Regulated Heat Shock Protein 27 Expression in Human Retinal Pigment Epithelium

Nataly Strunnikova,1 Judit Baffi,1 Adriano Gonzalez,1 Wesam Silk,1 Scott W. Cousins,2 and Karl G. Csaky1

PURPOSE. To examine the expression and regulation of an injury-related protein, heat shock protein (Hsp) 27, in retinal pigment epithelium (RPE), since RPE injury may be an important feature of age-related macular degeneration (ARMD).

METHODS. Retinal cross sections from eyes of Lewis rats were examined for Hsp27 in vivo by immunohistochemistry, and in vitro expression of Hsp27 in human ARPE-19 cells was determined by Northern and Western blot analysis. Oxidant-mediated injury was performed by exposing ARPE-19 cells to myeloperoxidase and hydrogen peroxide. Cell lines stably expressing green fluorescent protein (GFP) targeted to the cell membrane were used to study injury-induced membrane blebbing, and XTT conversion was used to detect cell viability.

RESULTS. High level of Hsp27 expression was detected in vivo in ganglion cells, RPE, and photoreceptor outer segments of rat retina. ARPE-19 cells also expressed high levels of Hsp27 in vitro. Oxidative injury in ARPE-19 cells resulted in transcriptional and translational activation of Hsp27 and induced extensive membrane blebbing. A high level of Hsp 27 protein was detected within membrane blebs. Increased expression of Hsp27 was also observed in differentiated ARPE-19 cells when compared with dividing cells. Higher Hsp27 levels in differentiated RPE cells correlated with increased viability and phenotypically different blebbing after exposure to the injury stimulus. In addition, sublethal injury doses caused a moderate amount of membrane blebbing, which was well tolerated by differentiated ARPE-19 cells.

CONCLUSIONS. These results indicate that Hsp27 may be an important component of the RPE injury response and may contribute to injury-induced membrane blebbing in differentiated RPE cells. It is hypothesized that Hsp27 levels may play a role in disease states in the retina, such as ARMD. (Invest Ophthalmol Vis Sci. 2001;42:2130–2138)

The retinal pigment epithelium (RPE) is a differentiated, cuboidal neuroepithelium lying between the photoreceptors and the choriocapillaris and is exposed to multiple cell stresses, including ultraviolet damage,1 inflammation,2 and oxidant injury.3,5 However, despite the exposure to various injury stimuli in vivo, the RPE rarely exhibits widespread death. Even in age-related macular degeneration (ARMD), a disease characterized by chronic RPE degeneration, regional death of the RPE, as seen in geographic atrophy, develops in only 8% of eyes over a 5-year period.6 Although programmed cell death has been implicated in the RPE in ARMD,7 it may be that various protective mechanisms are present that prevent the cell from entering into the death cascade.

Heat shock proteins (Hsps), originally identified on the basis of their increased synthesis after cellular exposure to high temperature,8 are ubiquitously expressed in multiple tissues. They are generally differentiated by their individual sizes. Several reports have demonstrated expression of multiple Hsps in the retina.9 Hsp60 has been detected in the retinal ganglion cells and photoreceptors,10 whereas the 70-, 84-, and 110-kDa forms of Hsps have been detected in the RPE.11 In addition, Hsp27 has been detected in the nerve fiber layer, ganglion cells, and photoreceptors.10

Hsp27 belongs to the family of small Hsps that includes αA- and αB-crystallins. In absence of cellular stress, Hsp27 is expressed in high amounts in the skin, stomach, intestine, colon, eye, and bladder.12 Recent data derived from many cell systems suggest that small Hsps, especially Hsp27, may be involved in microfilament organization,13 protection from apoptosis,14 and cell growth or differentiation.15,16 Studies on overexpression of Hsp27 indicate that this protein can protect against cell death induced by hyperthermia, anticancerous drugs, oxidative stress, and inflammatory cytokines.17–20

Two distinct biochemical functions have been implicated for Hsp27 in vitro: as a molecular chaperone and as a Factin cap-binding protein. In its native monomeric form, Hsp27 has been shown to prevent actin polymerization by capping its barbed-end filament.21,22 Alternatively, high-molecular-weight oligomers of Hsp27 may protect other proteins from cellular stress by aiding in the maintenance of their appropriate folded tertiary structure, thereby preserving biochemical function and protecting proteins from degradation.23,24

In the present study, we evaluated expression of Hsp27 in vivo in the normal rat retina and in vitro within RPE cells, specifically comparing two different functional phenotypes (actively proliferating or growth arrested, differentiated cells). Our data indicate that Hsp27 is highly expressed in the RPE and outer segments of the photoreceptors, and expression is greater in vitro in differentiated than in proliferating RPE. In addition, Hsp27 appears to be upregulated after exposure to an oxidant-mediated injury, and high Hsp27 levels correlate with resistance of RPE cells to injury, protection from cell death, and intensity of cell membrane blebbing. These data indicate that Hsp27 may help modulate the differentiation status of the RPE and may play a critical role in RPE stress response.

MATERIALS AND METHODS

Cell Culture

Human RPE cell primary cultures were established as previously described.25 ARPE-19 cells, a human retinal epithelial cell line,26 was a
kind gift of Laurence Hjelmeland (University of California, Davis, CA) and were maintained in Dulbecco’s modified Eagle’s medium-Ham’s F12 1:1 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS; Biowhittaker, Walkersville, MD) and 100 μM penicillin and streptomycin (Gibco-BRL, Grand Island, NY) at 37°C in a 95% air and 5% CO₂ environment (referred to as the proliferating cell condition). For all the experiments except when noted, ARPE-19 cells were plated at confluence and allowed to differentiate in DMEM/F12 medium supplemented with 1% FBS and 25 nM trans-retinoic acid (Sigma, St. Louis, MO) without antibiotics for a minimum of 15 days (referred to as the differentiated cell condition). Human aortic endothelial cells were grown in endothelial cell growth medium (EGM) supplemented with 20% FBS (Clonetics, San Diego, CA).

**Retroviral Vectors and Packaging Cell Lines**

All packaging cell line used were cultivated in high-glucose (4.5 g/ml) DMEM (Gibco-BRL) supplemented with 10% FBS (HyClone, Logan, UT) and 2 mM l-glutamine (Gibco-BRL).

The vector GCsamGFP-c-rRas, which does not contain a neogene,7 was constructed as follows: The coding sequence of enhanced green fluorescent protein (EGFP; Clontech, Palo Alto, CA) minus the TAG stop codon was amplified by PCR with the two primers 5’-CGATACATATCATGTTAAAGGCGAGG-3’ and 5’-CGATACATGTTAAAGGCGAGG-3’ and inserted in pSP72-GFP (pSP-GFP-c-rRas). The integrity of all constructs was confirmed by sequencing. The construct was then cloned into BamHI-HindIII sites of GCsam. In this vector, transcription is driven by a Moloney murine leukemia virus long terminal repeat (Fig. 1).

GCsamGFP-c-rRas was transfected into GP+E86 cells28 by calcium phosphate coprecipitation (Mammalian Transfection Kit; Stratagene, La Jolla, CA). Supernatant from transfected cells was harvested 16 hours later, passed through a 0.45-μm filter, and used to transduce PG-13 cells by a modified ping-pong procedure,29 as described.30 Transduced PG-13 cells, expressing GFP-c-rRas, were allowed to grow to confluence. Cells were then sorted on flow cytometer (FACStar Plus; Becton Dickinson, Franklin Lakes, NJ) with an argon laser. Cells marked with EGFP were excited with the 488-nm laser line and the emission collected with a 530/30 nm band-pass filter. Logical sort gates were set based on live cells using linear forward and side scatter and log fluorescence for positive fluorescent protein.

**Transduction of ARPE-19 Cells**

To ARPE-19 cells seeded the day before (1 × 10⁴ cells in each well of a six-well plate) was added 2 ml fresh retroviral supernatant every 4 hours for 2 days. Thereafter, the supernatant was removed, and the ARPE-19 cells were refed. Cells were allowed to grow, and 1 × 10⁵ cells were sorted according to the parameters used for the PG-13 cells.

**Western Blot Analysis of Hsp27**

Cells were washed twice with Dulbecco’s PBS (DPBS) and detached by scraping. Cells were pelleted at 1000 g, resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5] and 1% NP40) with protease inhibitors (Complete-Mini; Roche Molecular Biochemical, Indianapolis, IN) and lysed by repetitive disruption through a 20-gauge needle. The lysate was centrifuged at 12,000 g for 15 minutes at 4°C, and the protein concentration in the supernatant was determined using a protein assay kit (Protein Assay Reagent kit; Pierce, Rockford, IL). Proteins were separated on a 10% SDS-polyacrylamide gel (NuPAGE; Invitrogen, Carlsbad, CA). After electrophoresis, the proteins were transferred to a 0.45-μm nitrocellulose filter (Millipore, Bedford, MA), and duplicate gels were stained with an anti-Hsp27 rabbit polyclonal antibody, an anti-Hsp70 rabbit polyclonal antibody, an anti-Hsp90 rabbit polyclonal antibody (StressGen, Victoria, British Columbia, Canada), or an anti-actin antibody (Sigma) and developed by chemiluminescence (Western blot analysis kit; Roche Molecular Biochemical).

**Northern Blot Analysis**

Total RNA isolated using a commercial system (Atlas Pure Total RNA Labeling System; Clontech) was subjected to electrophoresis on formaldehyde denaturing gel and transferred to a membrane (Nytran; Schleicher & Schuell, Keene, NH). The membrane was then probed with 32P-labeled Hsp27 full-length or reduced glyceraldehyde-phosphate dehydrogenase (GADPH) cDNA probe and washed in 0.2× SSC and 0.1% SDS at 65°C for 30 minutes. Filters were then exposed to x-ray film (X-OMAT AR; Eastman Kodak, Rochester, NY) at −80°C and developed.

**Immunocytochemistry**

For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 30 minutes at 4°C and permeabilized with 0.6% Triton X-100. Hsp27 was detected by staining with a polyclonal rabbit antibody (StressGen) or an anti-ZO-1 rabbit polyclonal antibody (Zymed, San Francisco, CA) and appropriate CY3-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA).

Lewis rats, aged 8–12 weeks were obtained from the National Cancer Institute/Division of Cancer Treatment (NCI/DCT). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were killed by CO₂ asphyxiation and transcardially perfused with saline followed by 4% paraformaldehyde in PBS. The eyes were enucleated and postfixed overnight. Tissue was embedded in paraffin, 5-μm sections were cut, deparaffinized with xylene, rehydrated with graded dilutions of ethanol, washed in PBS, placed in PBS containing 3% H₂O₂, and incubated in 1% bovine serum containing 0.6% Triton X-100. Sections were then exposed overnight to a rabbit polyclonal anti-Hsp27 antibody (StressGen), followed by detection with a biotinylated anti-rabbit IgG, and were developed using the an ABC kit (Vectastain Elite; Vector Laboratories, Inc., Burlingame, CA). Immunocomplexes were localized after a 5- to 5-minute exposure to 0.05% diaminobenzidine containing ammonium-nickel sulfate and 0.01% H₂O₂. Slides were dried, mounted with synthetic resin (DePex; Fluca, Buchs, Switzerland) coverslipped, and viewed with a light epifluorescence microscope (model BX50; Olympus Optical Co., Melville, NY) equipped with a cooled charge-coupled device (CCD) camera or a dual-channel laser scanning confocal microscope (Leica, Exton, PA). All images were digitally acquired (Photoshop ver 5.0; Adobe, San Jose, CA) by computer.

**Injury and Induction of Membrane Blebbing**

ARPE-19 or ARPE-GFP-c-rRas cells were plated at a density of 10,000 to 20,000 cells/cm² for differentiation or 1,000 to 2,500 cells/cm² for

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**FIGURE 1.** Diagram of GCsamGFP-c-rRas retrovirus used in this study. LTR, long terminal repeat; SD, splice donor site; SA, splice acceptor site; ψ, retroviral packaging signal. Vector is not drawn to scale.
dividing conditions on either collagen-IV–coated eight-well chamber slides (LabTek, Naperville, IL) or 96-well plates (Becton Dickinson) at either subconfluence (proliferating condition) or at confluence and allowed to differentiate, as described earlier (differentiated condition). Cells were incubated with 10 mU myeloperoxidase (MPO; Alexis, San Diego, CA) per 10,000 cells for 90 minutes in DMEM/F12, followed by exposure to different concentrations of H2O2 for 5 hours to determine the extent of cell survival as measured by XTT-(sodium 3′-[1-phenylaminocarbonil]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay (Cell Proliferation Kit II; Roche Molecular Biochemicals). Data were analyzed using analysis of variance. From these values it was determined that 250 μM H2O2 represented a nonlethal dose under both culture conditions. Blebbing was assayed at 2 hours after the stimulus, a time point consistent with previous reports, and other injury responses were measured after 5 hours’ exposure.

**RESULTS**

To investigate the in vivo expression of Hsp27, sections of retinas from normal albino Lewis rats were subjected to immunohistochemistry with a polyclonal antibody against rat Hsp27. Figure 2 indicates that the Hsp27 was observed predominantly in the RPE, ganglion cells (Fig 2, GC), and photoreceptor outer segments (Fig. 2, OS). Hsp27 was absent from all other retinal cells, including the photoreceptor inner segments. The expression seen in the outer segments demonstrated spotty, variable expression, rather than the continuous high level of expression seen in the RPE and ganglion cell layer.

The next experiments were performed in vitro using ARPE-19 cells, an RPE cell line derived from normal eyes after death, to evaluate Hsp27 expression in normal RPE cultures. Total RNA and cell lysates from both ARPE-19 cells and endothelial cells were analyzed for expression of Hsp27. Endothelial cells are also known to be exposed to various injury stimuli and express high levels of Hsp27. As can be seen in Figure 3, ARPE-19 cells demonstrated comparable levels of Hsp27 at both the RNA and cell protein levels. To ensure that the level of Hsp27 in the ARPE-19 cell line is similar to that seen in primary human RPE cells, Western blot analysis was performed on these two cell lines. As can be seen in Figure 4A, comparable amounts of protein were present in undifferentiated ARPE-19 cells (Fig. 4A, lane b) and in primary human RPE cells (Fig. 4A, lane c).

In an attempt to reproduce the state of the RPE in vivo, ARPE-19 cells were allowed to differentiate in low serum in the presence of trans-retinoic acid, conditions that have previously been shown to promote differentiation. Cells demonstrated the typical cuboidal pattern of differentiated ARPE-19 cells as shown by the expression pattern of ZO-1, a membrane tight junction protein present in differentiated cells. Proliferating subconfluent RPE demonstrated elongated, spindle morphology and ZO-1 was absent (data not shown). Total cell lysates from proliferating, differentiated, and injured ARPE-19 cells were examined for expression of Hsp27. Compared with differentiated ARPE-19 cells (Fig. 4A, lane d), proliferating ARPE-19 cells demonstrated diminished intracellular expression of Hsp27 protein (Fig. 4A, lane b). Immunohistochemical localization of Hsp27 in differentiated ARPE-19 cells (Fig. 5) and in dividing ARPE-19 cells (data not shown) revealed homogeneous cytoplasmic staining without nuclear localization.

To study the effects of a stimulus implicated in the pathogenesis of ARMD, we evaluated Hsp27 expression after RPE cell membrane injury with a mild oxidizing stimulus in differentiated cells. Hsp27 expression 24 hours after MPO-mediated...
oxidant injury (Fig. 4A, lane c) was markedly increased compared with baseline levels in noninjured differentiated ARPE-19 cells (Fig. 4A, lane d). This increase was also seen in injured dividing cells (Fig. 4A, lane a), but at reduced levels when compared with injured differentiated cells (Fig. 4A, lane c).

To determine whether other Hsps were present in RPE cells and whether these Hsps were affected by culture conditions, total cell lysates from RPE cells were analyzed for the presence of Hsp70 and Hsp90. As can be seen in Figure 4B, Hsp70 was found in both primary proliferating human RPE (Fig. 4B, lane a) and ARPE-19 cells (Fig. 4B, lane c). However, under differentiating culture conditions (Fig. 4B, lane b), no change in Hsp70 expression was detected in ARPE-19 cells. Hsp90 levels, examined by Western blot analysis, revealed minimally detected levels under all conditions (data not shown).

To test whether increased Hsp27 expression in injured ARPE-19 cells was transcriptionally regulated, total RNA was extracted from differentiated ARPE-19 cells under baseline conditions 12 hours after MPO-mediated injury. Figure 6 demonstrates a several-fold increase in Hsp27 mRNA content within MPO-injured cells.

Hsp27 expression has been shown to protect cells from death cascades induced by oxidative stress. We evaluated the susceptibility of differentiated and proliferating ARPE-19 cells to death induced by MPO-mediated injury. As shown in Figure 7, proliferating ARPE-19 cells were significantly more susceptible to MPO-hydrogen peroxide–mediated XTT reduction than were differentiated ARPE-19 cells.

It is known that Hsp27 through its interaction with actin may be involved in membrane blebbing. To visualize the blebbing response in ARPE-19 cells, a retrovirus was generated expressing GFP targeted to the inner leaflet of the cell membrane through a palmitoylation site of inactive rRas. High-titer–producing cells can be generated with this construct through the use of fluorescent sorting. This technique also allows for selection of ARPE-19 clones with both a high degree and uniform expression of EGFP. Figure 8A shows a representative sample of differentiated GFP-c’-rRas–ARPE-19 cells grown on collagen-IV–coated slides, indicating membrane labeling in these cells. Membrane expression of GFP did not interfere with cell growth and no cell death was noted using the XTT assay (data not shown).

The GFP-c’-rRas–ARPE-19 cells were used to evaluate cell membrane blebbing after MPO-mediated injury. Figure 8B demonstrates the extensive blebbing that occurred in differentiated ARPE cells 2 hours after exposure to nonlethal concentrations of MPO and H2O2, as determined by XTT assay (Fig. 7). In contrast, proliferating GFP-c’-rRas ARPE-19 cells (Fig. 9A) demonstrated a different blebbing response, with less and phenotypically smaller blebbing seen at a similar nonlethal concentration of MPO and H2O2 (Fig. 9B). Blebbing was seen under both cell culture conditions at H2O2 doses greater than 100 μM. However, the difference in blebbing phenotype between differentiated and dividing cells was also seen at the higher doses of H2O2.

Immunohistochemistry for Hsp27 in injured RPE cells expressing GFP-c’-rRas revealed maintenance of the diffuse cyto-
plasmic location of the protein and localization of Hsp27 into extruded blebs (Fig. 10). Similar results were seen with Hsp27 labeling of blebs in ARPE-19 cells not expressing GFP-c-rRas (data not shown).

DISCUSSION

In this study, RPE expressed a high level of Hsp27 in vitro and in vivo. We also confirmed that ganglion cells and photoreceptor outer segments express Hsp27 in vivo.10 Our in vitro data suggest that levels of Hsp27 appear to be modulated by the differentiation state of the RPE with greater expression of Hsp27 in differentiated RPE than in proliferating RPE cells. Further, oxidant-mediated injury induced increase in level of Hsp27 in differentiated RPE cells and higher level of Hsp27 correlated with resistance to MPO-induced cell death and appeared to increase susceptibility to MPO-induced pathologic cell membrane blebbing. Finally, Hsp27 was retained within pathologic blebs, suggesting that high Hsp27 expression may be involved in assembly of the actin cytoskeletal filaments.

Hsp27 has been shown to be expressed in vivo during tissue differentiation, and this expression appears to be transcriptionally regulated.16 Differentiation of HL60 cells induced
by retinoic acid results in an increase in Hsp27 levels with an accumulation of larger oligomers.\(^\text{66}\) In contrast, Hsp70 has been shown not to be increased during differentiation, consistent with the involvement of this family of Hsps in mitogenic functions.\(^\text{37,38}\) Our study confirmed increased expression of Hsp27, but not of Hsp70, in differentiated RPE compared with proliferating cells, a finding that reinforces the need to carefully define the state of differentiation of cultured RPE cells before extrapolating experimental results to normal retina. For this study, the use of retinoic acid and serum conditions has been previously used to induce a differentiated phenotype characterized by functional tight junctions and the ability to phagocytose rod outer segments.\(^\text{26,39}\) These culture conditions have also been validated in other studies of differentiated ARPE-19 cells.\(^\text{39,40}\)

In vitro studies have also demonstrated that Hsp27 plays a critical role in the cell response to stress or injury through several different mechanisms. After cellular injury, Hsp27 often forms large oligomeric complexes ranging in size up to 1 kDa from its native monomer size of 27 kDa.\(^\text{66,67}\) Oligomeric Hsp27 appears to function as a chaperone molecule to protect other proteins from damage or aggregation induced by injury stimuli. Another proposed mechanism for Hsp27 protection from injury is its ability to increase intracellular glutathione,\(^\text{42}\) a property that may have special importance in RPE cells, because intracellular glutathione levels appear to play a protective role in the RPE response to oxidative stress.\(^\text{43}\) In addition, Hsp27 appears to directly inhibit programmed cell death initiated by cytotoxic agents,\(^\text{14,44}\) presumably by preventing cytochrome c-mediated activity of procaspase 9.\(^\text{9,44}\) The cytoplasmic localization of Hsp27 presented in the current study is consistent with previous studies,\(^\text{95-98}\) which have all detailed the cytoplasmic intracellular location of Hsp27. In addition, in RPE cells, which express relatively high levels of Hsp27, this protein remained cytoplasmic after cell injury, a finding that has also been demonstrated in other cell systems.\(^\text{66}\)

The use of the MPO-H\(_2\)O\(_2\) system as the injury stimulus in this study is based on the hypothesis that macrophages, known to be a source of MPO, are observed in the choriocapillaris during uveitis and in ARMD (in which they are often underlying the RPE at the edge of zones of geographic atrophy).\(^\text{49}\) Macrophage-derived mediators may initiate or potentiate the RPE injury response. MPO, in the presence of H\(_2\)O\(_2\) and certain amino acids, catalyzes the formation of powerful oxidants such as tyrosyl radicals, reactive halogens, and hypochlorous acid, all of which are capable of inducing injury to the cell membrane.\(^\text{50}\) The combination of MPO and H\(_2\)O\(_2\) is a well-characterized oxidative stress stimulus and has been used in multiple in vivo and in vitro systems.\(^\text{51-53}\) As was demonstrated in Figure 6, this injury transcriptionally upregulated Hsp27 expression, a finding that appears to be the result of activation of one or more heat shock transcription factors that bind to heat shock elements in the promoter region of Hsp27.\(^\text{54}\)

**Figure 9.** (A) Fluorescence microscopy image of dividing ARPE-19 cells stably expressing GFP-c\(^{-}\)-rRas grown on collagen-IV–coated slides demonstrating GFP targeted to the membrane. (B) Fluorescent microscopy of dividing ARPE-19 cells stably expressing GFP-c\(^{-}\)-rRas grown on collagen-IV–coated slides 2 hours after exposure to 10 mU MPO and 250 \(\mu\)M H\(_2\)O\(_2\) demonstrating smaller and less membrane blebbing. Magnification, \(\times\)400.

**Figure 10.** Confocal fluorescent image of differentiated ARPE-19 cells stably expressing GFP-c\(^{-}\)-rRas grown on collagen-IV–coated slides 2 hours after exposure to 10 mU MPO and 250 \(\mu\)M H\(_2\)O\(_2\), demonstrating extensive membrane blebbing with localization of GFP to the inner leaflet of the cell membrane (A) with detection of Hsp27 expression with Cy3 labeling (B). Homogeneous cytoplasmic expression was maintained in the absence of nuclear distribution of Hsp27 and localization to the membrane of the blebs. Magnification, \(\times\)650.
To interpret the differences observed in our cell system in the oxidative blebbing response between differentiated and dividing RPE cells, we hypothesize that increased Hsp27 expression in differentiated RPE cells promotes formation of pathologic blebs after injury, possibly by interfering with actin filament polymerization after injury-induced membrane extension. In contrast, in proliferating RPE cells, diminished Hsp27 levels promote efficient polymerization of actin filaments, a process crucial to cytoskeleton formation after successful cell division.55 However, the present study demonstrated only a correlational association, and direct proof awaits ongoing experiments that target the downregulation of Hsp27 expression directly, through the use of antisense oligonucleotides, as has been done in cells that express Hsp70.56

Pathologic cell membrane blebbing is a well-defined injury response after cellular exposure to a wide range of injury stimuli, including toxic drugs, oxidants, and physical agents.57-59 Much recent attention has been focused on lethal blebbing in association with caspase activation, nuclear fragmentation, and programmed cell death in a process called apoptosis.60 However, nonlethal blebbing is a normal cellular response to injury.61,62 Nonlethal blebbing may provide a beneficial mechanism by which an injured cell can discard damaged plasma membrane, organelles and cytosolic proteins. Moderate amounts of blebbing can be well tolerated by some cells, especially the RPE, as shown in this study. Current studies in the laboratory are underway to probe the differential mechanisms that regulate cascades separating nonlethal blebbing from programmed cell death in RPE cells.

The use of GFP to label the inner leaflet of the cell membrane is a novel and convenient method for the evaluation of pathologic blebbing, and was superior to the use of lipophilic membrane dyes, which failed to be retained in blebs during our pilot studies (data not shown). Because both the inner and outer leaflets of the plasma membrane are extruded in pathologic blebs, truncated rRas proteins, which anchor into the inner leaflet, are good choices for targeting labels to the cell membrane. Because rRas proteins insert into the inner membrane through palmitoylation sites present at the C-terminal end, it is straightforward to generate mutants with intact anchoring function but without the active site of the protein.65 The high expression of transgenes generated by a “neoless” GCsam construct, as seen in this study, is mediated, in part, by the higher integration efficiency of this vector.27 Also, fluorescent sorting allowed us to select high-GFP-expressing cells. Long-term cellular expression of GFP has been shown not to damage cells,66,67 and previous work has established the usefulness of the ARPE-19 cell line in the study of RPE function.26,40 Although we used a qualitative method to measure the amount of blebbing in this study, the GFP-c-rRas cell line will allow us to develop a more precise quantitative method based on measurement of fluorescence or measuring GFP protein itself in the blebs. We believe that our findings may have relevance to ARMD. First, the results suggest that differentiated RPE, compared with proliferating RPE, may have a significant capacity to resist cell death in response to injury. This finding may explain the absence of widespread RPE cell death until late in the course of ARMD. Nevertheless, relatively mild RPE injury can induce significant cellular responses, such as nonlethal blebbing. Further, it is possible that mild RPE injury, leading to upregulation of Hsp27, may induce a positive feedback loop to amplify resistance to cell death but may simultaneously increase susceptibility to membrane blebbing.

Finally, there are several pieces of evidence that nonlethal blebbing occurs in vivo and may be a common cellular injury response in certain diseases characterized by extracellular deposition accumulation, such as glomerulonephritis,68,69 and especially ARMD.70 For example, several investigators have observed RPE budding after various RPE injury stimuli in animals.71,72 Also, electron microscopic analysis of deposits in ARMD specimens have demonstrated various components potentially derived from blebs,73-74 including membranous debris, coiled bilayer membranes, spherical profiles, and, occasionally, even bleblike vesicles.75,76

However, while we postulate that repetitive nonlethal blebbing of the RPE in vivo might lead to the trapping of membrane and cytosolic constituents under the basal surface of the RPE, it is important to note that the present study did not address the question of whether blebbing can occur from the basal surface of a polarized RPE cell,65,77 an important step in further defining the role of blebbing in AMD. Future work will focus on this important question to determine the contribution of blebbing to the accumulation of membranous debris and cellular proteins within sub-RPE deposits and drusen formation.

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