

Retinal Pigment Epithelium Is Protected Against Apoptosis by α B-Crystallin

Claudia S. Alge,¹ Siegfried G. Priglinger,¹ Aljoscha S. Neubauer,¹ Anselm Kampik,¹ Markus Zillig,² Hans Bloemendal,³ and Ulrich Welge-Lüssen¹

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 α B-crystallin in the RPE in response to oxidative stress and to explore whether α B-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 α B-crystallin. Monolayer cultures of human RPE cells were stressed by heat shock (42°C for 20 minutes) or oxidant-mediated injury (50–300 μ M H₂O₂ for 1 hour). Induction of α B-crystallin and the corresponding mRNA was assessed by Western and Northern blot analyses. To study the cytoprotective effect of α B-crystallin, human RPE cells were transfected with either a neomycin-selectable expression vector containing α B-crystallin cDNA or a control vector without α B-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. α B-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 α B-crystallin mRNA and protein. Oxidant-mediated injury in RPE cells with baseline expression levels of α B-crystallin resulted in apoptotic cell death, as measured by caspase-3 activity, whereas RPE cells that had been stably transfected with α B-crystallin were more resistant to H₂O₂-induced cellular injury.

CONCLUSIONS. α B-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 α B-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)

From the ¹Department of Ophthalmology, Ludwig-Maximilians-University, Munich, Germany; the ²Department of Anatomy II, University of Erlangen-Nürnberg, Erlangen, Germany; and the ³Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

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Corresponding author: Ulrich Welge-Lüssen, Department of Ophthalmology Ludwig-Maximilians-University, Mathildenstrasse 8, 80336 Munich, Germany; ulrich.welge-luessen@ak-i.med.uni-muenchen.de.

Age-related macular degeneration (AMD) is the leading cause of severe visual impairment in elderly individuals.^{1,2} The complex pathogenesis of the disease is poorly understood, and there is no efficient therapy or prevention to date. Early AMD is characterized by changes in the pigmentation of the retinal pigment epithelium (RPE) and an accumulation of extracellular deposits underlying Bruch's membrane. The alterations finally lead to RPE cell loss, subsequent death of photoreceptors, and loss of vision.

As evidenced in human eyes obtained at autopsy, RPE cells in AMD may be lost by apoptosis.³ The ability of various cellular stress factors that may be implicated in pathogenesis of AMD, including blue light, hydrogen peroxide, ischemia, and treatment with the lipofuscin chromophore A2-E to induce apoptosis has been demonstrated in vitro.^{4–10} Although cellular damage can be initiated in various ways, the cell death program is probably executed by a family of cysteine proteases: the caspases.^{11–14} Caspases, which have multiple cellular substrates including protein kinases, structural components, or nucleic acids, may be directly responsible for the demise of the cell.

Many stress stimuli that are capable of triggering apoptosis, such as oxidative stress or heat shock, induce the synthesis of different heat shock proteins (HSPs) that confer a protective effect against a wide range of cellular stresses. Recent results indicate that HSPs also influence the cascade of apoptosis (for review, see Ref. 15). HSPs were originally identified on the basis of their increased synthesis after cellular exposure to elevated temperature¹⁶ and are ubiquitously expressed in various tissues. They are roughly grouped into several classes according to their sizes and sequence homologies. Most of them exert strong cytoprotective effects and behave as molecular chaperones for other proteins. Most classes of HSPs have been identified in the RPE.¹⁷

α B-crystallin, a member of the small HSP family, is constitutively expressed in many tissues, and it is particularly abundant in cells with minimal mitotic capacity, such as the lens, heart, and skeletal muscle.^{18–23} Similar to the RPE, most of these cells are in a postmitotic state and have to survive for the lifetime of the organism. Small HSPs form oligomeric complexes and function as molecular chaperones protecting other proteins from denaturation and destabilization.^{24–26} Recent data derived from many cell systems suggest that α B-crystallin may protect cultured cells from apoptosis induced by staurosporine,²⁷ okadaic acid,²⁸ UVA irradiation,²⁹ TNF α , and Fas.²⁷ Elevated expression levels of α B-crystallin have been described in various pathologic conditions, such as neurodegenerative disease (for review, see Ref. 30), autoimmune disease,^{31,32} and cancer.^{33,34} In the *rd* mouse, an animal model for inherited retinal degeneration, death of photoreceptors is accompanied by elevated levels of α B-crystallin.³⁵ The major purpose of the present study was to investigate the presence of α B-crystallin in the human RPE with emphasis on local differences between the macular and peripheral regions. Focusing on the possible role of chaperones in protecting RPE cells during pathologic conditions as found in AMD, we investigated the expression

and cytoprotective effect of α B-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-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 fine forceps. The eyecup was rinsed with Ca^{2+} and Mg^{2+} -free Hanks' balanced salt solution, and filled with 0.25% trypsin (GibcoBRL, Karlsruhe, Germany) for 30 minutes at 37°C. The trypsin was carefully aspirated and replaced with Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin, Germany) supplemented with 20% fetal calf serum (FCS; Biochrom). Using a pipette, the medium was gently agitated, releasing the RPE into the medium and avoiding damage to Bruch'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 suspended RPE cells were transferred to a 35-mm² Petri dish and 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 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 trephine 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 and centrifuged for 5 minutes at 800 rpm. After centrifugation, the supernatant was replaced with NP-40 cell lysis buffer (150 mM NaCl; 50 mM Tris [pH 8.0]; 1% NP-40) containing an appropriate amount of protease inhibitors (Complete Mini; Roche, Mannheim, Germany). Further RNA and protein extraction was performed as described. Cell preparations were snap frozen in liquid nitrogen and stored at -70°C for future use.

Human RPE Cell Culture

The RPE cell suspension was transferred to a 50-mL flask (Falcon, Wiesbaden, Germany) containing 20 mL DMEM supplemented with 20% FCS and maintained at 37°C in 5% CO₂. Epithelial origin was confirmed by immunohistochemical staining for cytokeratin with a pancytokeratin antibody (Sigma, Deisenhofen, Germany).³⁷ The cells were tested and found free of contaminating macrophages (anti-CD11; Sigma) and endothelial cells (anti-von Willebrand factor; Sigma) (data not shown). After reaching confluence, primary RPE cells were subcultured and maintained in DMEM supplemented with 10% FCS at 37°C in 5% CO₂. ARPE-19 cells, a human RPE cell line,³⁸ were purchased from ATCC (Manassas, VA) and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/Ham's F-12), supplemented with 10% FCS. Confluent primary RPE cells of passages 3 to 5 and ARPE-19 cells were used for the experiments. Heat shock treatment was performed at 42°C for 20 minutes in serum-free DMEM.

For 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 α B-crystallin is synthesized by the RPE, total RNA was isolated from freshly prepared donor RPE and cultured RPE cells by the guanidium thiocyanate-phenol-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 or absence of reverse transcriptase (Superscript 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 α B-crystallin sequence as described.³⁹ dNTPs were purchased from GibcoBRL, and 10 \times 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 a imager workstation (LAS-1000; RayTest, Pforzheim, Germany).

Northern Blot Analysis of α B-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). Pre-hybridizations 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, α B-crystallin-specific, 450-bp antisense riboprobe. Riboprobes were synthesized as described before.⁴⁰ After hybridization, the membrane was washed twice with 2 \times SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature (RT), followed by two washes in 0.1 \times 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 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 α B-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

centrifugation for 30 minutes at 19,000g in a microfuge (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, transferred with semidry blotting onto a polyvinylidene difluoride membrane (Roche) and probed with a rabbit anti- α B-crystallin antibody as described before.⁴⁰ 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 α B-Crystallin cDNAs

The full-length α B-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 α B-crystallin cDNA amplicons were created with the following oligonucleotide primers: 5'-GGCCGAATTCATGGACATCGCCATCCACAC-3' and 5'-GGCCCTCGAGCTATTTCTGGGGGCTGCGG-3'. Primers were purchased from Metabion (Munich, Germany). The PCR products were then digested with *Eco*RI and *Xba*I (Stratagene) and inserted into a pcDNA3 mammalian expression vector (Invitrogen, Karlsruhe, Germany) bearing the neomycin-resistance gene. Sequences were confirmed by automated DNA sequencing (Sequiseive, 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-resistant pcDNA3/ α B-crystallin plasmid (pcDNA3/ α B-crystallin). Control cells were transfected with the neomycin-resistant expression vector without insertion of α B-crystallin cDNA (pcDNA3/neo). Transfection complexes were prepared by preincubation of plasmid (7 μg) with liposome reagent (Lipofectamine; GibcoBRL) in 0.6 mL serum-free, antibiotic-free DMEM, according to the manufacturer's instructions. The reagent complex was then gently distributed over the cells and incubated at 37°C and 5% CO_2 . 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 48 hours, before G418 sulfate (Sigma), at a concentration of 650 $\mu\text{g}/\text{mL}$ for primary RPE cells and 700 $\mu\text{g}/\text{mL}$ 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 α B-crystallin by immunoblot analysis with anti- α B-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 300 μM H_2O_2 for 75 minutes. Cells were harvested, and aliquots of 2.5×10^6 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,

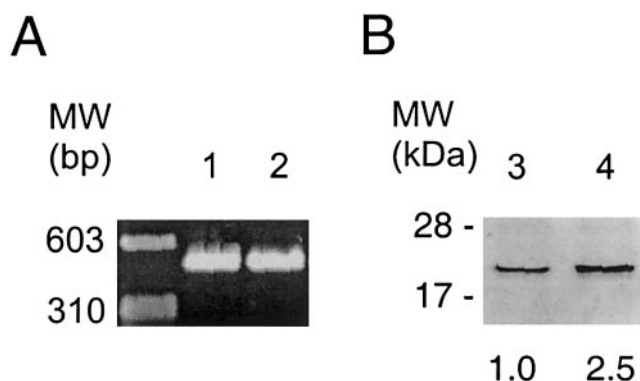


FIGURE 1. (A) RT-PCR analysis of α B-crystallin mRNA in human native (lane 1) and cultured (lane 2) RPE cells. Both cell types contained α B-crystallin transcripts of the appropriate size for the primer pairs used. All investigated RPE preparations and cultured RPE cells showed similar results. PCR performed on the negative control, when reverse transcriptase was omitted, was negative (data not shown). A DNA standard lane (MW) is shown to the left of the gel. (B) Western blot analysis of α B-crystallin protein levels in human extramacular (lane 3) and macular (lane 4) RPE. Native RPE was freshly isolated. Lysates containing approximately equal amounts of protein (1.5 μg) were separated by SDS-PAGE and blotted for immunochemical detection of α B-crystallin content. The number below each band shows the relative chemiluminescence measurement. MW, molecular weight.

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 300 μM H_2O_2 for 2 hours. For evaluation of cell viability, cells were washed in PBS and incubated with 2.0 $\mu\text{g}/\text{mL}$ propidium iodide and 1.0 $\mu\text{g}/\text{mL}$ Hoechst 33342 for 20 minutes at 37°C . Subsequently, cells were analyzed with an epifluorescence microscope (Axioskop; 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 α B-Crystallin Expression in Human RPE In Vivo

To evaluate the in vivo and in vitro expression of α B-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 α B-crystallin mRNA are present in the donor RPE as well as in cultured RPE cells. To determine the spatial distribution pattern of α B-crystallin expression in the retinal pigment epithelium, RPE was freshly isolated from extramacular and macular regions, respectively. Subsequent immunoblot analysis for α B-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.

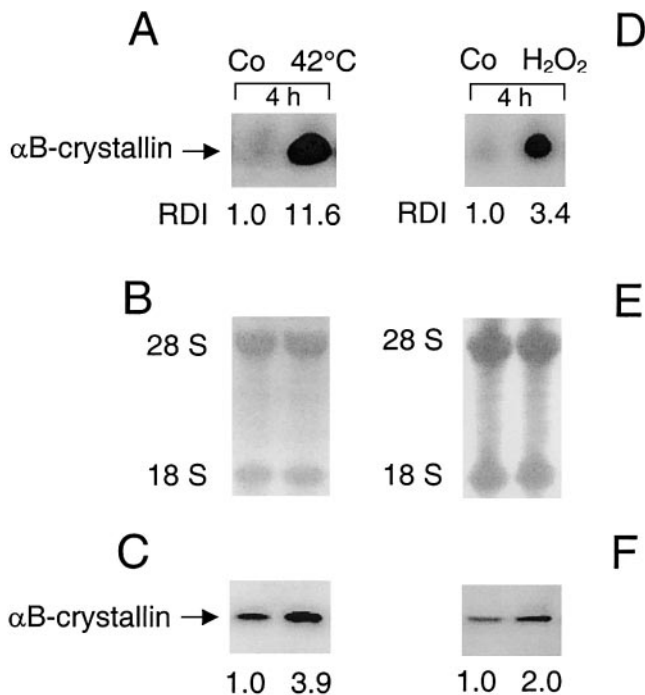


FIGURE 2. (A, D) Northern blot analysis of α B-crystallin mRNA in confluent human RPE cells (A) 4 hours after heat shock (42°C, 20 minutes) and (D) 4 hours after exposure to 200 μ M H₂O₂ for 1 hour. (B, E) Methylene blue staining of the 28S and 18S rRNA to demonstrate the relative integrity and even loading of the RNA. (C, F) Immunoblot of α B-crystallin in cultured RPE cells treated as described for Northern blot analysis. Lysates containing approximately equal amounts of protein (2.0 μ g) were separated by SDS-PAGE and blotted for immunochemical detection of α B-crystallin content. The number *below* each band depicts the relative chemiluminescence measurement. RDI, relative densitometric intensity (normalized to 28S rRNA); Co, control.

Induction of α B-Crystallin in Cultured RPE Cells by Cellular Stress

To find out whether α B-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 α B-crystallin mRNA at 4 hours after heat shock treatment (Fig. 2A). This effect was paralleled by an increased expression of α B-crystallin at the protein level (Fig. 2C). These results suggest that increased α B-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 α B-crystallin. In an attempt to reproduce such pathologic conditions, we chose to study the expression of α B-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 μ M H₂O₂ for 1 hour substantially increased α B-crystallin expression 4 hours after treatment (three- to fourfold; Fig. 2D). To determine whether the oxidative-injury-mediated increase in α B-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 α B-crystallin protein 4 hours after oxidative stress (Fig. 2F). For both types of stress, however, the increase in protein level was smaller than that of mRNA.

To determine the time course of α B-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 α B-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 α B-crystallin mRNA in response to oxidative stress were followed by a corresponding increase in protein synthesis.

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

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

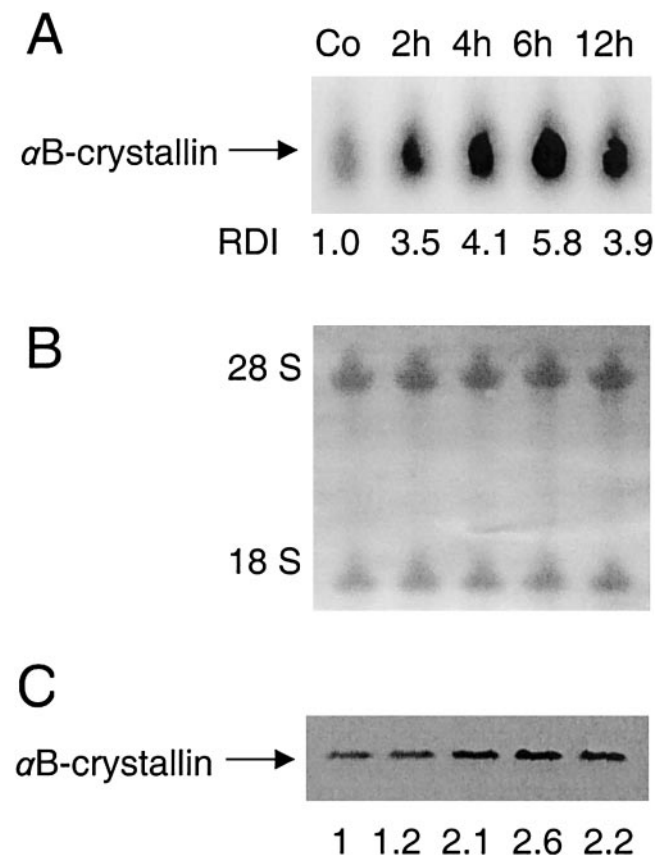


FIGURE 3. Time course of oxidative-stress-mediated induction of α B-crystallin mRNA (A) and protein (C). (A) Northern blot analysis of α B-crystallin mRNA in cultured human RPE cells 2, 4, 6, and 12 hours after treatment with 200 μ M H₂O₂ for 1 hour. (B) Methylene blue staining of ribosomal RNA to demonstrate the relative integrity and even loading of the RNA. (C) Western blot analysis of α B-crystallin in confluent cultured RPE cells under the same conditions as described in (A). Each lane was loaded with 2 μ g of protein. The number *below* each band depicts the relative chemiluminescence measurement. RDI, relative densitometric intensity (normalized to 28S rRNA); Co, control.

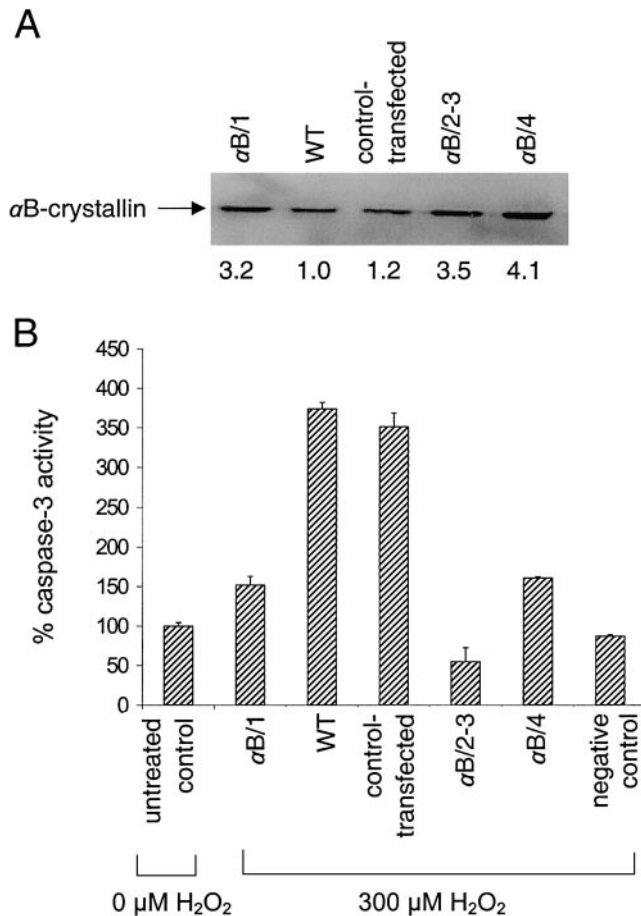


FIGURE 4. (A) Immunoblot analysis of α B-crystallin expression in wild-type (WT) nontransfected human RPE cells, the same cell line transfected with the pcDNA3/neo vector alone (control-transfected), and three independently derived clones transfected with pcDNA3/ α B-crystallin (α B/1, α B/2-3, and α B/4). Lysates from approximately equal amounts of protein (1.5 μ g) were separated by SDS-PAGE and blotted for immunochemical detection of α B-crystallin content. The number below each band depicts the relative chemiluminescence measurement. (B) Activation of caspase-3 in RPE cells after exposure to oxidative stress. Wild-type RPE cells, a control-transfected clone, and three α B-crystallin-transfected clones (α B/1, α B/2-3, and α B/4) were exposed to 300 μ M H_2O_2 for 75 minutes. Caspase-3 protease activity in RPE cells was determined as the release of pNA from the substrate and monitored colorimetrically at 405 nm. Bars indicate \pm SEM. Results are from three independent experiments performed in duplicate. The values were normalized to untreated cells (100%). Negative control: induced sample incubated with caspase-3 inhibitor before addition of substrate.

α B-crystallin and six clones transfected with the pcDNA3 vector (control transfectants) were screened for enhanced α B-crystallin expression by Western blot analysis. The immunoblot analysis of three of these clones is depicted in Figure 4A. Of the α B-crystallin transfectants, nine demonstrated greatly enhanced expression of α B-crystallin protein when compared with control transfected and parental RPE cells.

Oxidative injury of α B-crystallin-transfected, mock-transfected, and wild-type RPE cells was accomplished by treatment with 300 μ M H_2O_2 for 75 minutes. For analysis of apoptotic activity, we used a colorimetric assay for caspase-3. This assay uses the spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after its cleavage by activated caspase-3 from the labeled caspase-specific substrate. The pNA-chro-

mophore can be measured spectrophotometrically only after enzyme cleavage by the active form of caspase-3. Thus, exposure of wild-type and mock-transfected RPE cells to 300 μ M H_2O_2 resulted in a 3.5-fold increase of caspase-3 activity, compared with the untreated control. In contrast, RPE cells stably transfected with α B-crystallin (α B/1, α B/2-3, and α B/4) showed greater resistance to oxidative stress-induced caspase-3 activation. In the α B-crystallin-transfected cells, caspase-3 activity increased only 1.5-fold or remained at baseline, compared with nontreated control cells (Fig. 4B). Similar results were obtained when stably transfected ARPE-19 cells were used (data not shown).

Accordingly, when the viability of RPE cells was tested by labeling of the nuclei of nonviable cells with propidium iodide 9 hours after exposure to 300 μ M H_2O_2 , the α B-crystallin-overexpressing cells (α B/1 and α B/2-3) displayed a 48% to 69% reduction of nonviable cells compared with control-transfected or wild-type RPE cells (Fig. 5). Cell viability assays performed with stably transfected ARPE-19 cells yielded comparable results (data not shown).

DISCUSSION

The evidence for oxidative-stress-induced activation of caspase-3 and induction of α B-crystallin, together with the observation that overexpression of α B-crystallin reduces caspase-3 activity and cell death in RPE cells, indicates that α B-crystallin may exert an antiapoptotic effect on RPE cells. Our data show that in RPE derived from healthy human donor eyes, high levels of α B-crystallin were found in the macular RPE. Assuming that α B-crystallin functions as a marker for cellular stress, these results allude to increased cellular stress in the macular region. In this regard, much speculation in the literature suggests that macular RPE is exposed to higher levels of stress. Such cellular stress has been proposed to result from a higher photoreceptor-to-RPE cell ratio in the macular region,^{42,43} in turn yielding a higher photoreceptor turnover rate per RPE cell. Moreover, the constant exposure of the RPE to visible light and high local oxygen tension, together with the high metabolic activity and phagocytosis of photoreceptor outer segments, induce the generation of reactive oxygen species.

In our *in vitro* experiments, we showed that α B-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 α B-crystallin has been described for ciliary muscle cells and trabecular meshwork cells after treatment with transforming growth factor- β 2,^{40,44} and for neonatal cardiac muscle cells after oxidative damage and heat shock.⁴⁵ These findings suggest that the newly transcribed α B-crystallin mRNA may not be completely translated into protein, suggesting that posttranscriptional regulatory mechanisms may be involved in regulation of the α B-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 apoptosis¹¹⁻¹⁴: 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-

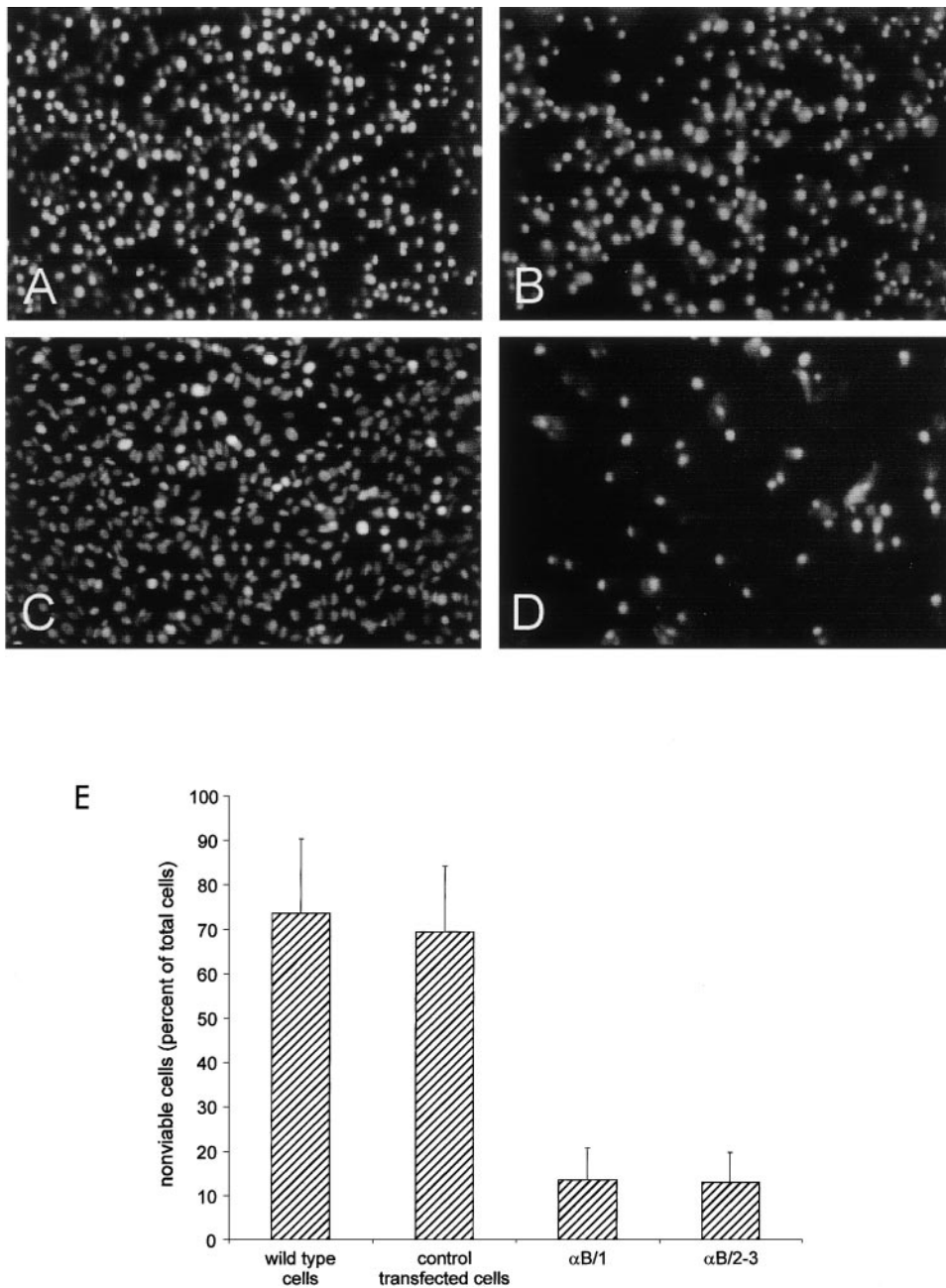


FIGURE 5. The death of RPE cells that were exposed to oxidative stress was inhibited in α B-crystallin-overexpressing cells. Wild-type cells, RPE cells stably expressing pcDNA3 vector alone (control-transfected) and pcDNA3/ α B-crystallin transfected cells (α B/1, α B/2-3) were treated with 300 μ M H_2O_2 for 2 hours. After exposure of the cells to oxidative stress, viability was determined at 9 hours by staining all nuclei with Hoechst 33342 and dead cells with propidium iodide. (A) Representative fluorescence photomicrograph of Hoechst 33342-stained, control-transfected RPE cells. (B) Nonviable cells in the corresponding field. Wild-type RPE cells yielded a similar result (data not shown). (C) Fluorescence photomicrograph of α B-crystallin-overexpressing RPE cells (α B-1) labeled with Hoechst 33342. (D) Nonviable α B-crystallin-overexpressing cells in the same field as in (C). (A–D) Magnification, $\times 90$. (E) Quantification of the effect of overexpression of α B-crystallin on the number of nonviable cells. The percentage of dead cells was scored by counting at least 700 cells in fluorescence photomicrographs of representative fields. Data (mean \pm SEM) are based on the sampling of 6 to 10 photomicrographs per condition in three independent experiments performed in duplicate.

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.^{15,46} For instance, HSP-70 and -90 bind to apoptotic protease activation factor 1 (Apaf-1) and therefore prevent activation of caspase-9,^{47–49} whereas HSP-27 has been shown to antagonize apoptosis by interaction with cytochrome *c*.⁵⁰ However, α B-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.⁴¹ In lens epithelial cells, transgene downregulation of the α B-crystallin gene attenuated the ability to resist H_2O_2 -induced apoptosis.⁵¹ In cardiomyocytes, transgene overexpression of α B-crystallin conferred protection against apoptosis during myocardial ischemia and reperfusion.⁵² In RPE cells, oxidative stress has been reported to induce apoptosis through activation of caspase-3.^{9,53}

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 α B-crystallin. This was in contrast to stably transfected RPE cells containing high amounts of α B-crystallin, in which caspase-3 activity remained low, despite oxidative injury. Furthermore, overexpression of α B-crystallin increased the viability of cultured RPE cells after oxidative injury. Taken together, our observations suggest that induction of α B-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 α B-crystallin allow cells to adapt to gradual changes in their environment and to survive otherwise lethal conditions.¹⁵ 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 α B-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.^{54,55} Particularly, the RPE of patients with AMD has repeatedly been reported to have decreased antioxidant activity.^{56,57} At the same time, with age the RPE is confronted with an abundance of endogenous photosensitizers, such as lipofuscin.^{42,58} 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 α B-crystallin in RPE cells may provide an additional mechanism to postpone cell death in age-related degenerative diseases.

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References

- Hawkins BS, Bird A, Klein R, West SK. Epidemiology of age-related macular degeneration. *Mol Vis.* 1999;5:26.
- Vingerling JR, Dielemans I, Hofman A, et al. The prevalence of age-related maculopathy in the Rotterdam Study. *Ophthalmology.* 1995;102:205-210.
- Xu GZ, Li WW, Tso MO. Apoptosis in human retinal degenerations. *Trans Am Ophthalmol Soc.* 1996;94:411-430.
- Jin GF, Hurst JS, Godley BF. Rod outer segments mediate mitochondrial DNA damage and apoptosis in human retinal pigment epithelium. *Curr Eye Res.* 2001;23:11-19.
- Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. *Invest Ophthalmol Vis Sci.* 2000;41:1981-1989.
- Sparrow JR, Cai B. Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by Bcl-2. *Invest Ophthalmol Vis Sci.* 2001;42:1356-1362.
- Seko Y, Pang J, Tokoro T, Ichinose S, Mochizuki M. Blue light-induced apoptosis in cultured retinal pigment epithelium cells of the rat. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:47-52.
- Wood JP, Osborne NN. The influence of zinc on caspase-3 and DNA breakdown in cultured human retinal pigment epithelial cells. *Arch Ophthalmol.* 2001;119:81-88.
- Cai J, Wu M, Nelson KC, Sternberg P Jr, Jones DP. Oxidant-induced apoptosis in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1999;40:959-966.
- Suter M, Reme C, Grimm C, et al. Age-related macular degeneration: the lipofuscin component N-retinyl-N-retinylidene ethanolamine detaches proapoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells. *J Biol Chem.* 2000;275:39625-39630.
- Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem.* 1999;68:383-424.
- Stennicke HR, Salvesen GS. Caspases: controlling intracellular signals by protease zymogen activation. *Biochim Biophys Acta.* 2000;1477:299-306.
- Miller DK. Activation of apoptosis and its inhibition. *Ann NY Acad Sci.* 1999;886:132-157.
- Wolf BB, Green DR. Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem.* 1999;274:20049-20052.
- Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun.* 2001;286:433-442.
- Ritossa F. A new puffing pattern induced by temperature and DNP in *Drosophila*. *Experientia.* 1962;18:571-573.
- Wong CG, Lin NG. Induction of stress proteins in cultured human RPE-derived cells. *Curr Eye Res.* 1989;8:537-545.
- Piatigorsky J. Lens crystallins and their genes: diversity and tissue-specific expression. *FASEB J.* 1989;3:1933-1940.
- Sax CM, Piatigorsky J. Expression of the α -crystallin/small heat-shock protein/molecular chaperone genes in the lens and other tissues. *Adv Enzymol Relat Areas Mol Biol.* 1994;69:155-201.
- Horwitz J. The function of α -crystallin in vision. *Semin Cell Dev Biol.* 2000;11:53-60.
- Fluegel C, Liebe S, Voorter C, Bloemendal H, Lütjen-Drecoll E. Distribution of α B-crystallin in the anterior segment of primate and bovine eyes. *Curr Eye Res.* 1993;12:871-876.
- Kato K, Shinohara H, Kurobe N, Inaguma Y, Shimizu K, Ohshima K. Tissue distribution and developmental profiles of immunoreactive α B-crystallin in the rat determined with a sensitive immun assay system. *Biochim Biophys Acta.* 1991;1074:201-208.
- Iwaki T, Kume-Iwaki A, Goldman JE. Cellular distribution of α B-crystallin in non-lenticular tissues. *J Histochem Cytochem.* 1990;38:31-39.
- Horwitz J. α -crystallin can function as a molecular chaperone. *Proc Natl Acad Sci USA.* 1992;89:10449-10453.
- Bennardini F, Wrzosek A, Chiesi M. α B-crystallin in cardiac tissue: association with actin and desmin filaments. *Circ Res.* 1992;71:288-294.
- Nicholl ID, Quinlan RA. Chaperone activity of α -crystallins modulates intermediate filament assembly. *EMBO J.* 1994;13:945-953.
- Mehlen P, Schulze-Osthoff K, Arrigo AP. Small stress proteins as novel regulators of apoptosis: heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *J Biol Chem.* 1996;271:16510-16514.
- Li DW, Xiang H, Mao YW, et al. Caspase-3 is actively involved in okadaic acid-induced lens epithelial cell apoptosis. *Exp Cell Res.* 2001;266:279-291.
- Andley UP, Song Z, Wawrousek EF, Fleming TP, Bassnett S. Differential protective activity of α A- and α B-crystallin in lens epithelial cells. *J Biol Chem.* 2000;275:36823-36831.
- van Rijk AF, Bloemendal H. α B-crystallin in neuropathology. *Ophthalmologica.* 2000;214:7-12.
- van Noort JM, van Sechel AC, Bajramovic JJ, et al. The small heat-shock protein α B-crystallin as candidate autoantigen in multiple sclerosis. *Nature.* 1995;375:798-801.
- Bajramovic JJ, Lassmann H, van Noort JM. Expression of α B-crystallin in glia cells during lesional development in multiple sclerosis. *J Neuroimmunol.* 1997;78:143-151.
- Strik HM, Weller M, Frank B, et al. Heat shock protein expression in human gliomas. *Anticancer Res.* 2000;20:4457-4462.
- Hermisson M, Strik H, Rieger J, Dichgans J, Meyermann R, Weller M. Expression and functional activity of heat shock proteins in human glioblastoma multiforme. *Neurology.* 2000;54:1357-1365.
- Jones SE, Jomary C, Grist J, Thomas MR, Neal MJ. Expression of α B-crystallin in a mouse model of inherited retinal degeneration. *Neuroreport.* 1998;9:4161-4165.
- Campochiaro PA, Jerdon JA, Glaser BM. The extracellular matrix of human retinal pigment epithelial cells in vivo and its synthesis in vitro. *Invest Ophthalmol Vis Sci.* 1986;27:1615-1621.
- Leschey KH, Hackett SF, Singer JH, Campochiaro PA. Growth factor responsiveness of human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1990;31:839-846.
- Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res.* 1996;62:155-169.
- Welge-Lussen U, Eichhorn M, Bloemendal H, Lütjen-Drecoll E. Classification of human scleral spur cells in monolayer culture. *Eur J Cell Biol.* 1998;75:78-84.
- Welge-Lussen U, May CA, Eichhorn M, Bloemendal H, Lütjen-Drecoll E. α B-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci.* 1999;40:2235-2241.
- Kamradt MC, Chen F, Cryns VL. The small heat shock protein α B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem.* 2001;276:16059-16063.

42. Feeney-Burns L, Hilderbrand ES, Eldridge S. Ageing human RPE: morphometric analysis of macular, equatorial, and peripheral cells. *Invest Ophthalmol Vis Sci.* 1984;25:195-200.
43. Dorey CK, Wu G, Ebenstein D, Garsd A, Weiter JJ. Cell loss in the ageing retina: relationship to lipofuscin accumulation and macular degeneration. *Invest Ophthalmol Vis Sci.* 1989;30:1691-1699.
44. Welge-Lussen U, Bloemendal H, Lütjen-Drecoll E. Transforming growth factor-beta: a growth factor inducing α B-crystallin expression in ciliary muscle cells. *Graefes Arch Clin Exp Ophthalmol.* 2000;238:993-997.
45. Van de Klundert FA, Gijsen ML, van den IJssel PR, Snoeckx LH, de Jong WW. α B-crystallin and hsp25 in neonatal cardiac cells: differences in cellular localization under stress conditions. *Eur J Cell Biol.* 1998;75:38-45.
46. Xanthoudakis S, Nicholson DW. Heat-shock proteins as death determinants. *Nat Cell Biol.* 2000;2:E163-E165.
47. Beere HM, Wolf BB, Cain K, et al. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol.* 2000 Aug;2:469-475.
48. Pandey P, Saleh A, Nakazawa A, et al. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J.* 2000;19:4310-4322.
49. Saleh A, Srinivasula SM, Balkir L, Robbins PD, Alnemri ES. Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol.* 2000;2:476-483.
50. Bruey JM, Ducasse C, Bonniaud P, et al. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol.* 2000; 2:645-652.
51. Mao YW, Xiang H, Wang J, Korsmeyer S, Reddan J, Li DW. Human bcl-2 gene attenuates the ability of rabbit lens epithelial cells against H₂O₂-induced apoptosis through down-regulation of the α B-crystallin gene. *J Biol Chem.* 2001;276:43435-43445.
52. Ray PS, Martin JL, Swanson EA, Otani H, Dillmann WH, Das DK. Transgene overexpression of α B-crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion. *FASEB J.* 2001;15:393-402.
53. Jin GF, Hurst JS, Godley BF. Hydrogen peroxide stimulates apoptosis in cultured human retinal pigment epithelial cells. *Curr Eye Res.* 2001;22:165-173.
54. Liles MR, Newsome DA, Oliver PD. Antioxidant enzymes in the ageing human retinal pigment epithelium. *Arch Ophthalmol.* 1991;109:1285-1288.
55. De La Paz MA, Zhang J, Fridovich I. Antioxidant enzymes of the human retina: effect of age on enzyme activity of macula and periphery. *Curr Eye Res.* 1996;15:273-278.
56. Cohen SM, Olin KL, Feuer WJ, Hjelmeland L, Keen CL, Morse LS. Low glutathione reductase and peroxidase activity in age-related macular degeneration. *Br J Ophthalmol.* 1994;78:791-794.
57. Frank RN, Amin RH, Puklin JE. Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration. *Am J Ophthalmol.* 1999;127:694-709.
58. Gaillard ER, Atherton SJ, Eldred G, Dillon J. Photophysical studies on human retinal lipofuscin. *Photochem Photobiol.* 1995;61:448-453.