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# Alterations of activity and intracellular distribution of the 20S proteasome in ageing retinal pigment epithelial cells

Yue Li [a], Yu-Sheng Wang [a], Xue-Feng Shen [b], Yan-Nian Hui [a,*], Jing Han [a], Wei Zhao [a], Jie Zhu [a]

[a] Department of Ophthalmology, Xijing Hospital, The Fourth Military Medical University and Eye Institute of PLA, Chang-le Road 17, Xi'an, Shaanxi 710032, China
[b] Institute of Neurosciences, The Fourth Military Medical University, Chang-le Road 17, Xi'an, Shaanxi 710032, China

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ABSTRACT

Age-related macular degeneration (AMD) remains high incidence and accounts for a main cause of blindness in ageing people, but its mechanism is still poorly understood. Ageing and associated dysfunction of retinal pigment epithelial (RPE) cells were believed to be the pathological onset of AMD. 20S proteasome has been tightly correlated with cell ageing due to its fundamental role in maintaining cellular homeostasis, but its implication in the ageing process of human RPE cells was seldom concerned. This study aimed to demonstrate the interconnections between 20S proteasome and ageing RPE cells by characterizing age-dependent alterations of the 20S proteasome in primarily cultured human RPE cells. For this purpose, a replicative ageing RPE cell model was established and validated through testing the cell viability, β-galactosidase activity and cellular autofluorescence. Decline in chymotrypsin-like, peptidylglutamyl-peptide hydrolase and trypsin-like activities of the 20S proteasome was detected in aged RPE cells through degradation of fluorogenic substrates. Immunofluorescence assay revealed that the 20S proteasome was concentrated in RPE nucleus, and redistributed partly to the peri-nuclear regions in old RPE passages. These age-dependent changes of the 20S complex were accompanied with a significantly increased fluorescent intensity of intracellular oxidized proteins. Further analysis of the proteasome-to-oxidized protein ratio indicated a preferred protection of the RPE nuclear proteins by the 20S proteasome, which also subsided remarkably as a function of the cell ageing. In conclusion, we demonstrated functional impairment and redistribution of the 20S proteasome with age in human RPE cells and supposed these alterations impactful on the process of RPE cell ageing and furthermore on the pathogenesis of AMD. Future researches on the mechanism of these alterations and the pathways to manipulate their effects are still strongly recommended.



## 1. Introduction

For the past two decades, evidences were collected to demonstrate a critical role of the proteasome pathway in cell ageing and senescence (Chondrogianni and Gonos, 2005; Szweda et al., 2003; Torres and Perez, 2008; Vernace et al., 2007). The proteasome system is a multicatalytic complex responsible for a large part of selective protein degradation in mammalian cells (in stead of the organelle lysosome, which was once assumed to degrade most of intracellular proteins) (Reinstein and Ciechanover, 2006). The catalytic core of proteasome system, termed 20S core, is a cylinder-shaped complex formed by 28 non-identical subunits disposed as 4 homologous rings with an overall molecular weight of about 700 kDa. Three specific proteolytic activities – peptidylglutamylpeptide hydrolyzing (PGPH), trypsin like (T-L), and chymotrypsin like (CT-L) activity, are located on the subunit $\beta_1$, $\beta_2$, and $\beta_5$, respectively. CT-L activity is the rate-limiting activity (Coux et al., 1996; Louie et al., 2002). When combined with the 19S regulator, the 20S core forms 26S proteasome. Both the 20S and 26S proteasome are implicated in elimination of oxidatively damaged proteins, however, the 20S core alone accomplishes most of cellular protein degradation independent of ubiquitin or energy (Chondrogianni et al., 2002; Hyun et al., 2003; Davies, 2001; Shringarpure et al., 2003). Moreover, the 20S proteasome exerts a complicated impact on cell survival through regulation of several essential cellular processes, including cell-cycle progression, gene repair and differentiation, and signal transduction (Powell et al., 2005; Reinstein and Ciechanover, 2006). Another type of proteasome is called immunoproteasome, with $\beta_1$, $\beta_2$, and $\beta_5$ subunits being substituted by $\beta_{1i}$, $\beta_{2i}$, and $\beta_{5i}$ subunits, respectively. Immunoproteasome and its association with the 11S complex are implicated in antigen presentation process.

Recent *in vitro* and *in vivo* studies underlined interactions among cell ageing, aggregation of oxidatively damaged proteins and compromised proteasomal function. Although data were not always consistent in various biological systems, evidence on four aspects revealed a remarkable influence of the 20S proteasome on cell ageing progression by virtue of its ability to forcefully

* Corresponding author. Tel.: +86 029 84775912; fax: +86 029 83292763.
*E-mail address:* fmmuhyn@fmmu.edu.cn (Y.-N. Hui).

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convert harmful protein aggregates into harmless refoldable or degradable polypeptide (Chondrogianni and Gonos, 2005). First, proteasomal dysfunction was observed in aged human tissues such as the lens and muscles (Ferrington et al., 2005; Viteri et al., 2004), and cells such as lymphocytes and fibroblast (Carrard et al., 2003; Hwang et al., 2007), as well as in several age-related pathologies like Cataract and Alzheimer's disease (Hope et al., 2003; Viteri et al., 2004). Second, specific inhibition of the 20S proteasome led to accelerated cell ageing, resulting in reduced cell vitality and senescence-like phenotype in young cell cultures (Awasthi and Wagner, 2006; Chondrogianni and Gonos, 2004; Chondrogianni et al., 2003; Stapnes et al., 2007). Third, stable over-expression of the 20S proteasome subunits enabled host cells to survive oxidative stress, resulting in delayed or alleviated senescence phenotype and even extended lifespan (Chondrogianni and Gonos, 2007; Hwang et al., 2007; Liu et al., 2007). Finally, the proteasomal pathway was proved to be one potential mechanism through which several agents promote or detain ageing process, examples like magnesium, oleuropein, aspirin and Cu, Zn-superoxide dismutases (Dikshit et al., 2006; Ferrè et al., 2007; Hyun et al., 2003; Katsiki et al., 2007). Given the fundamental importance of proteasome system in cell ageing, extensive study of this system would be very helpful in understanding the mechanism of several age-related pathologies, including age-related macular degeneration (AMD).

AMD is the leading cause of severe visual loss worldwide. While the etiology of this age-related disease largely remains a "black box", several independent lines of evidence supported a remarkable role of oxidative stress in the progression of AMD (Shen et al., 2007). Retinal pigment epithelial (RPE) cells are indispensable in maintaining normal retinal structure and function. It is supposed that age-related dysfunction and impairment in RPE cells are potential pathological onset of AMD (Boulton and Dayhaw-Barker, 2001). Although the significance of proteasome function in cell ageing has been emphasized repeatedly in a large variety of cell types and tissues, implication of this multi-functional system in RPE cell ageing remains unclear. In order to make a consolidated understanding of the actual role of proteasome system in ageing human RPE cells and further in the pathological mechanism of AMD, we investigated age-related alterations of the 20S proteasome in a human RPE cell model.

## 2. Materials and methods

### 2.1. Cell culture, maintenance and treatment

Primarily cultured human RPE cells were obtained from cell preservation in our lab (Han et al., 2001). RPE cells were planted on plastic flasks and grew to confluence in Dulbecco's minimal essential medium with high glucose (DMEM high glucose) supplemented with 10% fetal bovine serum and 100 U/ml penicillin G/streptomycin sulfate, in an incubator containing humidified ambient air plus 5% $CO_2$ at 37 °C. The medium was changed every 3 days. Cells were subcultured at an 85% ~ 90% confluence with a seeding density of $2\times10^5$/ml. Each subculture was deemed as one passage. All cell culture reagents were purchased from Invitrogen-Gibco (Grand Island, NY). Unless noted specifically, cells were maintained and subcultured in the same medium. According to our observation of the cell growth in preliminary experiments, passages 2 ~ 4, 12 ~ 14, and 22 ~ 24 were selected and defined as the young, middle and old passage groups, respectively.

### 2.2. Cell viability assay

The cell viability of RPE cells was measured with a *In Vitro* Toxicology Assay Kit (Sigma–Aldrich, USA), which performs a modified MTT test based on cleavage of the tetrazolium salt XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) to form an orange soluble dye (formazan) as a function of mitochondrial activities in living cells. According to the manufacturer's instruction, RPE cells of each passage group were planted in a 96 well plate with $2\times10^4$/ml density. The culture medium was replaced with 200 μl serum- and phenol red-free DMEM after being incubated overnight, in which 50 μl of reconstituted XTT was added. The plate was then returned to incubator for another 2 h. Formazan formation was determined with a Tecan Safire2™ microplate reader (Tecan, Switzerland) at 450 nm.

### 2.3. Detection of cell senescence

β-Galactosidase activity, another biomarker for cellular senescence, of RPE cells was detected with a Senescent Cell Staining Kit (Sigma–Aldrich, USA). The kit was designed to detect β-galactosidase activity in senescent cells, but not in quiescent, immortal, or tumor cells. The assay procedure was carried out according to the manufacture's instruction. Briefly, RPE cells were cultured in 6 well plates at the maintenance condition, then treated with fixation buffer and staining buffer. After $CO_2$-free incubation for 8 h, the cells were observed under a microscope immediately and the percentage of blue-stained cells (cells of active β-galactosidase) was calculated. Five visual fields of each passage group were chosen for analysis, each field containing at least 50 cells. The results were recorded as mean ± S.E.M. of the 5 counts.

### 2.4. Detection of intracellular oxidized/cross-linked proteins

The overall content of intracellular oxidized/cross-linked proteins was determined by measuring the cellular autofluorescence of yellow–green spectral range (563 ~ 607 nm) with a flow cytometry (Epics XL, Beckman Coulter, GMI, USA). As suggested by Sitte N (Sitte et al., 2000), the autofluorescence within this spectral range largely derived from oxidized/cross-linked proteins. About $1\times10^6$ RPE cells of each passage group were collected and resuspended in 500 μl DMEM, then immediately loaded in the flow cytometry for detecting the autofluorescence intensity.

### 2.5. Determination of the 20S proteasome activity

The fluorogenic peptides (Biomol, USA) LLE-AMC, suc-LLVY-AMC and LSTR-AMC were used to measure the PGPH, chymotrypsin-like and trypsin-like activities, respectively, as outlined (Bulteau et al., 2002; Kapphahn et al., 2007). About $2\times10^6$ RPE cells of each passage group were washed with PBS and lysed in 150 ml water containing 1 mM dithiothreitol (DTT) through vigorous shaking for 1 h at 4 °C, followed by centrifugating at 14,000g for 30 min to remove the non-lysed debris. The supernatant was immediately used to determine the proteolytic activities. After co-incubation with 100 mM of one of the fluorogenic peptides (dissolved in DMSO) for 1 h at 37 °C in a reaction system (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 20 mM KCl.), the proteolytic reaction was terminated by adding an equal volume of ice-cold ethanol. Concentrations of the substrates used for these detections were determined according to previous reports (Kapphahn et al., 2007; Louie et al., 2002) and our preliminary experiments (data not shown). Fluorescence generated in the reactions was detected at 380 nm excitation and 440 nm emission wavelengths for AMC with a fluorescence spectrophotometer. A standard curve of free AMC was prepared for quantification of the amount of substrates cleaved during the reaction. The procedure was performed either in absence or presence of the proteasome inhibitor MG-132 (100 mM) (Calbiochem, La Jolla, CA) to exclude non-proteasomal functions. Each activity was determined as the difference between the total activity in absence of MG-132 and the remaining activity in presence of MG-132.