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# Involvement of the ubiquitin-proteasome system in the expression of extracellular matrix genes in retinal pigment epithelial cells

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ABSTRACT

Emerging evidence suggests that dysfunction of the ubiquitin-proteasome system is involved in the pathogenesis of numerous senile degenerative diseases including retinal disorders. The aim of this study was to assess whether there is a link between proteasome regulation and retinal pigment epithelium (RPE)-mediated expression of extracellular matrix genes. For this purpose, human retinal pigment epithelial cells (ARPE-19) were treated with different concentrations of transforming growth factor-β (TGFβ), connective tissue growth factor (CTGF), interferon-γ (IFNγ) and the irreversible proteasome inhibitor epoxomicin. First, cytotoxicity and proliferation assays were carried out. The expression of proteasome-related genes and proteins was assessed and proteasome activity was determined. Then, expression of fibrosis-associated factors fibronectin (FN), fibronectin EDA domain (FN EDA), metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinases-1 (TIMP-1) and peroxisome proliferator-associated receptor-γ (PPARγ) was assessed. The proteasome inhibitor epoxomicin strongly arrested cell cycle progression and down-regulated TGFβ gene expression, which in turn was shown to induce expression of pro-fibrogenic genes in ARPE-19 cells. Furthermore, epoxomicin induced a directional shift in the balance between MMP-2 and TIMP-1 and was associated with down-regulation of transcription of extracellular matrix genes FN and FN-EDA and up-regulation of the anti-fibrogenic factor PPARγ. In addition, both CTGF and TGFβ were shown to affect expression of proteasome-associated mRNA and protein levels. Our results suggest a link between proteasome activity and pro-fibrogenic mechanisms in the RPE, which could imply a role for proteasome-modulating agents in the treatment of retinal disorders characterized by RPE-mediated fibrogenic responses.

## 1. Introduction

Age-related macular degeneration (AMD) is a progressive disease of the central retina-choroid tissue complex and one of the leading causes of blindness worldwide [1]. The retinal pigment epithelium (RPE), a polarized monolayer of epithelial cells that separates the neural retina from the vascularized choroid, has been implied to play an important role in the pathogenesis of the disease. Early AMD is characterized by focal drusen deposits in the macula, mostly located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane [2]. Drusen contain carbohydrates, zinc and nearly 150 proteins including vitronectin, apolipoproteins E and B, clusterin, connective tissue growth factor (CTGF) and complement system components [3,4]. Advanced AMD is divided into nonexudative or dry AMD which affects 8% of patients and is characterized by macular RPE atrophy and ensuing photoreceptor degeneration, and exudative or neovascular AMD (nAMD) which affects 5% of patients and is characterized by the development of choroidal neovascularization (CNV) [5]. CNV may ultimately lead to the development of a fibrous plaque or disciform scar that leads to secondary atrophy of the neurosensory retina and irreversible and untreatable loss of macular visual function [6–10]. The advent of anti-vascular endothelial growth factor (VEGF) therapy has

*Abbreviations:* AMD, age-related macular degeneration; ARPE-19, human retinal pigment epithelial cells; CNV, choroidal neovascularization; CTGF, connective tissue growth factor; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; FN, fibronectin; FN EDA, fibronectin EDA domain; IFNγ, interferon-γ; MMP-2, matrix metalloproteinase-2; nAMD, neovascular age-related macular degeneration; PPARγ, peroxisome proliferator-associated receptor-γ; RPE, retinal pigment epithelium; TIMP-1, tissue inhibitor of metalloproteinases-1; TGFβ, transforming growth factor-β; UPS, ubiquitin-proteasome system

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greatly improved the prognosis of nAMD patients, stabilizing or even improving visual function [11–13]. Subretinal fibrosis, however, is a common ensuing process of CNV membrane formation, occurring in approximately half of anti-VEGF treated eyes [7,10,14].

Fibrosis may be considered as a deregulated wound healing response to tissue damage [10,15,16]. Angiogenesis occurs in this process as an initial trigger for fibrin deposition, tissue repair, oxygen supply and recruitment of inflammatory cells to the wound [10,17]. In AMD, angiogenesis occurs in the subretinal or sub-RPE space, leading to exudation, hemorrhage and eventually fibrosis. During this process, various types of cells such as RPE cells, glial cells, fibroblasts, myofibroblast-like cells and macrophages infiltrate and/or proliferate, secreting pro-angiogenic and pro-fibrogenic factors that interact with inflammatory cytokines and growth factors. Prevention of visual loss in AMD may therefore depend on the development of successful therapeutic regimens that can halt subretinal fibrosis and preserve the RPE.

The fibrogenic response is stimulated by inflammatory-derived cytokines and growth factors, including transforming growth factor-β (TGFβ) [18], an ubiquitously expressed growth factor belonging to the large superfamily of activins/bone morphogenetic proteins [19] and connective tissue growth factor (CTGF), a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family of extracellular matrix (ECM) proteins, also known as CCN2 [20,21]. The expression of CTGF is regulated by TGFβ [22–24] and, likewise, CTGF has been shown to be an important mediator of TGFβ signaling and its effects in different cell types [24–32]. We and others have shown that both TGFβ and CTGF are major players in the fibrogenic response in the retina [4,16,21,33–40].

The ubiquitin-proteasome system (UPS), a multi-catalytic cytoplasmic and nuclear protein complex present in all eukaryotic cells, is responsible for non-lysosomal proteolysis and thus maintenance of a normal protein homeostasis in cells [41]. Mounting evidence suggests that UPS dysfunction is a major pathogenic mechanism in senile degenerative disorders [42], including AMD and other ophthalmic conditions [43–53]. Proteasomes diffuse rapidly in the cytoplasm and nucleus where they encounter intracellular proteins that are appropriately tagged or misfolded. Proteins are tagged by ubiquitination processes and as such recognized by the 19S regulatory particle of the proteasome [54]. Ubiquitin has been shown to be uniformly expressed in the RPE-Bruch's membrane complex of patients afflicted with AMD [53]. The 19S regulatory particle, combined with the 20S catalytic core, forms the standard proteasome. Within the proteasome core, specialized catalytic subunits are responsible for the cleavage of the carboxyl termini of proteins. There are 3 catalytic subunits in the standard proteasome: β1 for acidic amino acids, β2 for basic amino acids, and β5 for hydrophobic amino acids. The standard proteasome may in some instances undergo a change in configuration into the immunoproteasome. This is achieved upon replacement of the constitutive subunits in the standard proteasome by inducible subunits, β1i, β2i, and β5i [55,56]. Although uninjured RPE contains a baseline level of immunoproteasome subunits [52], cellular stress, such as retinal injury by cytotoxic T-lymphocytes [47], optic nerve trauma [57], aging mechanisms [44,48], complement overactivation [52], chronic oxidative stress [58] and exposure to pro-inflammatory cytokines [52,59] may increase the number of active immunoproteasome subunits. Therefore, the ratio between the nascent (β1, β2, and β5) and inducible subunits (β1i, β2i, and β5i) may be used as a marker of cellular stress [47,48,58].

The aim of the present study was to characterize the involvement of the proteasome pathway in TGFβ and CTGF-mediated expression of ECM genes in RPE cells. Likewise, potential anti-fibrogenic effects of the selective proteasome inhibitor epoxomicin were assessed in ARPE-19 cell cultures.

## 2. Materials and methods

### 2.1. Culture, maintenance and treatment of ARPE-19 cells

Experiments were conducted using ARPE-19 cells, a human RPE cell line that has structural and functional properties that are characteristic of RPE cells in vivo. Monolayers of cells cultured on transwell filters reached a transepithelial resistance of 30–40 omega $cm^2$ after 3 weeks of culture and expressed CRALBP, as detected by RT-PCR. Cells were cultured at 37 °C in 5% CO2 in gelatin-coated T75 cell culture flasks (Corning, Lowell, MA, USA) in Dulbecco Modified Eagle Medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA), low glucose, pyruvate in the presence of 1% penicillin/streptomycin and 10% fetal calf serum. Cell growth was monitored and medium was changed twice a week. For passaging of cells, TrypLE Express (Invitrogen, Carlsbad, CA, USA) was used and cell suspensions were diluted 3-fold. For experiments, cells were cultured in 6-well plates. Upon confluence, cells were washed once with phosphate-buffered saline (PBS), serum starved for 24 h and then treated with various concentrations of the selective and irreversible proteasome inhibitor epoxomicin (Sigma-Aldrich, St. Louis, MO, USA), rhCTGF (ProSpec-Tany TechnoGene, Rehovot, Israel), rhTGFβ1 (ProSpec) and interferon-γ (IFNγ) (PBL Biomedical, Piscataway, NJ, USA). All experiments were performed in triplicate and repeated at least twice.

### 2.2. Protein extraction

Cells were harvested using TrypLE Express (Invitrogen), collected in Eppendorf tubes and centrifuged for 10 min at 400*g*. Supernatant was removed and the pellet was suspended in TSDG buffer (10 mM Tris, pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 8% glycerol), 5 mM ATP and 1x protease inhibitor (Roche Applied Science, Penzberg, Germany).

Cells were lysed with 3 cycles of freezing in liquid nitrogen and thawed at room temp. After centrifugation (15 min, 10,000*g*), the protein concentration was determined using a Bradford protein assay (Serva, Heidelberg, Germany). All experiments were performed in triplicate and repeated at least twice.

### 2.3. Cell cycle and cell viability assays

To assess the viability of healthy ARPE-19 cells and to assess the toxic effects of different stimulants used throughout assays, the PrestoBlue cytotoxicity assay (Invitrogen) was performed according to the manufacturer's instructions. The assays were carried out in 96-well plates (roughly 10,000–25,000 cells per well). After cells were conditioned and washed, PrestoBlue reagent was added to each well. The plates were subsequently incubated at 37 °C for the recommended time period (20–30 min). After incubation, the solution containing PrestoBlue reagent from the wells of the assay plates was transferred to new wells in a 96-well plate, and absorbance was read on a plate reader (Bio-Rad, Hercules, CA, USA) with the excitation/emission wavelengths set at 570/600 nm.

To evaluate the effects of different stimulants on cell proliferation, the Click-iT EdU Alexa Fluor 488 imaging kit (Invitrogen) was applied according to the protocol provided by the manufacturer. Briefly, ARPE-19 cells at 30–50% confluence were treated with EdU (10 μM). EdU was added 2 h prior to the addition of CTGF and TGFβ (both 24 h incubation) and epoxomicin (16 h incubation). Subsequently, cells were fixed, permeabilized, and click-labeled. As a negative control, untreated cells were used. Following incubation, fluorescence readout was determined using a FACS LSRII (Becton Dickinson, Breda, The Netherlands) to determine percentages of EdU-proliferative cells in S and M phase and EdU-negative quiescent cells in G0 and G1 phase. The experiment was performed in triplicate and repeated twice (N = 2).