Redox Biology 13 (2017) 255–265

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

Altered bioenergetics and enhanced resistance to oxidative stress in human retinal pigment epithelial cells from donors with age-related macular degeneration

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ARTICLE INFO

Keywords:
6 max) Age-related macular degeneration
Retinal pigment epithelium
Mitochondrial function
Glycolytic function
Antioxidants
Oxidative stress

ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness among older adults. It has been suggested that mitochondrial defects in the retinal pigment epithelium (RPE) underlies AMD pathology. To test this idea, we developed primary cultures of RPE to ask whether RPE from donors with AMD differ in their metabolic profile compared with healthy age-matched donors. Analysis of gene expression, protein content, and RPE function showed that these cultured cells replicated many of the cardinal features of RPE *in vivo*. Using the Seahorse Extracellular Flux Analyzer to measure bioenergetics, we observed RPE from donors with AMD exhibited reduced mitochondrial and glycolytic function compared with healthy donors. RPE from AMD donors were also more resistant to oxidative inactivation of these two energy-producing pathways and were less susceptible to oxidation-induced cell death compared with cells from healthy donors. Investigation of the potential mechanism responsible for differences in bioenergetics and resistance to oxidative stress showed RPE from AMD donors had increased PGC1α protein as well as differential expression of multiple genes in response to an oxidative challenge. Based on our data, we propose that cultured RPE from donors phenotyped for the presence or absence of AMD provides an excellent model system for studying "AMD in a dish". Our results are consistent with the ideas that (i) a bioenergetics crisis in the RPE contributes to AMD pathology, and (ii) the diseased environment *in vivo* causes changes in the cellular profile that are retained *in vitro*.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in elderly individuals, affecting ~28% of individuals 75–85 years [29] It is predicted that 196 million people will be living with macular degeneration by 2020. As the generation of "baby boomers" grow older, this number will rise dramatically to 288 million by 2040 [46]. The number of individuals suffering from macular degeneration

Abbreviations: AMD, Age Related Macular Degeneration; ANOVA, Analysis of Variance; ARBP, 60S Acidic Ribosomal Protein P0; BEST1, Bestrophin; CAT, Catalase; cDNA, Complementary DNA; CMST, Cell Mito Stress Test; CRABP, Cellular Retinoic Acid Binding Protein; CRALBP, Cellular Retinaldehyde Binding Protein; Cyt b, Cytochrome B; CYTC, Cytochrome C; ECAR, Extra Cellular Acidification Rate; FBS, Fetal Bovine Serum; FCCP, Carbonyl Cyanide-4-(trifluoromethoxy)phenylhydrazone; FITC, Fluorescein-5-Isothiocyanate Isomer I; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GPX1, Glutathion peroxidase 1; GSH, Glutathione; GST, Glycolytic Stress Test; GSTπ, Glutathion-S-Transferase pi; HO-1, Heme-Oxygenase; MCT3, Monocarboxylate Transporter 3; MGS, Minnesota Grading System; miRNA, Micro-RNA; MITF, Microphthalmia-associated Transcription Factor; mRNA, Messenger RNA; mtDNA, Mitochondrial DNA; NQO-1, NAD(P)H Quinone Dehydrogenase; NRF2, Nuclear Factor E2-Related Factor; OCR, Oxygen Consumption Rate; OS, Outer Segments; OxPhos, Oxidative Phosphorylation; PEDF, Pigment Epithelium-Derived Factor; PGC-1α, Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1α; PMEL17, Pre-melanosome Protein 17; PPARα, Peroxisome Proliferator-Activated Receptor Alpha; PPARγ, Peroxisome Proliferator-Activated Receptor Gamma; PRDX3, Peroxiredoxin; qRT-PCR, Quantitative Reverse Transcriptase Polymerase Chain Reaction; RBP1, Retinol Binding Protein 1; RDH11, Retinal Dehydrogenase; RPE, Retinal Pigment Epithelium; rRNA, Ribosomal RNA; SOD1, Cytosolic Superoxide Dismutase; SOD2, Mitochondrial Superoxide Dismutase; SRXN1, Sulfiredoxin 1; TRYP1, Tyrosinase Related Protein

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http://dx.doi.org/10.1016/j.redox.2017.05.015
Received 17 April 2017; Accepted 23 May 2017
Available online 01 June 2017

and other visual impairments comes with a cost. In 2013, it was estimated that the cost of visual impairment due to retinal disorders in the U.S. alone was $8.7 billion [1]. The combination of personal and public costs, as well as the large number of individuals afflicted, creates an urgent need to develop effective treatments.

AMD destroys the macula, a part of the retina supplying high acuity central vision. Patients that develop this disease lose their ability to read, drive, and recognize faces as AMD progresses. There are two forms of AMD, “wet” and “dry”, with about 10% of cases being “wet” AMD. Currently, there are treatments available for those suffering from “wet” AMD, which occurs when abnormal blood vessels grow into the retina, leading to rapid vision loss [41]. There are currently no effective treatments for “dry” AMD, characterized by loss of the retinal pigment epithelium (RPE). The RPE forms the outer blood-retinal barrier and has several key functions. RPE transport nutrients to the outer retina, absorb light and protect against photo-oxidation, regenerate the visual pigment in rhodopsin, phagocytose the tips of photoreceptors, and secrete factors required for preserving the structural integrity of the retina [44]. Because the RPE perform functions that are essential for maintaining retinal homeostasis, the loss of RPE results in photoreceptor death and blindness. A better understanding of how the environment of the diseased retina affects RPE function will provide valuable insight into disease mechanism and drive discovery of new clinical treatments that either prevent AMD or stop its progression.

The central dogma of AMD pathology has included a role for oxidative stress and oxidative damage in the retinal degeneration associated with AMD [6]. This idea has been supported by later studies in mouse models that include either global knockout of the antioxidant CuZn superoxide dismutase (SOD1) or the RPE-specific elimination of the mitochondrial manganese superoxide dismutase (SOD2) [20,22]. The elevated retinal oxidative stress in these mouse models had detrimental effects on retinal function and caused retinal degeneration that was reminiscent of AMD. An emerging hypothesis, evolved from the idea that oxidative stress contributes to AMD pathology, involves mitochondrial dysfunction in the RPE as a prominent player in AMD pathogenesis. Strong supporting evidence from studies of human donors with AMD include the reported decrease in mitochondrial mass with disruptions in mitochondrial architecture, and an altered mitochondrial proteome evidenced by lower content of mitochondrial electron transport chain proteins [10,33,34] Additionally, enhanced mitochondrial DNA (mtDNA) damage has been reported in human donor RPE at stages of AMD preceding macular degeneration and vision loss [24,45]. The ramifications of mitochondrial dysfunction include a reduced capacity for energy production, as well as detrimental effects on redox signaling and subsequent changes in gene expression.

A major limitation in understanding AMD pathology is the complexity of this multifactorial disease, which manifests in individuals over 60 years and is influenced by both environmental and genetic factors. While there are a number of good animal models available for studying specific pathways that are predicted to be involved in AMD, none of these models fully replicate all of the key features of AMD. Thus, additional model systems for studying AMD disease mechanism are needed. In this study, primary cultures of RPE from human donors with and without AMD were used to test the hypothesis that mitochondrial defects in the RPE underlie AMD pathology. We compared the bioenergetic profiles of two major energy pathways, mitochondrial oxidative phosphorylation and glycolysis. We also tested the response of these cells to an oxidative challenge. Our results show major differences in bioenergetics and oxidative stress response comparing cells from donors with or without AMD.

2. Materials and methods

2.1. Human eye procurement and grading for AMD

De-identified donor eyes were obtained from the Minnesota Lions Eye Bank (Saint Paul, MN). Eyes are obtained with the written consent of the donor or donor's family for use in medical research in accordance with the Declaration of Helsinki. The Minnesota Lions Eye Bank is licensed by the Eye Bank Association of America (accreditation #0015204) and accredited by the FDA (FDA Established Identifier 3000718538). Donor tissue is exempt from the process of Institutional Review Board approval.

Tissue handling, storage and donor exclusion criteria are as outlined previously [24,45]. Evaluation of the presence or absence of AMD was determined by a Board Certified Ophthalmologist (Sandra R. Montezuma) from stereoscopic fundus photographs of the RPE using the criteria (RPE pigment changes and the presence, size and location of drusen) established by the Minnesota Grading System [35,7]. Records from the Minnesota Lions Eye Bank provided demographics (age, gender, time and cause of death) for the donors used to generate RPE primary cultures (Supplement Table 1).

2.2. Cell culturing

Eyes obtained within 24 h of death were dissected and processed as described [4]. RPE cells were isolated from human donor eyecups by gently dislodging cells from Bruch's membrane with a glass rod following incubation (15 min) with 0.125% trypsin pre-heated to 37 °C. Cells were suspended in MEM and 15% serum for transport to the Ferrington laboratory, then processed for cultures as described [42] using a series of filters (70 and 40 μm) to reduce contamination of cultures with cell debris. Cells were placed in one Primaria T25 Flask and cultured in Minimum Essential Medium Eagle alpha medium (MEM-α; Sigma-Aldrich) supplemented with 15% Fetal Bovine Serum (FBS)(Atlanta Biologicals), 1 mM sodium pyruvate (Gibco), 1% non-essential amino acid (Cellgro), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco). The serum was gradually reduced to 10% FBS on day 2% and 5% FBS on day 7. Media was changed twice per week. Cells were passaged using trypsin when they reached confluence. All cell cultures were maintained in a humidified atmosphere of 95% air containing 5% CO_2 at 37 °C.

Cells in passage 2 or 3 were used for characterization and functional assays. For functional assays, pigmented cells were grown to confluence in a T75 flask for approximately three months, then transferred to either 96 or 6-well plates and allowed to grow for 2–7 days. Optimal cell number and timing for procedures was experimentally determined for each assay. Cell number and condition are indicated under each experimental protocol.

2.3. Western immunoblotting

Cells (2.5×10^5) were grown in 6-well plates and washed with PBS and lysates were extracted on ice with either RPE lysis buffer (20 mM KCl, 50 mM Tris pH 7.8, 5 mM EDTA, 1% NP-40, 20% Glycerol) or RIPA lysis buffer (Thermo Fisher). Protein concentration was determined using a BCA assay kit (Thermo Fisher). Western blots were performed as described [31]. Proteins were resolved on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were incubated overnight with primary antibodies (Supplement Table 2). The optimal protein load for each antibody was determined from initial experiments where the linear range of detection was determined. Appropriate secondary antibodies conjugated to horseradish peroxidase were used to visualize immune reactions using chemiluminescence with Super Signal West Dura Extended Duration substrate (Thermo Fisher). Immune reactions were imaged using a ChemiDoc XRS (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

2.4. Immunofluorescence

Cells were grown on either fibronectin-coated chamber slides (Nunc) or transwell filters (Costar). Paraformaldehyde-fixed cells were