{6,12-14} However, functional studies of human caspase-12 have excluded its direct involvement in ER stress-induced apoptosis,¹ as no differences have been observed in the expression of caspase-12L, the longer form of caspase-12, and caspase-12S in response to various apoptotic stimuli.⁷ In contrast, humans expressing caspase-12L show impaired responses to bacterial and viral infections.^{7,15} As a result, these individuals have an increased risk of severe sepsis and sepsis-related mortality.^{6,7,16} These observations suggest that human caspase-12L plays a major role in inflammation and functionally is differentiated from murine caspase-12 because it has no functional role in apoptosis. Because of the loss of the large and small subunits, the most common CARD-only form of human caspase-12, caspase-12S, has been considered to be a functionally inactive decoy of caspase-12 through natural selection. The C terminus-truncated caspase-12S gives humans an advantage in fighting microbial infections.^{5,15,17,18}

Although caspase-12S has been thought to be a functionally null gene,^{6,17,18} all known CARD-only proteins, including *ICEBERG*, *COP* and *INCA*¹⁹⁻²¹; caspase-9 short form²²; and NOD2-S, the short form of NOD,²³ function as dominant neg-

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ative regulators in caspase signaling pathways.³ The CARD-CARD interactions have also been known to participate in NF- κ B signaling pathways in innate and adaptive immune responses.² Furthermore, it has been shown that the LPS-induced activation of NF- κ B is reduced in the cells transiently expressing caspase-12S, although the reduction is weak compared with caspase-12L.⁷

We hypothesized that the CARD-only human caspase-12S may be functionally active in human retinal pigment epithelial (hRPE) cells and that its expression may be regulated in response to inflammatory stimuli. Such regulation of the human caspase-12 gene has not yet been reported in any cell type. In this study, we examined expression and regulation of caspase-12S in hRPE cells. Caspase-12S was subject to regulation by many pro- or anti-inflammatory stimuli, implying a functional role in innate immune responses of hRPE cells.

MATERIALS AND METHODS

Recombinant human IL-1 β , TNF- α , IFN- γ , and IL-10 were purchased from R&D Systems (Minneapolis, MN). Human tissue cDNAs were purchased from BD-Clontech (Mountain View, CA). A cell and tissue lysate homogenizing kit [QIashredder] and an RNA isolation kit [RNeasy Mini Kit] were purchased from Qiagen (Valencia, CA); the reverse transcription system was obtained from Invitrogen (Carlsbad, CA). RQ1 RNase-free DNase from Promega (Madison, WI); the real-time RT-PCR detection system (Cycler IQ) from Bio-Rad (Hercules, CA); SYBR green I dye from Invitrogen-Molecular Probes (Eugene, OR); and the rabbit anti-MCP-1 antibody was purchased from R&D Systems. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Isolation and Culture

The hRPE cells were isolated within 24 hours of death from the donor eyes obtained from the Midwest Eye Bank, as previously described.²⁴ 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 (DMEM/Ham's F12), containing 15% fetal bovine serum, penicillin G (100 U/mL), streptomycin sulfate (100 μ g/mL), and amphotericin B (0.25 μ g/mL) in culture plates (Primaria; BD-Falcon, Bedford, MA) to inhibit fibroblast growth. The hRPE monolayers exhibited uniform immunohistochemical staining for cytokeratin 8/18, fibronectin, laminin, and type IV collagen in the chicken-wire meshwork distribution characteristic of these epithelial cells. Cells were subcultured, grown to reach confluence, and used for experiments. The cells were in culture up to four to six passages. RPE cells and monocytes were each obtained from three donors for use in the study. For each experiment at least two donors were used.

Monocyte Isolation and hRPE-Monocyte Coculture

Human monocytes were freshly isolated from the peripheral blood of healthy volunteers, as described previously.²⁵ In brief, 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 an ionic density gradient (1.068 g/mL Fico-Lite; Atlanta Biologicals, Lawrenceville, GA) for the enrichment of monocytes. The isolated cells were then washed, cytospun onto a glass slide, stained (Diff-Quik; Fisher Scientific, Pittsburgh, PA), and differentially counted. The purity of the cells was >97%. For hRPE cell-monocyte coculture, enriched monocyte populations (3×10^5) were overlaid onto untreated or pretreated near-confluent hRPE cultures (2×10^5) for 6 hours. After coculture, the human monocytes were removed, and hRPE cells were subjected to further analyses.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

The total cellular RNA was isolated from the hRPE cells (QIashredder and RNeasy Mini Kit; Qiagen), according to the manufacturer's protocol. The cDNA synthesis reaction was set up according to the protocol for the reverse transcription system. Briefly, 5 μ g of RNA was added to the reaction mixture with 200 U/ μ L transcriptase (RT; Superscript III; Invitrogen) and 1 μ L Oligo d(T)₂₀ (0.5 μ g/ μ L), for a total volume of 20 μ L RT-PCR for each product, was performed with three different cycles: 15, 25, and 35. The RT-PCR reactions were accepted as semi-quantitative when individual amplicates were performed in the mid-linear portion of the response curve. Specific cDNA was amplified by 32, 28, and 20 cycles for caspase-12, MCP-1, and β -actin, respectively. For tissue expression profiling, 0.1 μ g cDNA from each tissue was used for PCR. The reaction was initiated by adding 0.15 μ L of *Taq* DNA polymerase (5 U/ μ L) to a final volume of 20 μ L. The primer sequences for human caspase-12 genes were as follows: 5'-GCCATGGCTGATGAGAAACC-3' (sense, primer 1), 5'-GTGTTCCGGTCCACATGGTGAAG-3' (sense, primer 3), 5'-CTGAGTTGCTTCTATGAG-3' (antisense, primer 2), and 5'-CAAACCTGCTTAGTGCTGTTTC-3' (antisense, primer 4). The synthetic oligonucleotide primers for human MCP-1 were 5'-GCTCATAGCAGCCACCTTCATTC-3' (sense) and 5'-GTCTTCGGAGTTGGGT-TTGC-3' (antisense). Human β -actin sense (5'-GTGGGGCGCCCCA-GGCACCA-3') and antisense (5'-GCTCGGCCGTGGTGGTGAAGC-3') primers were used in parallel, to ensure that an equal amount of templates was used in each amplification reaction. The following conditions were used in RT-PCR reaction for caspase-12, MCP-1, and β -actin: denaturation at 95°C for 45 seconds (caspase-12) or 1 minute (MCP-1 and β -actin), annealing at 65°C for 45 seconds (caspase-12) or 1 minute (MCP-1) or 62°C (β -actin) for 1 minute, and extension at 72°C for 1 minute (caspase-12) or 2 minutes (MCP-1 and β -actin) for 32 (caspase-12), 28 (MCP-1), or 20 (β -actin) cycles. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Quantitative Real-Time RT-PCR

Isolation of the total cellular RNA and synthesis of cDNA were similar to the procedure for semiquantitative RT-PCR. To ensure the complete removal of genomic DNA, we introduced an additional treatment with RQ1 RNase-free DNase. Real-time RT-PCR was performed by using a real-time RT-PCR detection system (Cycler IQ; Bio-Rad) to measure the fluorescence produced by SYBR Green I dye that intercalates into RT-PCR product. The RT-PCR reactions were performed in triplicate on each cDNA template along with triplicate reactions of the housekeeping gene, β -actin. Negative control was obtained by performing real-time RT-PCR without cDNA. The proprietary synthetic oligonucleotide primers for human caspase-12 (Bioscience SuperArray, Frederick, MD) and 5'-GTGGGGCGCCCCAGGCA CCA-3' (sense) and 5'-GCTCGGC-CGTGGTGGTGAAGC-3' (antisense) for human β -actin were used. The thermal cycling conditions were 3 minutes at 95°C, followed by 35 cycles at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. All RT-PCR reaction products were verified by melting-curve analysis and agarose gel electrophoresis. The caspase-12 mRNA expression levels were quantified by calculating the average value of triplicate reactions, normalized by the average value of triplicate reactions for β -actin.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of immunoreactive MCP-1 in the hRPE supernatants were determined by modification of a double-ligand ELISA method, as previously described.²⁶ Briefly, 96-well microtiter plates were coated with rabbit anti-MCP-1 antibody for 20 hours at 4°C. Nonspecific binding sites were blocked with 2% bovine serum albumin. Diluted supernatants from hRPE cultures (50 μ L) were added and incubated for 1 hour. The plates were then subjected to sequential incubations with biotinylated rabbit anti-cytokine (1:1000) for 45 minutes and streptavidin-

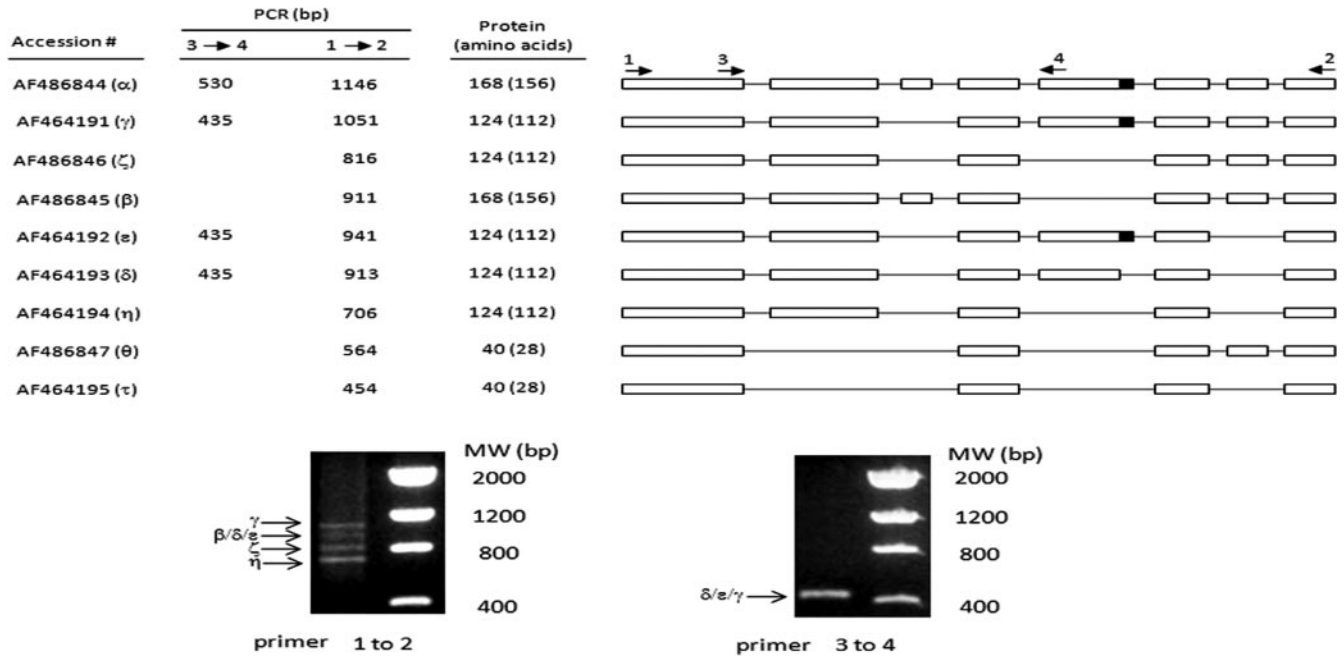


FIGURE 1. Exon structure and expression of human caspase-12S splice variants in hRPE cells. The splice variants are named as described by Fischer et al.¹¹ The accession numbers of each variant are based on GenBank data. *Top left:* predicted lengths of RT-PCR products and proteins. The protein amino acid lengths are predicted assuming the existence of one nucleotide 651G deletion plus 724T>C point mutation,¹¹ or existence of only 724T>C point mutation⁷ as shown in the parenthesis. *Top right:* exon structure shown as *open rectangles*. For convenience, the introns are shown as lines that are not proportional to their real sizes. The primers 1 to 4 are marked by corresponding numbers with *arrows* indicating the transcription directions. Typical RT-PCR products are shown with primers 1 to 2 (*bottom left*) and 3 to 4 (*bottom right*).

peroxidase conjugate for 30 minutes. A chromogen substrate (OPD) was added, the plates were incubated to the desired extinction, and the reaction was terminated with 3 M H₂SO₄. Absorbance for each well at 490 nm was read in an ELISA reader. Standards included half-log dilution of corresponding cytokine concentrations ranging from 1 pg to 100 ng/well. This ELISA method consistently detected cytokine concentrations higher than 10 pg/mL in a linear fashion.

Assays for Cell Survival and Apoptosis

The cell viability after treatment with tunicamycin was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay based cell counting kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). The hRPE cells were plated in 96-well plates at 1×10^4 cells per well in the growth medium. At the times indicated, absorbance at 450 nm was measured. The percentage of cell survival was determined by estimating the value of untreated cells at the indicated times as 100%. Apoptosis was quantified 24 or 48 hours after challenge with ELISA (Cell Death Detection ELISA kit; Roche Applied Science, Indianapolis, IN). The hRPE cells were seeded and grown in 96-well plates until the cells were close to confluence. After treating the cells with various concentrations of tunicamycin for 24 or 48 hours, we measured apoptosis according to the manufacturer's protocol. This immunoassay provides a relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) out of the cytoplasm of cells during apoptosis. Apoptosis was expressed as absorbance difference between A_{405nm} and A_{490nm}.

Statistical Analysis

Various assay conditions were compared by using ANOVA and *t*-test (Statview; SAS, Cary, NC) and $P < 0.05$ was considered to be statistically significant. Values represent means \pm SEM and are symbolized in the figures for $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Tissue Distribution of Human Caspase-12 mRNA

Two sets of primers were designed for RT-PCR analysis. The predicted sizes using primer pairs 1 to 2 are shown in Figure 1 (top left). As shown, four major bands were visualized on agarose gels. According to a previous report,¹¹ these four bands represent the variants γ (1051 bp), δ and ϵ (913 and 941 bp), ζ (816 bp), and η (706 bp), respectively. Because of low abundance, the two exon 3-containing variants α (1146 bp) and β (911 bp) are reported as undetectable when using primers that are similar to primers 1 and 2 in our study.¹¹ In agreement with this previous report, the largest variant α was not seen on the gels when using either primer pair 1 and 2 (1146 bp) or 3 and 4 (530 bp; Fig. 1). Located below the four major bands were two barely detectable bands, corresponding to variants θ (564 bp) and τ (454 bp).

Sequence analysis of the single band detected by primer pair 3 and 4 confirmed that the band represents the sequence common among variants γ , ϵ , and δ (435 bp; Fig. 1). These data are consistent with the previous report of Fischer et al.,¹¹ showing that caspase-12 contains both a 651G deletion and a 724T>C point mutation. Sequencing results confirmed that the hRPE cells used in the experiments were from subjects expressing only the short forms of caspase-12 (caspase-12S). Since the expressions of hRPE RT-PCR products from cells subjected to different treatments as detected by primer pair 3 and 4 were very similar to those detected by primer pair 1 and 2, we elected to use primer pair 3 and 4 in the RT-PCR analyses reported in this study.

RT-PCR analysis of caspase-12S expression by various human tissues showed that human caspase-12S was highly expressed in pancreas, prostate, small intestine, lung, spleen, and kidney; moderately in colon and testis; and barely in heart,

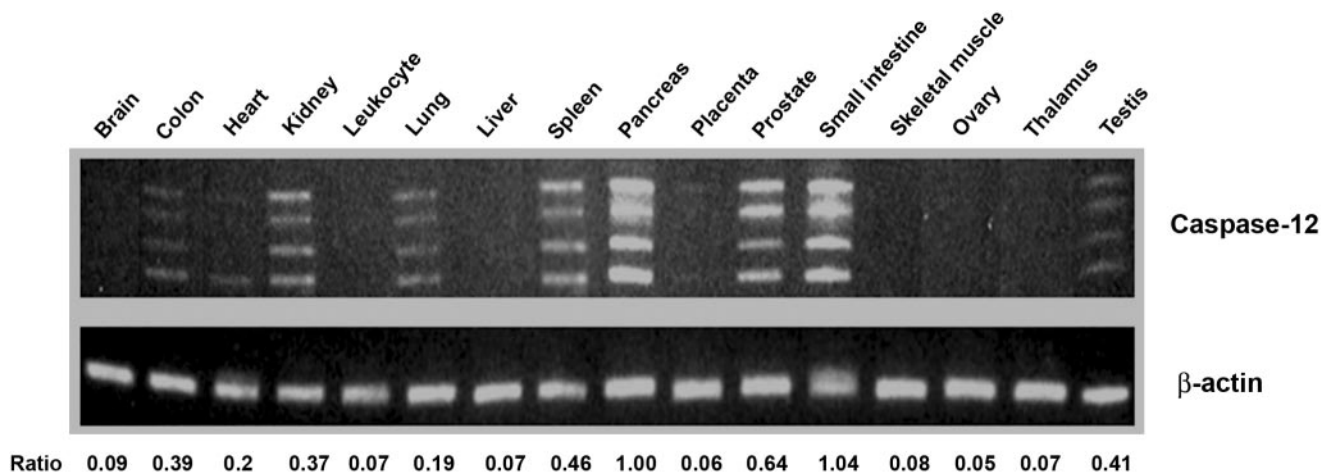


FIGURE 2. Tissue distribution of human caspase-12S mRNA. RT-PCR was performed with primers 1 to 2 for human caspase-12S and the primers for β -actin. The changes are expressed as ratios of densities of caspase-12S to those of β -actin mRNA, with the highest ratio for pancreas assigned as 1.

placenta, brain, leukocyte, liver, skeletal muscle, ovary, and thalamus (Fig. 2).

Downregulation of Caspase-12S mRNA Expression by IL-1 β , TNF- α , LPS, and Monocyte Coculture

Previous studies have shown that cells expressing full-length caspase-12L also exhibit impaired inflammatory responses.^{7,8} To determine whether caspase-12S is responsive to inflammatory stimuli, we examined caspase-12S mRNA levels in the hRPE cells treated with proinflammatory cytokines IL-1 β and TNF- α , exposed to LPS or cocultured with freshly isolated human monocytes. These treatments have been shown to be able to induce proinflammatory responses in hRPE cells.^{25,27-29} Chosen were concentrations of IL-1 β (2 ng/mL), TNF- α (20 ng/mL), and LPS (1000 ng/mL) and conditions for monocyte-hRPE coculture that we have shown to maximally stimulate proinflammatory responses in hRPE cells. As shown by semiquantitative RT-PCR (Fig. 3) and quantitative real-time RT-PCR (Fig. 4), all these agents and coculture with monocytes significantly reduced caspase-12S mRNA expression. As determined by quantitative real-time RT-PCR, treating hRPE cells with IL-1 β , TNF- α , LPS, and monocytes inhibited caspase-12S

mRNA expression by 76% \pm 9%, 71% \pm 4%, 62% \pm 8%, and 86% \pm 16%, respectively. However, treatment with interferon- γ (IFN- γ) at 500 U/mL for 6 hours appeared to elicit only mild reductions of hRPE caspase-12S mRNA expression, either as the sole stimulant or in addition to simultaneous TNF- α -mediated suppression of caspase-12S gene expression (Fig. 3).

Inhibition by inflammatory cytokines appeared to be transient (Fig. 5). At 3 and 6 hours post IL-1 β treatment, the caspase-12S mRNA expression was reduced by 30% and 50%, respectively, but returned to near control levels after 24 hours of incubation (90%).

Upregulation of Caspase-12S Expression by Cyclosporine (Cys) and IL-10

Because proinflammatory agents transiently inhibited caspase-12S mRNA expression, we surmised that immunomodulating agents such as Cys and anti-inflammatory cytokine IL-10 would have an opposite effect on caspase-12S mRNA expression. In our previous studies, we have demonstrated that Cys and IL-10

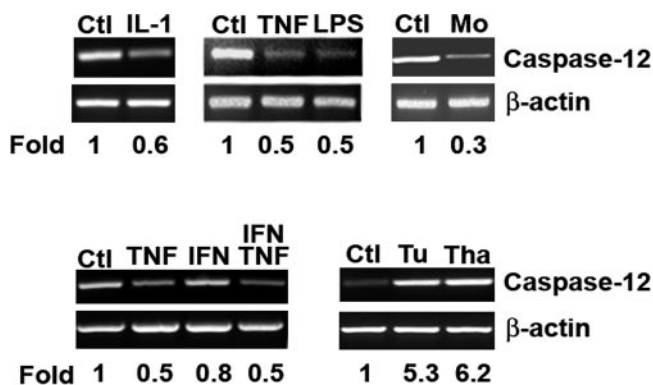


FIGURE 3. RT-PCR analysis of caspase-12S mRNA expression in hRPE cells. After hRPE cells were treated with selected agents for 6 hours, the total RNA was isolated and subjected to RT-PCR analysis. The changes (x -fold) were analyzed by relative density after normalization with β -actin. Ctl, untreated control; IFN, interferon- γ (500 U/mL); IL-1, IL-1 β (2 ng/mL); LPS, (1 μ g/mL); Mo, co-culture with monocytes; Tha, thapsigargin (25 nM); TNF, TNF- α (20 ng/mL), Tu, tunicamycin (3 μ M).

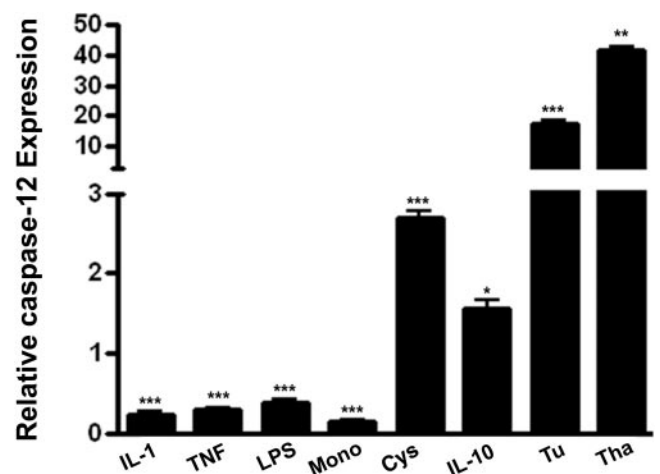


FIGURE 4. RT-PCR analysis of caspase-12S mRNA expression in hRPE cells. After hRPE cells were treated with selected agents for 6 hours, total RNA was isolated and subjected to quantitative real-time RT-PCR. * P < 0.05; ** P < 0.01; *** P < 0.001. CSA, cyclosporine (30 ng/mL); IL-1, IL-1 β (2 ng/mL); IL-10 (100 U/mL); LPS (1 μ g/mL); Mono, co-culture with monocytes; Tha, thapsigargin (25 nM); TNF, TNF- α (20 ng/mL), Tu, tunicamycin (3 μ M).

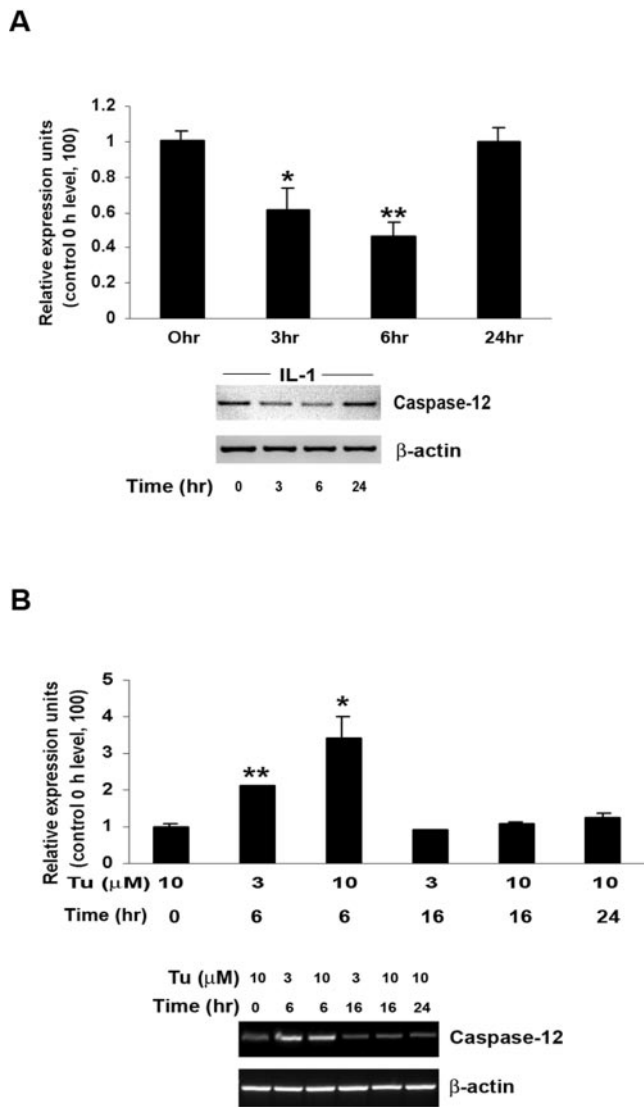


FIGURE 5. Time course of suppressed (A) and induced (B) human caspase-12S expression in hRPE cells. The hRPE cells were treated with 2 ng/mL of IL-1 β (A) and 3 or 10 μ M tunicamycin for the times indicated. Total mRNAs was isolated and subjected to semiquantitative and real-time RT-PCR analyses for caspase-12S; results are shown as bar graphs and on agarose gels, respectively. * $P < 0.05$; ** $P < 0.01$. Tu, tunicamycin.

exhibit anti-inflammatory actions by inhibiting stimulated chemokine production in hRPE cells.^{30,31} When hRPE cells were treated with Cys (30 ng/mL) and IL-10 (100 U/mL) for 6 hours, caspase-12S mRNA was, in fact, significantly induced. The stimulation by Cys and IL-10, as determined by quantitative real-time RT-PCR, was 2.7 ± 0.1 - and 1.6 ± 0.1 -fold, respectively (Fig. 4).

ER Stress Activator–Enhanced Caspase-12S Expression and Concomitant Suppression of IL-1 β - and TNF- α -Induced hRPE MCP-1

ER stress has been implicated in inflammatory responses. Thus, it is plausible that ER stress activators also alter caspase-12S gene expression. Two commonly used ER stress activators, tunicamycin and thapsigargin, were selected to determine their effects on hRPE caspase-12S expression. Treatment of hRPE cells with 3 μ M tunicamycin and 25 nM thapsigargin

resulted in markedly enhanced caspase-12S mRNA expression (Figs. 3, 4). The stimulated increases by tunicamycin and thapsigargin, as measured by quantitative real-time RT-PCR, were 17.3 ± 1.2 - and 41.8 ± 1.2 -fold, respectively (Fig. 4). As with the pattern observed for proinflammatory agents, stimulation of caspase-12S mRNA expression by these stress activators was also transient. Figure 5 shows that the maximum caspase-12S mRNA expression reached a peak at tunicamycin concentrations as low as 3 to 10 μ M after 6 hours' incubation. The stimulated expression, however, returned to the basal level when the cells were treated longer than 16 hours. Under the experimental conditions of this study, results by RT-PCR and real-time RT-PCR were about the same. Similarly, thapsigargin at concentrations of 25 and 100 nM resulted in about the same levels of stimulation in caspase-12S mRNA expression (data not shown).

Tunicamycin has also been known to cause ER stress-mediated apoptosis in many cell types. In this study, treating hRPE cells by 10 μ M tunicamycin for 48 hours also caused significant dose-dependent apoptosis as determined by an ELISA kit (Fig. 6A). To examine whether the reduced caspase-12S mRNA expression at 16 hours after treatment was due to

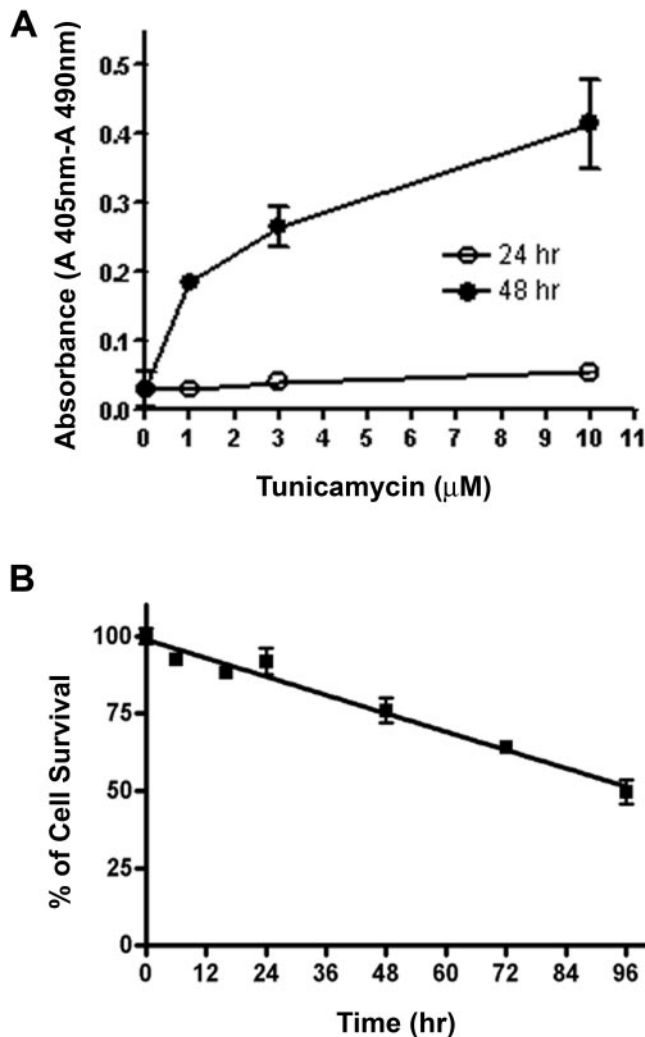


FIGURE 6. Cell survival and apoptosis after treating hRPE cells by tunicamycin. (A) Dose-dependent induction of apoptosis in hRPE cells by tunicamycin. (B) Time-dependent cell death by treating hRPE cells with 10 μ M tunicamycin. Apoptosis and cell survival were determined by an apoptosis detection ELISA kit and cell counting.

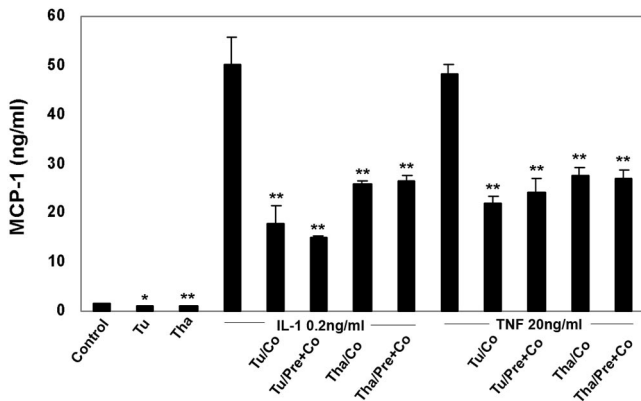


FIGURE 7. Inhibition of TNF- α - and IL-1 β -induced MCP-1 protein production and by tunicamycin and thapsigargin in hRPE cells. The cells were treated with IL-1 β (0.2 ng/mL) or TNF- α (20 ng/mL) by pre- or coincubation with tunicamycin (10 μ M) or thapsigargin (100 nM) for 1 hour or 24 hours, respectively. The media were collected for ELISA analysis of MCP-1 protein. * P < 0.05; ** P < 0.01. Co, co-incubation; IL-1, IL-1 β ; pre, preincubation; Tha, thapsigargin; TNF, TNF- α ; Tu, tunicamycin.

cell death, we assessed the time course of cell survival in the presence of tunicamycin (Cell Counting Kit-8; Dojindo Molecular Technologies). As seen in Figure 6B, the cell survival percentage dropped in a linear fashion. At 6 hours, when tunicamycin-induced caspase-12S mRNA reached its peak (Fig. 5), the cell death rate was 8%. At 16 hours, when the levels of caspase-12S mRNA returned to basal levels (Fig. 5), the cell death rate was only slightly higher than that at 6 hours (12%), suggesting that the reduced caspase-12S expression under persistent stimulation by tunicamycin was mainly due to transient protective responses similar to that observed in the presence of proinflammatory agents.

Most previous studies have demonstrated that ER stress induces proinflammatory responses.³² The results from treating the hRPE cells by tunicamycin and thapsigargin appeared contradictory to this notion, because these two agents were similar to the anti-inflammatory agents in stimulating caspase-12S expression. To test whether these two ER stress activators induce anti-inflammatory rather than proinflammatory responses in hRPE cells, we examined the effect of ER stress activation on MCP-1 expression and caspase-12S mRNA expression. Our previous studies demonstrated that proinflammatory IL-1 β and TNF- α each strongly stimulate MCP-1 mRNA expression and protein production in hRPE cells.^{27,28} In the present study, we used stimulation of MCP-1 expression as an indicator of hRPE proinflammatory response to the ER stress activators. Tunicamycin and thapsigargin both had a small, but statistically significant, inhibitory effect on constitutive hRPE MCP-1 secretion (Fig. 7). In contrast, when hRPE cells were exposed to tunicamycin and thapsigargin before and during incubation with IL-1 β or TNF- α or to these ER stressors only during incubations with IL-1 β or TNF- α , MCP-1 secretion was markedly inhibited. MCP-1 induced by IL-1 β was $70.1\% \pm 1.1\%$ and $64.6\% \pm 14.0\%$ reduced by tunicamycin and $47.3\% \pm 2.3\%$ and $48.4\% \pm 1.3\%$ by thapsigargin (pre- and co-incubation, respectively), whereas MCP-1 induced by TNF- α was $50.0\% \pm 6.0\%$ and $44.0\% \pm 2.6\%$ reduced by tunicamycin, and $54.5\% \pm 3.9\%$ and $42.7\% \pm 2.4\%$ by thapsigargin (pre- and co-incubation, respectively; Fig. 7).

Consistent with inhibition of the stimulated MCP-1 secretion, 25% inhibition by tunicamycin of IL-1 β -stimulated MCP-1 mRNA production was also observed (Fig. 8A). To further investigate the link between the protective anti-inflammatory response by tunicamycin and caspase-12S expression, we de-

termined whether caspase-12S mRNA was involved in hRPE cells treated by tunicamycin in combination with TNF- α and IL-1 β . As demonstrated in Figure 8B, combined stimulation by tunicamycin (which stimulates caspase-12S expression) with IL-1 β or TNF- α (which inhibit caspase-12S expression) only slightly reduced the enhanced caspase-12S mRNA by tunicamycin alone. The net induction of caspase-12S expression by co-incubation of IL-1 β or TNF- α with tunicamycin suggests that tunicamycin dominates the controlled expression of caspase-12S mRNA and acts like anti-inflammatory agents.

DISCUSSION

Progressive cell loss is a key feature of many ocular diseases. Since murine caspase-12 plays an important role in ER stress-induced apoptosis,¹² this finding has stimulated several studies on activation of caspase-12 in various ocular tissues^{33,34} and cells, including RPE cells,^{35,36} retinal photoreceptor cells,³⁷ retinal neurosensory cells,³⁸ retinal ganglion cells,³⁹ retinal pericytes,⁴⁰ lens epithelial cells,⁴¹ and keratoconus corneal fibroblasts.⁴² Most of these studies have been performed in rodent models. The current knowledge about caspase-12 functional differences between humans and rodents suggests that the results from rodent models may not translate to humans.⁷ Accordingly, human caspase-4 and -5 have been proposed as the candidates, corresponding to mouse caspase-12, that mediate the ER stress-induced cell death of human cells.^{1,14}

Although the relatively high level of expression of caspase-12S in lung agreed with previous reports in human and mouse,^{11,43} our results demonstrate additional differences in tissue expression and regulation of human caspase-12S compared with those reported for mouse caspase-12. In contrast to the undetectable expression in eyes and high level expression in skeletal muscle of mouse caspase-12,⁴³ we found expression of caspase-12S mRNA to be high in hRPE cells, but undetectable in human skeletal muscle, the latter finding similar to that reported by Fischer et al.¹¹ We also found IL-1 β , TNF- α , and LPS to reduce and IFN- γ to mildly reduce hRPE caspase-12S gene expression, even though IFN- γ , LPS, and TNF have been shown to stimulate mouse caspase-12 mRNA production in

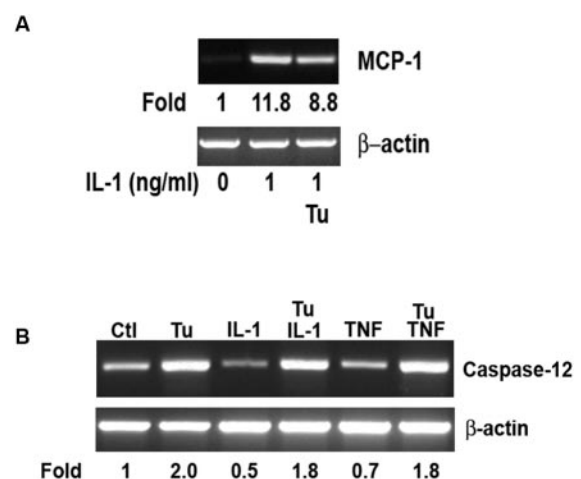


FIGURE 8. Inhibition of MCP-1 mRNA expression induced by IL-1 β (A) and of caspase-12S mRNA expression induced by tunicamycin in combination with IL-1 β or TNF- α (B). Human RPE cells were stimulated with 2 ng/mL of IL-1 β or 20 ng/mL of TNF- α in the presence or absence of tunicamycin (10 μ M) for 6 hours. The total RNA was isolated and subjected to RT-PCR analysis. The relative expression levels were estimated by normalization with β -actin mRNA. IL-1, IL-1 β ; TNF, TNF- α ; Tu, tunicamycin.

B16/B16 cells. Therefore, caution must be exercised when translating results of rodent caspase-12 studies to humans.

It should be noted that activation of caspase-12 in human ocular cells has been the subject of several reports. The methods used in these studies include use of carboxyfluorescein apoptosis detection kits (FLICA; Immunochemistry Technologies LLC, Bloomington, MN),³⁶ fluorochrome inhibitor,⁴² and anti-caspase-12 antibodies.^{35,40,41} However, the carboxyfluorescein FLICA apoptosis detection kits are not made to detect human caspase-12. The fluorochrome inhibitor (BioVision Research Products, Mountain View, CA) was designed based on the key amino acid residues ATAD immediately upstream from the autocleavage site at the large-small subunit junction of rodent caspase-12.⁴⁴ In human caspase-12L, the corresponding four amino acids are ASAD, not identical with that of rodent caspase-12. Moreover, this inhibitor has not been tested against human caspase-12L protein or shown differentially acting on caspase-12L, but not caspase-12S-positive cells. Regarding anti-caspase-12 antibodies, all the commercially available antibodies were raised against mouse caspase-12. However, the ER stress-activated, cross-reactive proteins that were detected in these reports were probably not derived from the known human caspase-12 gene. First, the most commonly used antibodies, for example, are made against mouse caspase-12 amino acids 2-17 and 100-116, regions that show no homology to human caspase-12. Second, the truncated short forms of caspase-12 (caspase-12S) encode proteins with molecular weights not higher than 20 kDa, not matching the molecular weight of 50 to 60 kDa assigned for caspase-12 in those reports. The full-length, high-molecular-weight human caspase-12L is not common and is confined to a small population of African descent.^{7,17,18} Third, individuals with the wild-type (caspase-12S/S), heterozygous (caspase-12S/L), and homozygous (caspase-12L/L) genotypes do not show differences in ER stress-mediated apoptosis.⁷ Therefore, the identity of the ER stress-activated human proteins recognized by the commercially available antibodies to caspase-12 has yet to be determined.

Despite the current notion that human caspase-12 is not directly involved in ER stress-induced apoptosis,¹ the inhibition of NF- κ B activation by human caspase-12L and caspase-12S⁷ suggests their potential roles in ER stress-induced apoptosis because activation of NF- κ B is known to play an important role in this type of apoptosis.^{45,46} Indeed, we found that blockade of NF- κ B activation in hRPE cells significantly accelerated tunicamycin-induced cell death (data not shown).

Inflammatory cytokines are essential mediators of the innate immune response. Cytokines play a critical role in noninfectious and infectious retinal diseases, such as proliferative vitreoretinopathy,⁴⁷ age-related macular degeneration,⁴⁸ uveitis,⁴⁹ and endophthalmitis.⁵⁰ It is believed that excessive cytokine levels set the stage for capillary leakage and tissue injury. As hRPE cells are known to be major sources of these cytokines, hRPE cells are thought to participate actively in propagating these retinal diseases. Recent results from caspase-12L studies have revealed the dual nature of cytokines.¹⁶ The inhibitory effect of human caspase-12L on caspase-1 signaling pathways may occur in a manner similar to its mouse homologue via autoproteolysis at its Asp³¹⁹ within the caspase-1 complex.⁴⁴ The activation of caspase-12L may be dominant and detrimental to the small number of individuals expressing caspase-12L, as full-length human caspase-12 leads to suppressed cytokine production and increased susceptibility to infections and severe sepsis.⁷ The close link between susceptibility to microbial infection and expression of the full-length caspase-12L is underscored by the fact that bacterial clearance and sepsis resistance are enhanced in caspase-12-deficient mice, possibly due to the dominant-negative suppressive effect of caspase-12 on caspase-1.⁸ The functional difference between

caspase-12L and caspase-12S can also be seen in human hepatitis C virus (HCV) clearance that is impaired by expression of the former.¹⁵ Although caspase-12S has been taken as loss-of-function mutant of human caspase-12L, the transient up- and downregulation of caspase-12S gene expression by anti- and proinflammatory agents that we show in hRPE cells suggests that human caspase-12S is unlikely to be a null gene. Instead, it may function as a dominant negative regulator in a way similar to many other CARD-only proteins.⁵ To our knowledge, the regulation pattern of caspase-12S expression we report in hRPE cells has not been reported in any cell type; it suggests that caspase-12S may be a mediator, that has the capacity to facilitate both pro- and anti-inflammatory responses.

Although ER stress is known to stimulate certain inflammatory cytokines, we found that proinflammatory hRPE responses are attenuated during early ER stress induced by tunicamycin and thapsigargin. This effect was shown by the ability of these two ER stressors to block TNF- α - and IL-1 β -induced hRPE MCP-1. The anti-inflammatory effects of tunicamycin and thapsigargin that we observed are consistent with several previous reports.⁵¹⁻⁵³ Since homozygosity for caspase-12S has evolved as the dominant form of caspase-12 in most human populations, the counterpart of human caspase-12S acting in human cells is unlikely to be the full-length caspase-12L as it is for other caspase pairs. Caspase-12S probably inhibits molecules yet to be identified. As NOD1/2 and RIP2 interaction is an important event upstream from caspase-1 signaling and NF- κ B activation, it has been hypothesized that caspase-12 is upregulated by LPS stimulation and inhibits cytokine production by dominantly inhibiting the CARD-CARD interaction between NOD and RIP2.¹ This assumption is attractive because it accounts for the roles of caspase-12 in inhibiting both caspase-1-mediated pro-IL-1 and pro-IL-18 cleavage and NF- κ B activation. However, a recent pull-down study shows that mouse caspase-12 coimmunoprecipitates with RIP2 independent of the CARD domain,⁵⁴ suggesting that the potential role in immunomodulation played by the CARD-only human caspase-12S may mechanistically differentiate it from human caspase-12L-mediated antimicrobial peptide production and mucosal immunity. As the CARD amino acid sequence of caspase-12 is more closely related to caspase-1 than any other CARD-containing proteins, direct interaction between caspase-12 and -1 cannot be ruled out. Microbial infections of the eye are the common cause of various inflammatory ocular diseases, such as infectious keratitis, onchocerciasis, bacterial endophthalmitis, viral retinitis, and infectious uveitis.⁵⁵ In small populations of African descent who express caspase-12L, caspase-12L could be a therapeutic target for infectious ocular diseases, as well as for the prevention of sepsis.⁵ Based on the present study, it is reasonable to postulate that caspase-12S is functionally similar to caspase-12L, but with reduced immunosuppressive activity. Therefore, for most populations who express caspase-12S, modulation of caspase-12S expression may also be beneficial. Further delineating the role of caspase-12S in proinflammatory signaling cascades in human tissues and cells will shed light into the functional role of human caspase-12 and may provide novel pharmacologic interventions for inflammatory ocular diseases.

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