Cytokine 62 (2013) 369–381

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Parainflammation associated with advanced glycation endproduct stimulation of RPE *in vitro*: Implications for age-related degenerative diseases of the eye

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ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form 14 March 2013
Accepted 26 March 2013
Available online 17 April 2013

Keywords:
Microarray
Age-related macular degeneration
Vitreous
Pathway analysis
CXCL11

ABSTRACT

Age related macular degeneration (AMD) is one of the leading causes of blindness in Western society. A hallmark of early stage AMD are drusen, extracellular deposits that accumulate in the outer retina. Advanced glycation endproducts (AGE) accumulate with aging and are linked to several age-related diseases such as Alzheimer's disease, osteoarthritis, atherosclerosis and AMD. AGE deposits are found in drusen and in Bruch's membrane of the eye and several studies have suggested its role in promoting oxidative stress, apoptosis and lipofuscin accumulation. Recently, complement activation and chronic inflammation have been implicated in the pathogenesis of AMD. While AGEs have been shown to promote inflammation in other diseases, whether it plays a similar role in AMD is not known. This study investigates the effects of AGE stimulation on pro- and anti-inflammatory pathways in primary culture of human retinal pigment epithelial cells (RPE). Differential gene expression studies revealed a total of 41 up- and 18 down-regulated RPE genes in response to AGE stimulation. These genes fell into three categories as assessed by gene set enrichment analysis (GSEA). The main categories were inflammation (interferon-induced, immune response) and proteasome degradation, followed by caspase signaling. Using suspension array technology, protein levels of secreted cytokines and growth factors were also examined. Anti-inflammatory cytokines including IL10, IL1ra and IL9 were all overexpressed. Pro-inflammatory cytokines including IL4, IL15 and IFN-γ were overexpressed, while other pro-inflammatory cytokines including IL8, MCP1, IP10 were underexpressed after AGE stimulation, suggesting a para-inflammation state of the RPE under these conditions. Levels of mRNA of chemokine, CXCL11, and viperin, RSAD2, were up-regulated and may play a role in driving the inflammatory response via the NF-kB and JAK-STAT pathways. CXCL11 was strongly immunoreactive and associated with drusen in the AMD eye. The pathways and novel genes identified here highlight inflammation as a key response to AGE stimulation in primary culture of human RPE, and identify chemokine CXCL11 as putative novel agent associated with the pathogenesis of AMD.

1. Introduction

Age-related macular degeneration (AMD) is a complex multifactorial disease in which aging, genetic and environmental factors all play a role in its pathogenesis. It is the leading cause of irreversible blindness in the elderly [1]. One hallmark of AMD is the presence of macular extracellular deposits called drusen, found between the basal lamina of the retinal pigment epithelium (RPE) and the inner layer of Bruch's membrane (BM). Proteomic studies revealed that drusen contain proteins associated with the immune response and inflammation (e.g. immunoglobulins), acute phase molecules (e.g. vitronectin, amyloid P, fibrinogen), apolipoproteins, complement factors (e.g. C5, C5b-9), and complement regulatory molecules (clusterin, complement receptor 1). These molecules suggest an inflammatory process associated with drusen, but what activates or triggers the inflammation or the mechanism by which these molecules deposit in drusen remains unknown [2–5].

There is little direct evidence to support a causal relationship between the presence of drusen and AMD disease progression. Nevertheless, it is known that drusen deposits occur in early stage AMD [6,7], and the size and number of drusen are correlated with

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http://dx.doi.org/10.1016/j.cyto.2013.03.027

the risk of vision loss. Drusen are composed of many peptides, some of which are known to be harmful to cells, such as amyloid beta and advanced glycation endproducts. Thus, it seems plausible that drusen, or individual components of drusen, may promote pathological degenerative processes in the RPE [8].

Here we focus on the effects of advanced glycation endproducts (AGE), a constituent of drusen and Bruch's membrane in the aging retina [6], on gene expression patterns in primary culture of human RPE cells. We hypothesize that AGE stimulation of primary culture of RPE cells will induce pro-inflammatory gene changes. Our studies are the first in primary culture of RPE to validate and extend the studies of AGE stimulation on bovine [7] and mouse [8] RPE as well as RPE cell lines [9,10]. Knowing the effects of AGE stimulation in primary culture of human RPE, arguably a better *in vitro* model than a cell line, is especially important for translational studies and treatment strategies for AMD that target human genes and their products.

In this study, we identify the cellular pathways activated by AGE stimulation by exploring the changes in the genome wide gene expression patterns, and validated several gene products by suspension array technology. Together with our earlier study on amyloid beta stimulation of RPE *in vitro* [11], a clearer picture of the *in vitro* response of primary culture of human RPE cells to drusen components, such as amyloid beta and AGE, is formed and includes activation of pro-inflammatory, proteasome degradation and caspase signaling pathways, all of which trigger important early events in the pathogenesis of AMD [12].

2. Materials and methods

2.1. Cell culture of human RPE cells

Methods for securing human tissue were humane and included proper written informed consent from each participant involved in the study. This study complied with the Declaration of Helsinki and was approved by the Clinical Review Ethics Board of the University of British Columbia. Human fetal donor eyes were used under the guidelines and regulation of the Clinical Research Ethics Board at the University of British Columbia, Vancouver, Canada. Human RPE cells were isolated from fetal donor eye tissues as described previously [11]. The eyes were cut circumferentially, the vitreous removed and the neuroretina gently detached from the RPE cell layer. The choroid/RPE complex was placed in 2% Dispase (Gibco, Madison, WI) in Hanks' balanced salt solution (HBSS) (Invitrogen) for 25 min at 37 °C. The RPE layer was then removed in fragments and passed through 70-μm and 40-μm nylon mesh filters (Falcon Plastics, Oxnard, CA). Only the fragments that were left behind were retained. After centrifugation at 1500 rpm for 5 min, the fragments were gently dissociated and seeded onto laminin-coated 6-well plates (Falcon Plastics, Oxnard, CA). RPE cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco), 100 μg/ml penicillin (Sigma, St. Louis, MO), 100 μg/ml streptomycin (Sigma) and 2 mM L-glutamine (Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. At confluence, the cells were detached using 0.05% trypsin/0.02% EDTA (Invitrogen), collected by centrifugation and expanded. This study used passage 2–4 cells.

AGEs were obtained by modification of bovine serum albumin (BSA; BioVision) using a Maillard reaction from published protocols [13–17]. Briefly, essential fatty-acid-free grade of BSA especially purified for cell culture application was used to decrease low molecular weight contaminants such as LPS and endotoxin. This BSA was incubated with 0.01873 M glycolaldehyde and phosphate buffered saline (PBS, pH 7.4) under sterile conditions at 37 °C for 7 days. The unbound material was purified by extensive dialysis against PBS. The major AGEs formed are pentosidine and carboxymethyl lysine (CML). Quantification of pentosidine by HPLC based on this synthesis was previously described [14]. Endotoxin levels in the AGE-BSA were measured by manufacturer to be <0.2 pg/ml. Fluorescence of the AGE was confirmed by fluorescence spectrophotometry using 370 nm excitation/440 nm emission. Glycated AGE-BSA shows a 7000% increase in fluorescence when compared to control (unmodified) BSA.

2.2. Quantification of N(epsilon)–carboxymethyllysine (CML) in AGE-BSA

The quantification of CML level in our samples was performed using CML ELISA kit (Cat#STA-316, Cell Biolabs, INC, San Diego, CA) following manufacturer's protocol. Briefly, CML-BSA standards, AGE-BSA, and BSA samples (10 μg/ml) are adsorbed onto a 96-well plate overnight at 4 °C. The CML protein adducts present in the sample or standard are probed with an anti-CML antibody, followed by an HRP conjugated secondary antibody. The CML protein adduct content in our sample is determined by comparing with a standard curve that is prepared from CML-BSA standards.

2.3. Cell viability assay

RPE cells (2×10^4 cells/well) were seeded in 200 μl DMEM containing 10% FBS medium on a 96-well culture plate and incubated for 24 h. The medium was then discarded and the cells were washed three times with phosphate buffered saline (PBS). RPE cells were stimulated with a wide range of concentrations of AGE (0.01–100 μg/ml) in 1% FBS in DMEM. After AGE stimulation, RPE cell viability was measured by the addition of 50 μl of 3-(4,5-dimethylthiazol-2-yle)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/ml) to each well, and incubation at 37 °C for 4 h. Mitochondrial and cytosolic dehydrogenases of living cells reduced the yellow tetrazolium salt (MTT) to a purple formazan dye that was then detected by spectrophotometry. After 4 h, the MTT solution was aspirated and 150 μl of dimethylsulfoxide (DMSO) was added for a period of 20 min. Optical densities of the supernatant were read at 550 nm using a microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Absorbances were normalized to the untreated control cultures that were incubated in 1% FBS DMEM, which represented 100% viability. Two independent experiments in quadruplicate were performed in this study. The data were analyzed using mean ± standard deviation (SD). To facilitate data interpretation, RPE cells grown in DMEM media (without serum) or unmodified BSA served as negative controls, while RPE cells grown in DMEM and 1% FBS served as a standard for normalization.

2.4. AGE stimulation

For stimulation studies, confluent RPE cultures were used. Prior to stimulation, the cells were washed 3 times with PBS. The RPE cells were then treated with AGE (described above) at a concentration of 10 μg/ml for 24 h in 1 ml of DMEM containing 1% FBS. Untreated RPE cells in only DMEM containing 1% FBS were used as an untreated positive control. RPE stimulation with 10 μg/ml of AGE was selected from physiologic level of AGEs in human serum [20] which was estimated to be approximately 8–10 μg/ml of AGE-BSA.

2.5. Microarray and data analysis

The total cellular RNA was isolated from cultured human RPE cells according to manufacturer's recommendations and as described previously [11] (TRIzol; Invitrogen). RNA samples were then subsequently treated with TURBO DNA-free (Ambion, Streetsville, ON, Canada) to remove any DNA contamination.