Original Contribution

Prematurely senescent ARPE-19 cells display features of age-related macular degeneration

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Abstract

The etiology of age-related macular degeneration (AMD), the leading cause of blindness in the developed world, remains poorly understood, but may be related to cumulative oxidative stress. The prime target of the disease is the retinal pigmented epithelium (RPE). To study the molecular mechanisms underlying RPE degeneration, we investigated whether repetitive oxidative stress induced premature senescence in RPE cells from the human ARPE-19 cell line. After exposure to 8 mM *tert*-butylhydroperoxide (*tert*-BHP) for 1 h daily for 5 days, the cells showed four well-known senescence biomarkers: hypertrophy, senescence-associated β-galactosidase activity, growth arrest, and cell cycle arrest in G1. A specific low-density array followed by qRT-PCR validation allowed us to identify 36 senescence-associated genes differentially expressed in the prematurely senescent cells. Functional analysis demonstrated that premature senescence induced amyloid β secretion, resistance to acute stress by *tert*-BHP and amyloid β, and defects in adhesion and transepithelial permeability. Coculture assays with choroidal endothelial cells showed the proangiogenic properties of the senescent RPE cells. These results demonstrate that chronic oxidative stress induces premature senescence in RPE cells that modifies the transcriptome and substantially alters cell processes involved in the pathophysiology of AMD. Oxidative stress-induced premature senescence may represent an in vitro model for screening therapeutics against AMD and other retinal degeneration disorders.
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Age-related macular degeneration (AMD) is a retinal degenerative disease and the leading cause of vision loss in Western countries, affecting 5 to 10% of the population older than 60 years [1]. The prime target of its early development is the retinal pigmented epithelium (RPE), a monolayer of quiescent cells that perform various processes essential for maintaining photoreceptor (PR) functions and survival. The RPE is essential for the retinoid cycle and for maintaining the blood–retina barrier (BRB). The RPE degenerates during AMD and leads to BRB disturbance and PR apoptosis. The neovascular form of AMD is characterized by newly formed vessels that originate in the choroidal vascular endothelial cells and break through Bruch's membrane into the subretinal pigment epithelial space. Many genetic and environmental factors

play a role in AMD but may also confound the role of other factors. RPE cell dysfunction, inflammatory processes, and oxidative stress have been proposed as pathogenic pathways. The recent finding that oxidative stress modulates the expression of the complement factor H (*CFH*) gene, which is associated with AMD, suggests interactions between AMD risk factors, the strongest of which is advanced age [2].

For several reasons, the retina is an ideal environment for the generation of reactive oxygen species (ROS) and oxidative damage [3]: (1) it consumes much more oxygen than any other tissue; (2) it is subject to high levels of cumulative irradiation; (3) membranes of the PR outer segments are rich in polyunsaturated fatty acids; (4) the PR and RPE contain an abundance of photosensitizers; and (5) phagocytosis of rod outer segments by the RPE is itself an oxidative stress and generates ROS. Chronic and cumulative oxidative stress may also be involved in the pathogenesis of AMD [3–5]. The free radical theory of aging, which proposes that aging and age-related disorders result from cumulative damage from reactions involving ROS and oxidative damage, may thus help explain AMD.

Premature senescence can be induced by the hyperactivation of oncogenes or by chronic exposure to cellular stress, such as DNA-damaging agents or oxidative stress [6–8]. Age-related changes in the transcriptional profile of RPE have previously been analyzed with microarray and PCR in mice and rats [9–11].

In this study, we investigated whether ARPE-19 cells undergo premature senescence after exposure to a direct oxidative agent at sublethal concentrations. We also used a low-density DNA array, quantitative PCR analyses, and various biological assays to assess whether this premature senescence affects RPE-specific functional properties through a transcriptional process. We show that chronic oxidative stress induces premature senescence, affects the expression of RPE-specific genes, and alters several RPE functional properties essential for retinal homeostasis.

Materials and methods

Cultures and cell treatments

ARPE-19 (generously provided by Dr. Hjelmeland, University of California, Davis, CA, USA), a nontransformed human RPE cell line that displays many differentiated properties typical of RPE in vivo, was cultured in DMEM:F12 (Invitrogen), supplemented with 10% serum, 2 mM glutamine, and 15 mM Hepes (complete culture medium). Premature senescence was induced by oxidative stress on ARPE-19 cells, as previously described [12]. Briefly, confluent human ARPE-19 cells were incubated daily in the presence of 8 mM *tert*-BHP for 1 h for 5 days (Fig. 1). The medium contained serum to avoid serum depletion-associated oxidative stress [13]. After each stress, the cells were thoroughly washed with HBSS and provided with complete culture medium for 24 h. Complete culture medium was replaced daily in parallel cultured control cells during the 5-day period (Fig. 1). After the fifth stress, the cells were allowed to recover for 3 days before we conducted further experiments. The cells were then replated at various densities, ranging from 10,000 to 40,000 cells/cm^2, depending on the particular assay.

Choroidal endothelial cells (CEC) were isolated from bovine eyes and purified as previously described [14]. They were cultured in EGM-2MV medium (Cambrex) on 50 μg/ml fibronectin-coated dishes (Invitrogen) and cocultured with ARPE-19 cells as follows: ARPE-19 cells were seeded on permeable-insert-system membranes (Transwell, Costar) at 30,000 cells/cm^2 (day 0) and cultured for 24 h. CEC (passage 4 to 7) were then seeded on fibronectin-coated wells at 5000 cells/cm^2 (day 1) and placed 24 h later under the ARPE-19 insert in minimum culture medium (without serum, FGF, or VEGF) (day 2).

Analysis of cell viability and senescence

ARPE-19 cell viability was assessed by (1) counting trypan blue-excluding cells after adding 0.5% trypan blue, (2) using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) staining (Sigma), and (3) monitoring lactate dehydrogenase (LDH) release into the culture medium, with a cytotoxicity detection kit (Roche). Senescence was investigated with the senescence-associated β-galactosidase (SA β-gal) staining kit (Cell Signaling), according to the manufacturer's instructions.

Measurement of intracellular oxidation

We measured intracellular ROS levels with a 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) probe (Interchim). Cells were incubated with 1 μM H$_2$DCFDA for 15 min at 37°C, collected in 500 μl 1% PAF, and analyzed by flow cytometry (Epics ALTRA; Beckman Coulter). Intracellular glutathione (GSH) concentrations were determined by colorimetric assays according to the manufacturer's instructions (GT10; Oxford Biomedical Research).

Cell cycle analysis

DNA content was determined by staining cells with propidium iodide (PI). Cells were washed in PBS and fixed in 70%
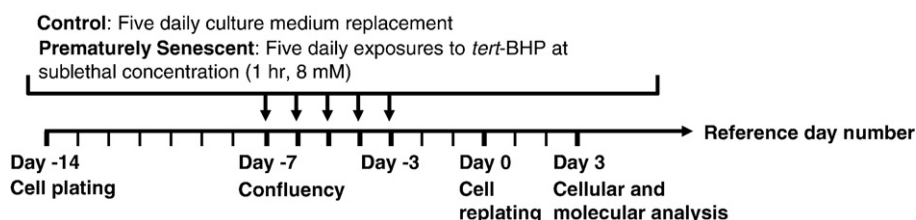

Control: Five daily culture medium replacement
Prematurely Senescent: Five daily exposures to *tert*-BHP at sublethal concentration (1 hr, 8 mM)

Reference day number

Day -14
Cell plating

Day -7
Confluency

Day -3

Day 0
Cell replating

Day 3
Cellular and molecular analysis

Fig. 1. Experimental design and reference time frame.