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Increased 26S proteasome non-ATPase regulatory subunit 1 in the aqueous humor of patients with age-related macular degeneration

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Age-related macular degeneration (AMD) is the leading cause of blindness in the world. Evidence indicates that the suppression of the ubiquitin-proteasome system (UPS) contributes to the accumulation of toxic proteins and inflammation in retinal pigment epithelium (RPE), the functional abnormalities and/or the degeneration of which are believed to be the initiators and major pathologies of AMD. To identify new protein associations with the altered UPS in AMD, we used LC-ESI-MS/MS to perform a proteomic analysis of the aqueous humor (AH) of AMD patients and matched control subjects. Six UPS-related proteins were present in the AH of the patients and control subjects. Four of the proteins, including 26S proteasome non-ATPase regulatory subunit 1 (Rpn2), were increased in patients, according to semi-quantitative proteomic profiling. An LC-MRM assay revealed a significant increase of Rpn2 in 15 AMD patients compared to the control subjects, suggesting that this protein could be a biomarker for AMD. [BMB Reports 2014; 47(5): 292-297]

INTRODUCTION

Age-related macular degeneration (AMD), an important cause of blindness in elderly people, is the progressive degeneration of the retinal pigment epithelium (RPE), retina, and choriocapillaris (1). AMD is classified into two major categories: dry (atrophic) and wet (neovascular or exudative). In neovascular AMD, the secretion of angiogenic factors, including vascular endothelial growth factor (VEGF), by RPE cells leads to new vessel formation from the choroid. This status induces subretinal fluid accumulation, hemorrhage, exudation, and scarring, resulting in vision loss (2).

Currently, the mechanism of AMD progression is elusive. Considerable evidence implicates retinal oxidative stress and inflammation as critical pathogenic events (3, 4). Oxidative stress is the progressive cellular damage caused by reactive oxygen species, contributing to protein misfolding and functional abnormalities in RPE cells (5). The ubiquitin-proteasome system (UPS), which plays a crucial role in removing detrimental proteins, is suppressed in oxidative-stressed RPE cells, resulting in the accumulation of toxic proteins and irreversible damage (6). The inhibition of proteasome machinery also induces angiogenic factors, such as VEGF, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and inflammatory factor IL-8 (7, 8).

The UPS is a highly selective proteolysis sequence that is crucial for protein quality control, cell cycle control, proliferation, differentiation, and signal transduction (9). Ubiquitin-activating enzyme (E1) first activates and transfers ubiquitin to a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) attaches the ubiquitin to a target protein (10). The continuous conjugation of ubiquitin on substrates, known as polyubiquitination, facilitates the recognition of the substrate by the 26S proteasome (10).

The aqueous humor (AH) is a complex mixture of electrolytes and proteins that fills the anterior segment of the eye (11). The proteins in the AH arise from plasma as the result of filtration through fenestrated capillaries of the ciliary body (12); however, various eye diseases, including both anterior segment (13) and posterior segment disorders (14), can change the composition of the AH. VEGF in the AH could be related to AMD disease activity and reflect expression in the vitreous fluid (15). Because the UPS is associated with AMD pathogenesis, searching for proteins related to the UPS in the AH could reveal biomarkers other than VEGF and could help us to better understand AMD pathogenesis.

In this study, we profiled and characterized the AH proteome from patients with neovascular AMD and identified proteins related to UPS. We used liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to identify whole UPS components in the AH. For the quantita-

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tive verification of a selected protein marker candidate, liquid chromatography-multiple reaction monitoring (LC-MRM) was applied to another 15 AH sample sets.

RESULTS

LC-ESI-MS/MS proteomic profiling of the AH from two patients with neovascular AMD

Four AH samples from 2 AMD patients before and after treatment (intravitreal injection of 0.5 mg ranibizumab) and matched controls (6 samples total) were analyzed. The clinical characteristics of patients are summarized in Table 1. We identified 292 and 223 proteins in the AH of the two patients (Supplementary Table 1). In the two matched controls, 161 and 144 proteins were detected. Many of the identified proteins were also present in previous analyses of AH proteins (16-18).

First, we screened for UPS-related proteins from the identified proteins. Six proteins were commonly found in the patients and control subjects. 26S proteasome non-ATPase regulatory subunit 1 (regulatory particle non-ATPase 2, Rpn2) and E3 ubiquitin-protein ligase SNF2 histone-linker PHD-finger RING-finger helicase (SHPRH) were increased in both patients, as determined by a semi-quantitative analysis. E3 ubiquitin-protein ligase ring finger protein 123 (RNF123) and ubiquitin specific protease17 (USP17) were increased in patient 2 compared to the matched control. G2/M phase-specific E3

Table 1. Summary of the demographic characteristics of age-related macular degeneration (AMD) patients and control subjects

Property	Sample set 1: profiling of total proteomes in AH[a]			Sample set 2: LC-MRM of AH		
	AMD[b]		Control	AMD		Control
	Before[c]	After[d]		Before	After	
No. of AH samples	2	2	2	15	15	15
Age (mean ± SD, years)	70.5 ± 12.0		72.0 ± 7.1	69.8 ±7.2		70.3 ± 8.0
Sex (men:women)	1 : 1		1 : 1	8 : 7		8 : 7
Diabetes mellitus (No.)	0		0	3		2
Hypertension (No.)	1		1	6		9

[a]AH: aqueous humor; [b]AMD: age-related macular degeneration; [c]Before: before treatment with ranibizumab; [d]After: one month after treatment with ranibizumab.

Table 2. Proteins that were differently expressed in the AH of AMD patients compared to controls

Accession no. (GI number)	Protein Name	MW[a]	Biological process	Localization	Expression level[b]	Patient
25777600	26S proteasome non-ATPase regulatory subunit 1 isoform 1	105768.7	Involved in substrate binding, gate opening and 19S proteasome attachment to the base to the lid	Cytoplasm	Increased	Both
289547541	E3 ubiquitin-protein ligase SHPRH isoform a	192955.8	Upon genotoxic stress, polyubiquitinates PCNA and promotes damage avoidance pathways	Nucleus	Increased	Both
37588869	E3 ubiquitin-protein ligase RNF123	148420	Required for poly-ubiquitination and proteasome-mediated degradation of CDKN1B during the G1 phase of the cell cycle Also involved in the degradation of the ATR kinase in lamin misexpression	Cytoplasm	Increased	2
153792150	Ubiquitin-specific protease 17	59580.73	Regulates cell proliferation by inhibiting RCE1 and SDS3 Promotes cell-cycle progression by stabilizing CDC25A Regulates cell migration through the activation of the Rho family GTPases. Stimulates the inflammatory response to viral infection	Cytoplasm	Increased	2
33620749	G2/M phase-specific E3 ubiquitin-protein ligase	80452	Essential in early embryonic development to prevent apoptotic death	Nucleoli	Decreased	1
122114651	Ubiquitin-specific protease 36	122831.5	Stabilizes nucleophosmin/B23 and fibrillarin, which are involved in rRNA processing machinery Stabilizes SOD2, which protects cells from oxidative stress	Nucleoli/ mitochondria	Decreased	1

[a]MW: molecular weight, [b]Expression level: protein level change in the AH of AMD patients compared to that of the controls based on the results obtained from LC-ESI-MS/MS.

ubiquitin-protein ligase (G2E3) and ubiquitin specific protease36 (USP36) were decreased in patient 1 compared to the control. The proteins and their biological functions are listed in Table 2. Interestingly, except for Rpn2, UPS-related proteins were the first identified AH proteins in this study.

LC-MRM of 15 sets of AH samples from patients and their controls

To identify potential biomarkers for AMD, we selected Rpn2, which exists in human plasma (19). Because blood sampling is a less invasive procedure than accessing the AH, finding a serum biomarker for AMD would be beneficial for future analyses.

We conducted a LC-MRM analysis of Rpn2. The selection of unique tryptic peptides for a target protein with good MS signals is critical; therefore, we used the MRMPilot™ software (ABSCIEX, Foster City, CA) to select for multiple tryptic peptides from the given target protein. The MRM transition was optimized for 1 (Q1 mass, 820.41; Q3 mass, 1187.62; peptide sequence, EPEPNFQLLDNPAR; fragment type, 2/y10 ion; collision energy, 41) peptide of the target protein.The selected peptide was examined using a QTRAP 5500 triple quadrupole/linear ion trap mass spectrometer. The level of Rpn2 was increased by more than 3-fold compared with the control groups, as determined by a t-test analysis (Fig. 1A). The level of Rpn2 was decreased to various degrees in most patients after ranibizumab treatment.

Further evaluation of this protein as a biomarker was conducted using a receiver operating characteristic (ROC) curve analysis, which is widely used in case-control studies. We performed ROC analysis using triplicate intensities. The ROC analysis revealed an area under the curve (AUC) of 0.610 (95% CI, 0.505-0.708) (Fig. 1B).

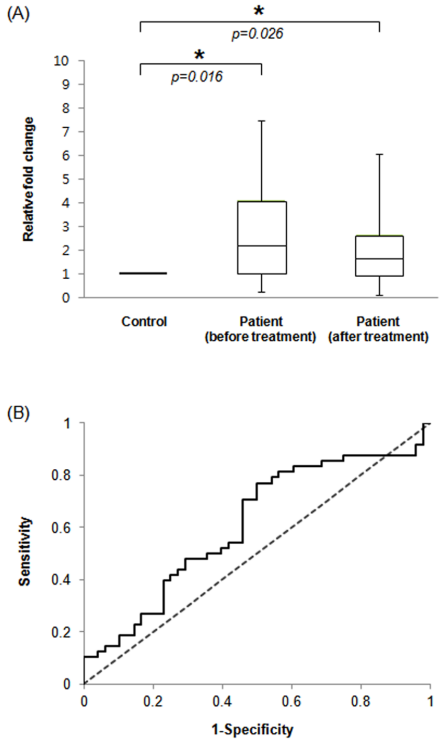

Fig. 1. (A) Box plots of results from LC-MRM analysis in an independent sample set of 15 AH samples of AMD before and after treatment with ranibizumab and 15 matched AH samples of control. The relative abundance of Rpn2 was elevated in patient samples compared to control samples and was decreased after ranibizumab treatment, as determined by a t-test analysis. The relative level of Rpn2 was adjusted by setting the level of the matched control to a value of 1. (B) ROC curve of Rpn2. The AUC value was 0.610 (95% CI, 0.505-0.708).

Western blot analysis of Rpn2

Western blot analysis was performed to determine whether RPE cells, in culture, could secrete Rpn2 under oxidative stress conditions that mimic the heightened oxidative stress of the cellular environment in AMD (Fig. 2A, B). The increased level of Rpn2 in the conditioned medium of the ARPE-19 cell culture that was exposed to oxidative stress (400 μM paraquat, 24 hr) suggested that the presence of Rpn2 in the AH could result from the secretory activity of the RPE of the patients.

DISCUSSION

Our results revealed that several UPS-related proteins were differentially expressed in the AH of AMD patients compared to control subjects. In the AH of AMD patients, Rpn2, SHPRH, RNF123, and USP17 were increased and USP36 and G2E3 were decreased.

The presence of ubiquitin and proteasome subunits, including Rpn2, were reported in the AH in previous proteomic studies (20, 21); however, there has been no validation of the UPS proteins as potential AMD biomarkers. Rpn2 quantification using LC-MRM showed an increase in the average level of Rpn2

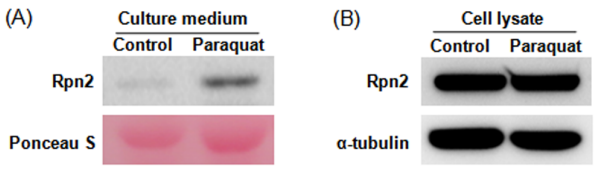

Fig. 2. Rpn2 secretion is increased by oxidative stress compared to control cultures in ARPE-19 cells. (A) ARPE-19 cells were cultured with or without 400 μM paraquat for 24 hours. Equal amounts of proteins (15 μg) from conditioned media (CM) were subjected to Western blot with an Rpn2 antibody. Rpn2 levels in the CM treated with paraquat increased compared to the CM from control cultures. Ponceau S staining was used as a loading control for CM. (B) Cell lysates from the ARPE-19 cells that produced the CM (15 μg) were immunoblotted with Rpn2. There was no significant difference between the control and paraquat lane. α-Tubulin was the loading control.

in AMD patients. Rpn2 is a non-ATPase subunit of a 26S proteasome complex (22). The function of Rpn2 includes binding to the polyubiquitinated substrate, attaching to the 19S base of the lid and gate opening of the proteasome (23). Furthermore, the secretion of Rpn2 increased in RPE cell cultures under the oxidative stress condition, as determined by Western blot analysis. The presence of a circulating proteasome in plasma was reported, which implies that the proteasome could be excreted (19). The increase of Rpn2 in the AH may identify the relationship between UPS and AMD pathogenesis, suggesting that Rpn2 secreted by RPE could be a biomarker for AMD.

LC-MRM analysis showed a higher average level of Rpn2 in patients than in the controls; however, its level was decreased in some patients. This finding may indicate variability in the personal adaptive response. The level and activity of the proteasome is increased when RPE was exposed to mild oxidative stress (24). In contrast, extensive oxidative stress impairs the functions of all of the components of UPS (25). Thus, the variable personal adaptive response of UPS and the severity of oxidative stress highlight the potential efficacy of personalized therapy in which the status of UPS activity is identified for each individual. High concentrations of UPS-related proteins in the AH could be part of the defense mechanism of the RPE against AMD. Therefore, the pharmacological manipulation of the UPS or related signaling pathways could be a therapeutic strategy for AMD.

The UPS-related proteins RNF123, SHPRH, and USP17 as well as Rpn2 were increased in AMD patients. RNF123 is an E3 ubiquitin-protein ligase and is involved in cell cycle progression. Cyclin-dependent kinase inhibitor $p27^{Kip1}$ should be degraded by RNF123 during the G0/G1 cell cycle transition (26). The increase of RNF123 in AMD patients may be related to the cell proliferative condition induced by increased levels of growth factors in AMD. SHPRH is important for the maintenance of genomic stability (27). Increased SHPRH could result from the demand of DNA repair in AMD patients because DNA instability frequently occurs in AMD (28). USP17 inhibits cell proliferation, leading to apoptosis (29), and is involved in the inflammatory response (30). Because apoptosis and inflammatory reactions are important components of AMD pathogenesis, USP17 could be a novel molecular link between UPS and AMD.

USP36 and G2E3 were decreased in the AH of patient 1. Reduced levels of these proteins may be associated with oxidative stress and the apoptotic condition involved in AMD (31-33).

In conclusion, we identified 6 UPS-related proteins that were related to protein degradation, cell cycle regulation, apoptosis, and inflammatory reactions. Among these proteins, Rpn2 was significantly increased in AMD patients. Because there are limited options for AMD treatment, early identification of AMD susceptibility and preventive measures are important therapeutic strategies. Our study identified new potential biomarkers for neovascular AMD and UPS-related proteins that are altered in AMD patients. These results will serve as the basis for future studies to determine target proteins involved in the protection of the eye against AMD.

MATERIALS AND METHODS

Subjects and the AH sample collection

AH samples were collected at the Department of Ophthalmology, Konkuk University Medical Center, Seoul, Korea. from Dec. 1, 2011 to Dec. 31, 2012, 17 patients with untreated neovascular AMD and 17 age- and sex-matched patients undergoing cataract surgery (controls) were enrolled in this study. The 17 sets of samples analyzed consisted of control samples as well as samples from patients before treatment (an intravitreal injection of ranibizumab) and samples from patients at 1 month after the first treatment, for a total of 51 AH samples. The 17 patients had not received treatment for neovascular AMD before their inclusion in the study. Patients with other ophthalmic diseases (e.g., glaucoma, uveitis, or progressive retinal disease), uncontrolled systemic diseases (e.g., uncontrolled diabetes mellitus or arthritis), or who had undergone laser or intraocular surgery were excluded. AH samples from patients undergoing cataract surgery for visual rehabilitation were used as a control. We matched the ages of the patients ($\pm$ 5 years) with those of the control subjects, and the extent of the cataracts in each individual corresponded to the patient's age. The control subjects did not have eye diseases other than cataracts. Control samples were obtained immediately before cataract surgery. Samples from neovascular AMD patients were obtained before performing the first intravitreal injection of 0.5 mg ranibizumab and 1 month after the injection (before performing the second intravitreal injection of 0.5 mg ranibizumab). Two sets of samples (6 samples) were used for the whole-protein profiling by LC-ESI-MS/MS analysis. Finally, data were acquired for 15 sets of samples (45 samples) and analyzed by LC-MRM.

All sample collections and intravitreal injections were performed using standard sterile procedures, and AH samples were obtained by anterior chamber paracentesis using a 30-gauge needle. No complications were encountered after paracentesis of the anterior chamber. AH samples (50-100 μl) in safe-lock microcentrifuge tubes (1.5 ml) were immediately frozen at -80^{o}C and stored until they were needed for analysis. The guidelines of the Declaration of Helsinki were followed, and informed written consent was obtained from all patients and control subjects. The procedure for AH collection was approved by the Institutional Review Board of Konkuk University Medical Center, Seoul, Korea.

Nano-LC-ESI-MS/MS analysis and database searching

Trypsin-digested peptides were loaded onto a fused silica microcapillary column (12 cm x 75 μm) packed with C18 resin (5 μm, 200 Å). LC separation was conducted under a linear gradient as follows: 3-40% solvent B (0.1% formic acid in 100% ACN) gradient with a flow rate of 250 nL/min for 60

min. The column was directly connected to a LTQ linear ion-trap mass spectrometer (Finnigan, CA) equipped with a nano-electrospray ion source. The electrospray voltage was 0.95 kV, and the threshold for switching from MS to MS/MS was 500. The normalized collision energy for MS/MS was 35% of the main radio frequency amplitude (RF), and the duration of activation was 30 ms. Spectra were acquired in data-dependent scan mode. Each full MS scan was followed by MS/MS scans of the five most intense peaks. The repeat peak count for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was 180 s and width of exclusion mass of $\pm$1.5 Da. The list size of dynamic exclusion was 50. We analyzed the 6 AH samples in triplicate and selected proteins that were identified in at least two replicate analyses.

The LC-ESI-MS/MS spectra were analyzed using the BioWorksBrowser™ (version Rev. 3.3.1 SP1, Thermo Fisher Scientific Inc., CA) with the SEQUEST search engine, which searches the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) non-redundant human protein database. The searching conditions were as follows: trypsin enzyme specificity, permissible level of two missed cleavages, peptide tolerance of $\pm$2 amu, mass error of $\pm$1 amu on fragment ions and fixed modifications for carbamidomethylation on cysteine (+57 Da) and oxidation on methionine (+16 Da) residues. To compare protein expression, a label-free quantification method, which counts the number of spectra used to identify each protein, was used.

LC-MRM

AH samples (50 μg) from 15 patients were resolved in 6 M urea and 50 mM ammonium bicarbonate (pH 7.8) in HPLC-grade water. Denatured AH proteins were reduced with 30 mM DTT for 2 h, followed by 1 h of 55 mM iodoacetamide treatment in the dark for alkylation. Alkylated AH samples were digested in solution with sequencing grade modified trypsin (Promega, Madison, WI) overnight at 37°C. Formic acid was added to the sample to stop the digestion. MRM mode was performed on a QTRAP 5500 hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems / MDS Sciex, Carlsbad, CA) equipped with a nanospray ionization source for the quantitative analysis of specific peptides. A given MRM Q1/Q3 ion value (precursor / fragment ion pair) was monitored to select a specifically targeted peptide corresponding to a candidate protein. The MRM scan was performed in a positive mode with ion spray voltages in the 1800-2100 V range. The MRM mode settings were as follows: curtain gas and spray gas at 10 and 20 psi, respectively, and the collision gas at unit resolution. The declustering potential (DP) was set to 100 V. The mass resolution was set to unit using an advanced MS parameter. For the correct LC-MRM, the monitoring of the selected peptide by enhanced product ion (EPI) scan was performed with threshold switching of 100 counts and the selection of rolling collision energy. In the positive mode, a product of 30, scan range 100-1000 Da, and two scans were used. In the advanced MS tab, the quadrupole resolution was set to low, the scan speed was 10,000 amu/s, and a dynamic fill time was selected.

ARPE-19 cell cultures and the cell culture supernatant (conditioned medium)

Human retinal pigment epithelial ARPE-19 cells were cultured in DMEM/F-12 supplemented with 10% FBS and 1% antibiotics. Approximately 5×10^6 cells were plated in each 100-mm culture dish and maintained at 37°C in a 5% CO_2 incubator to allow proliferation. When cell confluence reached ~90%, the cells were washed three times in PBS and treated with 400 μM paraquat (Sigma) at 37°C for 24 h under serum-free conditions. At the same time, total cell lysates were prepared from the cells that produced the CM. A total of 100 ml of cell culture supernatant (CM) was collected and centrifuged at 480 $\times$ *g* for 10 min and then at 1,900 $\times$ *g* for 10 min to remove dead cells and cell debris. The CM was concentrated to ~1 ml using an Amicon® Ultracel-10K molecular-weight cutoff centrifugal filter device (Millipore) for Western blot analysis.

Western blot analysis

The protein concentration was determined by a modified Bradford Assay (Bio-Rad Laboratories). Cell lysates and CM sample preparations containing 15 μg protein were loaded per well. The membrane was blocked with 5% nonfat milk for 1 h and incubated overnight at 4°C with anti-26S proteasome non-ATPase subunit 1 (LSBio) and α-tubulin (Sigma-Aldrich Co.) antibody. Ponceau S solution (Biochemical) was used as loading control for CM. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling) secondary antibodies were used. A chemiluminescence substrate (ECL Prime, Amersham) was used to visualize the immunoreactive protein.

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