Retinal Pigment Epithelium Is Protected Against Apoptosis by αB-Crystallin

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PURPOSE. The degeneration of retinal pigment epithelial (RPE) cells is considered to be a crucial event in the pathophysiology of age-related macular degeneration (AMD). Cumulative oxidative damage has been implicated in the development of the changes seen in AMD. The present study was undertaken to evaluate the expression of the small heat shock protein αB-crystallin in the RPE in response to oxidative stress and to explore whether αB-crystallin expression confers an antiapoptotic cytoprotective effect on RPE cells.

METHODS. Native human RPE cells from the macula and retinal periphery were analyzed by RT-PCR and Western blot analysis for expression of αB-crystallin. Monolayer cultures of human RPE cells were stressed by heat shock (42°C for 20 minutes) or oxidant-mediated injury (50–300 μM H2O2 for 1 hour). Induction of αB-crystallin and the corresponding mRNA was assessed by Western and Northern blot analyses. To study the cytoprotective effect of αB-crystallin, human RPE cells were transfected with either a neomycin-selectable expression vector containing αB-crystallin cDNA or a control vector without αB-crystallin cDNA. Caspase-3 activity was determined by observing the cleavage of a colorimetric peptide substrate. Cell viability was quantified by combined propidium iodide and Hoechst 33342 staining.

RESULTS. αB-crystallin is constitutively expressed in RPE under in vivo and in vitro conditions. Western blot analysis of freshly isolated RPE showed greater baseline expression levels in RPE derived from the macular area than in that from the more peripheral regions. Heat shock treatment and oxidative stress caused a significant increase in αB-crystallin mRNA and protein. Oxidant-mediated injury in RPE cells with baseline expression levels of αB-crystallin resulted in apoptotic cell death, as measured by caspase-3 activity, whereas RPE cells that had been stably transfected with αB-crystallin were more resistant to H2O2-induced cellular injury.

CONCLUSIONS. αB-crystallin may function as a stress-inducible antiapoptotic protein in human RPE and is inducible by oxidative stress, a condition implicated in the pathogenesis of AMD. Overexpression of αB-crystallin may be an important mechanism for the RPE to prevent apoptotic cell death in response to cellular stress. (Invest Ophthalmol Vis Sci. 2002;43:3575–3582)

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and cytoprotective effect of αB-crystallin in RPE cells in response to oxidative damage.

**Materials and Methods**

**Isolation of Human RPE Cells**

Eyes of 12 human donors were obtained from the Munich University Hospital Eye Bank and processed within 4 to 16 hours after death. The donors ranged in age between 18 and 79 years. None of the donors had a known history of eye disease. Methods for securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee. Human RPE cells were harvested after the procedure as described previously.36 In brief, whole eyes were thoroughly cleansed in 0.9% NaCl solution, immersed in 5% poly(1-vinyl-1-2-pyrrolidone)-iodine (Jodobac; Bode-Chemie, Hamburg, Germany), and rinsed again in sodium chloride solution. The anterior segment from each donor eye was removed, and the posterior poles were examined with the aid of a binocular stereomicroscope to confirm the absence of gross retinal disease. Next, the neural retinas were carefully peeled away from the RPE-choroid-sclera with forceps. The eyeball was cut in the equator plane and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) solution into the eyecup. The suspended RPE cells were transferred to a 50-ml flask (Falcon, Becton Dickinson, Heidelberg, Germany) and grown in 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM; Biochrom, Berlin, Germany) supplemented with 20% fetal calf serum (FCS; Biochrom). A pipette, the medium was gently agitated, releasing the RPE into the medium and avoiding damage to Bruchi’s membrane.

For RT-PCR analysis, the RPE cells from three donors were released from Bruch’s membrane by gently pipetting ice-cold phosphate-buffered saline (PBS; pH 7.4) solution into the eyecup. The RPE cells were transferred to a 2.0-ml microcentrifuge tube and centrifuged for 5 minutes at 800 rpm. After centrifugation, the supernatant was removed and replaced by RNA extraction solution.

For preparation of protein lysates the posterior poles from eyes of five different donors were prepared as described earlier. Then, 5-mm tissue punches were taken from the macular and extramacular regions. Specimens were placed in a 35-mm² Petri dish containing ice-cold PBS. Under visual control, a stream of PBS was pipetted over the specimen surface to mechanically dislodge the RPE cells from the basement membrane. The RPE cell suspension was checked by microscope for cross-contamination. Cell suspensions were then transferred to a 2.0-ml microcentrifuge tube and centrifuged for 5 minutes at 800 rpm. After centrifugation, the supernatant was replaced with NP-40 cell lysis buffer. After centrifugation, the supernatant was removed and replaced by RNA extraction solution.

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**Northern Blot Analysis of αB-Crystallin**

After RNA isolation, total RNA (2 µg) was denatured and size fractionated by gel electrophoresis in 1% agarose gels containing 2.2 M formaldehyde. The RNA was then vacuum blotted onto a nylon membrane (Roche) and cross-linked (1600 µJ, Stratalinker; Stratagene). To assess the amount and quality of RNA, the membrane was stained with methylene blue, and images were obtained (LAS-1000; RayTest). Prehybridizations were performed at 68°C for 1 hour. Hybridizations were performed at 68°C overnight in prehybridization solution (Dig Easy Hyb; Roche) containing 50 ng/ml digoxigenin-labeled, αB-crystallin-specific, 450 bp antisense riboprobe. Riboprobes were synthesized as described before. After hybridization, the membrane was washed twice with 2X SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature (RT), followed by two washes in 0.1X SSC, 0.1% SDS, for 15 minutes at 68°C. After hybridization and posthybridization washes, the membrane was washed for 5 minutes in washing buffer (100 mM maleic acid [pH 7.5] and 150 mM NaCl, 0.3% Tween-20) and incubated for 60 minutes in blocking solution. The blocking solution contained 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 1% blocking reagent (Roche). Anti-digoxigenin alkaline phosphatase (Roche) was diluted 1:10,000 in blocking solution and the membrane incubated for 30 minutes. After an additional four washes in washing buffer (each 15 minutes), the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl [pH 9.5]) for 5 minutes. For fluorescence detection a chemiluminescence substrate (CDP-Star; Roche) was diluted 1:100 in blocking solution and the membrane incubated for 30 minutes. After an additional four washes in washing buffer (each 15 minutes), the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl [pH 9.5]) for 5 minutes. For fluorescence detection a chemiluminescence substrate (CDP-Star; Roche) was diluted 1:100 in detection buffer and the filter incubated for 5 minutes at RT. After air-drying, the semidry membrane was sealed in a plastic bag. Chemiluminescence was detected with the imager (LAS-1000; RayTest). Exposure times ranged between 5 and 40 minutes. Quantification of the chemiluminescence signal was performed on computer (AIDA software; RayTest).

**Protein Extraction and Western Blot Analysis of αB-Crystallin**

Cells grown on 35-mm² tissue culture dishes were washed twice with ice-cold PBS, collected, and lysed in NP-40 cell lysis buffer. After induction of oxidative stress, cells were washed three times with serum-free and phenol red-free medium (GibcoBRL) and incubated with 200 to 300 µM H2O2 in this medium for 1 to 2 hours. Thereafter, the medium was changed and replaced by regular medium.

**RNA Isolation and RT-PCR of Native and Cultured RPE Cells**

To determine whether αB-crystallin is synthesized by the RPE, total RNA was isolated from freshly prepared donor RPE and cultured RPE cells by the guanidium thiocyanate-pheno chloroform extraction method (Stratagene, Heidelberg, Germany). After confirming the structural integrity of the total RNA samples by electrophoresis on 1% agarose gels, 1 µg of RNA was reverse transcribed using oligo(dT)12-18 (GibcoBRL) as a primer in the presence of absence of reverse transcriptase (Supercript II; GibcoBRL) according to the manufacturer’s instructions. PCR was then performed in a total volume of 50 µL using cDNA as a template in the presence of primer pairs derived from the human αB-crystallin sequence as described.39 Oligonucleotides were purchased from GibcoBRL, and 10X reaction buffer and Taq polymerase were obtained from Eppendorf (Hamburg, Germany). Hot-start PCR was used. cDNA was melted at 94°C for 10 minutes to denature the DNA, and the reaction was then run through 36 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds in a thermocycler (Mastercycler Gradient; Eppendorf). After the last cycle, the polymerization was extended for 10 minutes to complete all strands. PCR performed on each sample of RNA that had not been reverse transcribed to cDNA was used as a negative control and showed no amplified product. PCR amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization with an imager workstation (LAS-1000; RayTest, Pforzheim, Germany).
centrifugation for 30 minutes at 19,000g in a microtube (5810R; Eppendorf) in the cold, the supernatant was transferred to fresh tubes and stored at −70°C for future use. The protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Denatured proteins (1–2 µg) were separated under reducing conditions by electrophoresis using a 5% SDS-polyacrylamide stacking gel and a 12% SDS-polyacrylamide separating gel. Blots were transferred with semidry blotting onto a polyvinyl difluoride membrane (Roche) and probed with a rabbit anti-aB-crystallin antibody as described before.8 Chemiluminescence was detected with the imager (LAS-1000; RayTest). Exposure times ranged between 1 and 10 minutes. Quantification was performed on computer (AIDA software; RayTest).

**Construction of aB-Crystallin cDNAs**

The full-length aB-crystallin cDNA was PCR amplified from human RPE cDNA and cloned into a mammalian expression vector bearing the neomycin resistance gene, as described elsewhere.41 In brief, full-length aB-crystallin cDNA amplicons were created with the following oligonucleotide primers: 5′-GGCGCAATTCATGCATGGCATCCACGAC- CAC-3′ and 5′-GGGCCCTCGAGATTTTCTGGGGTCTGG-GG-3′. Primers were purchased from Metabion (Munich, Germany). The PCR products were then digested with EcoRI and XhoI (Stratagene) and inserted into a pcDNA3 mammalian expression vector (Invitrogen, Karlsruhe, Germany) bearing the neomycin-resistance gene. Sequences were confirmed by automated DNA sequencing (Sequiserve, Vaterstetten, Germany).

**Establishment of Stably Transfected Cell Lines**

To ensure consistency in the results, stable transfection experiments were undertaken on cultured primary RPE cells of passage 3 and on the commercially available immortalized human RPE cell line ARPE 19. Cells were grown in 60-mm dishes to 70% to 80% confluence and stably transfected with the neomycin-resistance pcDNA3/aB-crystallin plasmid (pcDNA3/aB-crystallin). Control cells were transfected with the neomycin-resistant expression vector without insertion of aB-crystallin cDNA (pcDNA3/neo). Transfection complexes were prepared by pre-incubation of plasmid (7 g) with liposome reagent (Lipofectamine; Invitrogen) according to the manufacturer’s instructions. The reagent complex was then gently distributed over the cells and incubated at 37°C and 5% CO2. After 2 hours, 150 µL serum-free, antibiotic-free DMEM was added, and incubation was continued for another 4 hours. Then, 3 mL antibiotic-free DMEM containing 5% FCS was added to each dish. Cells were allowed to recover in DMEM with 10% FCS for 12 hours, before G418 sulfate (Sigma), at a concentration of 650 µg/mL for primary RPE cells and 700 µg/mL for ARPE-19 cells, was added to begin selection. After an additional 3 days, cells were replated on a 10-cm dish. Medium containing G418 sulfate was renewed twice weekly, and after 3 weeks, individual colonies were isolated with cloning rings, subcultured, and expanded. Individual G418 sulfate-resistant clones were examined for expression of aB-crystallin by immunoblot analysis with anti-aB-crystallin as described.

**Caspase-3 Assay**

Caspase-3 activity was determined with a colorimetric assay (ApoAlert; Clontech, Heidelberg, Germany) according to the manufacturer’s protocol. In this assay the capacity of the cellular caspase-3 to cleave the labeled substrate DEVD-p-nitroaniline (DEVD-pNA) was measured spectrophotometrically. In brief, apoptosis was induced by 200 to 500 µM H2O2 for 75 minutes. Cells were harvested, and aliquots of 2.5 × 104 cells were used for each reaction. Cell lysates were incubated in the presence or absence of 50 µM DEVD-pNA for 1 hour at 37°C. Absorbance was measured at 405 nm in a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA). Uninduced and induced cells without substrate served as the background control. Additional induced cells were incubated with DEVD-CHO, an inhibitor of caspase-3, to correlate protease activity and signal detection. Samples from three independent experiments were assayed in duplicate.

**Cell Viability Assay**

Cell viability was quantified based on a two-color fluorescence assay in which the nuclei of nonviable cells appear red because of staining by the membrane-impermeable dye propidium iodide (Sigma), whereas the nuclei of all cells were stained with the membrane-permeable dye Hoechst 33342 (Intergen, Purchase, NY). Confluent cultures of RPE cells growing on coverslips in 24-well tissue culture plates were exposed to 500 µM H2O2 for 2 hours. For evaluation of cell viability, cells were washed in PBS and incubated with 2.0 µg/mL propidium iodide and 1.0 µg/mL Hoechst 33342 for 20 minutes at 37°C. Subsequently, cells were analyzed with an epifluorescence microscope (Axiophot; Zeiss, Göttingen, Germany). Representative areas were documented on film (Fujichrome 400; Fuji Film, Tokyo, Japan). The labeled nuclei were then counted in fluorescence photomicrographs, and dead cells were expressed as a percentage of total nuclei in the field. The data are based on counts from three experiments performed in duplicate wells, with three to five documented representative fields per well.

**Results**

**Regional Differences of aB-Crystallin Expression in Human RPE In Vivo**

To evaluate the in vivo and in vitro expression of aB-crystallin, retinal pigment epithelium (RPE), derived from freshly prepared human donor eyes as well as cultured human RPE, was analyzed by RT-PCR. Figure 1A indicates that the 450-bp amplicons representing aB-crystallin mRNA are present in the donor RPE as well as in cultured RPE cells. To determine the spatial distribution pattern of aB-crystallin expression in the retinal pigment epithelium, RPE was freshly isolated from extramacular and macular regions, respectively. Subsequent immunoblot analysis for aB-crystallin showed a 2.5-fold higher amount of the 22-kDa protein in macular compared with extramacular RPE (Fig. 1B). Experiments were performed with macular and peripheral RPE of five eyes from individual donors and showed similar results.
Induction of αB-Crystallin in Cultured RPE Cells by Cellular Stress

To find out whether αB-crystallin functions as a stress-inducible protein or marker for cellular stress in RPE, the next experiments were performed in vitro on human RPE cells of passages 3 to 5. RPE cells were subjected to elevated temperature, the classic stimulus for induction of HSPs. Northern blot analysis revealed a strong induction of αB-crystallin mRNA at 4 hours after heat shock treatment (Fig. 2C). This effect was paralleled by an increased expression of αB-crystallin at the protein level (Fig. 2F). These results suggest that increased αB-crystallin expression may result from increased cellular stress in the RPE.

Next, we sought to determine whether a stimulus implicated in the pathogenesis of AMD leads to an increased expression of αB-crystallin. In an attempt to reproduce such pathologic conditions, we chose to study the expression of αB-crystallin after RPE injury with a mild oxidizing stimulus. Northern blot analysis of untreated RPE showed a faint band that was approximately 0.8 kb in length. Exposure of RPE cells to 200 μM H2O2 for 1 hour substantially increased αB-crystallin expression 4 hours after treatment (three-to fourfold; Fig. 2D). This increase in protein level was smaller than that of mRNA.

To determine whether the oxidative-injury-mediated increase in αB-crystallin mRNA was associated with corresponding changes at the protein level, RPE cells were incubated in the same conditions as described earlier and analyzed by SDS-PAGE. Western blot analysis showed a two to threefold increase in αB-crystallin protein 4 hours after oxidative stress (Fig. 2E). For both types of stress, however, the increase in protein level was smaller than that of mRNA.

To determine the time course of αB-crystallin mRNA induction, RPE cells were harvested 2 to 12 hours after induction of oxidative injury. Under these experimental conditions, Northern blot analysis revealed a marked increase of αB-crystallin mRNA after 2 hours. This induction reached a peak at 6 hours after treatment (five-to sixfold). Twelve hours after oxidative stress mRNA levels had declined to two to four times the baseline level (Fig. 3A). Western blot analysis demonstrated similar, although less-pronounced, changes at the protein level, which occurred with a longer latency (Fig. 3C). Thus, the increases in αB-crystallin mRNA in response to oxidative stress were followed by a corresponding increase in protein synthesis.

Effect of Increased Expression Levels of αB-Crystallin on Oxidative Stress-Induced Activation of Caspase-3 and Cell Death in RPE Cells

To evaluate a possible functional role of αB-crystallin induction in RPE, we stably transfected primary RPE cells and ARPE-19 cells with either a neomycin-selectable expression plasmid containing human αB-crystallin cDNA (pcDNA3/αB-crystallin) or a control neomycin-selectable expression vector containing no αB-crystallin cDNA (pcDNA3/neo). Thirteen clones independently derived from RPE cells transfected with pcDNA3/
Effect of αβ-Crystallin on Apoptosis in RPE

In our in vitro experiments, we showed that αβ-crystallin in cultured RPE was inducible by raising the temperature and oxidative stress, demonstrating its role as a stress-inducible protein in this cell type. However, for both types of stress, stimulation at the mRNA level was not paralleled by a similar effect at the protein level. A similar discrepancy between mRNA and protein expression of αβ-crystallin has been described for ciliary muscle cells and trabecular meshwork cells after treatment with transforming growth factor-β, and for neonatal cardiac muscle cells after oxidative damage and heat shock. These findings suggest that the newly transcribed αβ-crystallin mRNA may not be completely translated into protein, suggesting that posttranscriptional regulatory mechanisms may be involved in regulation of the αβ-crystallin protein level. Further studies are necessary to elucidate the underlying molecular mechanisms responsible for these findings.

The events of cellular stress and cell death are linked, and HSPs induced in response to stress appear to function at key regulatory points in the control of apoptosis. There are two principal pathways leading to apoptosis: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. The caspase family of cysteine proteases are critical effectors of apoptosis that selectively cleave key proteins at aspartate residues. The mitochondrial and death receptor-me-
diated pathways activate distinct apical caspases (caspase-9 and -8, respectively) that converge on the proteolytic activation of the downstream executioner, caspase-3. Many HSPs are anti-apoptotic and directly inhibit activation of caspase. For instance, HSP-70 and -90 bind to apoptotic protease activation factor 1 (Apaf-1) and therefore prevent activation of caspase-9, whereas HSP-27 has been shown to antagonize apoptosis by interaction with cytochrome c. However, αB-crystallin has been reported to antagonize both the mitochondrial (cytochrome c/Apaf-1-dependent) and the death receptor (caspase-8-dependent)-mediated activation of caspase-3, by inhibiting its autoproteolytic maturation. In lens epithelial cells, transgene downregulation of the αB-crystallin gene attenuated the ability to resist H$_2$O$_2$-induced apoptosis. In cardiomyocytes, transgene overexpression of αB-crystallin conferred protection against apoptosis during myocardial ischemia and reperfusion. In RPE cells, oxidative stress has been reported to induce apoptosis through activation of caspase-3. In keeping with this, we were able to demonstrate an increase of caspase-3 activity upon oxidative stress in RPE cells with baseline expression of αB-crystallin. This was in contrast to stably transfected RPE cells containing high amounts of αB-crystallin, in which caspase-3 activity remained low, despite oxidative injury. Furthermore, overexpression of αB-crystallin increased the viability of cultured RPE cells after oxidative injury. Taken together, our observations suggest that induction of αB-crystallin in RPE may, at least in part, function to protect the RPE from a rash induction of cell death.

We believe that our findings are relevant to the understanding of degenerative diseases with prolonged progression, such as AMD. HSPs such as αB-crystallin allow cells to adapt to gradual changes in their environment and to survive otherwise lethal conditions. Proapoptotic stimuli, delivered below a threshold level, such as moderate oxidative stress, can elicit protective responses, amplifying resistance to cell death. Elevated constitutive expression levels of αB-crystallin in macular...
RPE may reflect an increased need for cellular protection in this particular region of the eye. However, with age, the intrinsic antioxidant capacity of the RPE decreases. Particularly, the RPE of patients with AMD has repeatedly been reported to have decreased antioxidant activity. At the same time, with age the RPE is confronted with an abundance of endogenous photoperoxidizers, such as lipofuscin. At this point, alternative protective cellular mechanisms, such as induction of stress proteins, may become relevant. To what extent chronic low-level damage and decreased antioxidant activity contribute to development of AMD is not yet clear. Although fully speculative, it is tempting to hypothesize that an overexpression of stress proteins such as αB-crystallin in RPE cells may provide an additional mechanism to postpone cell death in age-related degenerative diseases.

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References


