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Increased plasma levels of 20S proteasome α-subunit in glaucoma patients: an observational pilot study

Kerstin Wunderlich,[1,2] Olga Golubnitschaja,[3] Mona Pache,[1] Alex-Niklaus Eberle,[2] Josef Flammer[1]

Departments of [1]Ophthalmology and [2]Research, University of Basel, Basel, Switzerland; [3]Department of Radiology, University of Bonn, Bonn, Germany

Purpose: Several reports have shown that proteasome levels may change during pathological processes. Here we investigated whether altered gene expression of the α-subunit of 20S proteasomes in leukocytes of glaucoma patients at the level of mRNA, shown by us earlier, is reflected by the level of protein synthesis.
Methods: Western blot analysis was performed to determine the protein levels of the 20S proteasome α-subunit in leukocytes of 12 glaucoma patients (6 HTG and 6 NTG) and 6 healthy controls.
Results: Plasma levels of the 20S proteasome α-subunit showed a 3.4±0.47 (mean±SD; $p<0.0001$) fold increase in glaucoma patients when compared to healthy controls. In addition, normal tension glaucoma patients revealed a more pronounced α-subunit expression ($p<0.05$) when compared to patients with high tension glaucoma.
Conclusions: During the pathological process of glaucoma, proteasome protein levels increase. This indicates that proteasome protein levels may be a diagnostic or eventually a prognostic marker for glaucomatous damage.

The 20S proteasome is a multicatalytic endopeptidase with a high molecular mass of 700 kDa. This multimeric proteinase occurs in all types of eukaryotic cells and in some archaea and eubacteria [1,2]. Proteasomes are found both in the nucleus and cytoplasm and play a major role in the ubiquitin-dependent and ubiquitin-independent non-lysosomal pathway of intracellular protein degradation. They are also involved in the turnover of various regulatory proteins, like rate limiting enzymes [3], proteins for cell cycle control [4], or transcriptional regulation [5]. 20S proteasomes are also essential for antigen processing [6], cell differentiation [7,8] and apoptosis [9,10], as well as, under pathological conditions, for accelerated degradation of proteins in muscles associated with certain diseases including sepsis [11,12]. Proteasomes are often overexpressed in malignant tumors, in the tissue surrounding a tumor [13,14], and in the serum of tumor patients [15]. There is also an increasing interest in the proteasome activity due to its potential role in neuron degeneration and cell death [16].

Glaucomatous optic nerve neuropathy (GON) is characterized by death of retinal ganglion cells, at least through apoptotic processes [17], and by atrophy of the optic nerve head (ONH). The development of the ONH excavation is due to an additional loss of glial tissue including capillaries, and from compression, stretching and rearrangement of the cribriform plates of the lamina cribrosa. Although the clinical picture of glaucoma is well described, the exact mechanism leading to this specific type of damage is not yet clear. Beside a mechanical stress, GON may also result from ischemia/reperfusion injury (I/R), provoked by a vascular dysregulation, which interferes with the autoregulation of ocular perfusion. While the clinical impact might be confined to the eyes, vascular dysregulation may simultaneously lead to subclinical I/R in other organs, as demonstrated by increased prevalence of silent myocardial ischemia in such patients. I/R can also cause an inflammatory response. Depending on the severity of such a response of the post ischemic tissue, inflammatory reactions may occur in distant organs. Leukocytes exposed to ischemic tissue may reenter the systemic circulation in an activated state after reperfusion. In glaucoma the repeated I/R injury to the ONH might usually be so subtle that it does not lead to microinfarctions, and the resulting inflammatory response may remain clinically undetectable. However, using molecular methods like subtractive hybridization, Golubnitschaja-Labudova et al. [18] demonstrated that in isolated leukocytes an altered expression of several genes including XAPC7, which encodes the 20S α-subunit, parallels the appearance of a glaucomatous damage in NTG patients.

Since proteasomes play a central role in the turnover of various regulatory proteins, we investigated if the increased level of mRNA of 20S proteasomes in glaucoma patients, described previously, corresponds with an increased concentration of the accompanying 20S proteasome protein.

METHODS

Blood samples: Blood samples were collected from 12 glaucoma patients (6 high tension glaucoma [HTG], 6 normal tension glaucoma [NTG]) and 6 healthy controls. All subjects were adjusted for gender and age (Table 1). A detailed medical and ophthalmological history was taken, and all subjects completed an ophthalmological examination. All glaucoma patients had bilateral typical glaucomatous optic nerve head cupping and visual field defects. In NTG patients, intraocular pressure (IOP) never exceeded 21 mm Hg, but after local cool-

Correspondence to: Josef Flammer, University eye clinic, Mittlere Strasse 91/PO Box, CH-4012 Basel, Switzerland; Phone: +41-61-265-8651; FAX: +41-61-265-8652; email: Josef.Flammer@uhbs.ch

ing of the fingers all these NTG patients exhibited a stop in blood flow for more than 20 s, which was detected by nailfold capillaromicroscopy (indicative of vasospasm). In contrast, HTG patients exhibited an IOP higher than 21 mm Hg, but no vasospastic response. Ophthalmologic examination of healthy controls yielded unremarkable results and also no vasospastic response. No patient had received either a systemic or a locally applied ocular therapy at least four weeks before blood draw. Exclusion criteria were a history of alcohol or drug abuse and a history of systemic disease. The study conformed to the Declaration of Helsinki for research involving human subjects. Healthy control subjects were chosen from a group of voluntary individuals who are employed at the University Eye Clinic.

Isolation of mononuclear cells: Mononuclear cells were isolated from 10 ml heparinized whole blood of 6 NTG patients, 6 HTG patients and 6 healthy controls by density centrifugation as described by Maurer et al. [19] using a lymphocyte separating medium (Lymphodex; Innotrain, Kronberg, FRG). After an extensive washing procedure (three times) with PBS cells were incubated for 20 min on ice in an erythrocyte lysing buffer (ECB) containing 150 mM NH_4Cl, 100 mM $KHCO_3$ and 100 mM EDTA, pH 7.4, to avoid contamination with proteasomes derived from erythrocytes. After an additional washing step with PBS, mononuclear cells were disrupted in 1 ml of a buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3) with 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and a protease inhibitor mixture (complete protease inhibitor tablet, Roche Molecular Biochemicals, Basel, Switzerland) for 1 h on ice as described by Andersson et al. [10]. The lysate was then centrifuged at 18,000x g for 15 min at 4 °C and the supernatant was immediately frozen in aliquots on dry ice and stored at -75 °C.

Protein determination: After thawing, the protein concentration was determined in duplicates using the colormetric DC Protein assay (Bio-Rad, Munich, FRG) with bovine serum albumin as standard. Absorbance was measured at 750 nm in a microplate reader (Versamax; SOFTmax version 2.01; Molecular Devices/Bucher Biotec, Basel, Switzerland).

Table 1. Information about healthy subjects and glaucoma patients

Group Number	HTG subject age	NTG subject age	Control subject age	Gender
1	62	62	59	F
2	46	47	44	F
3	56	53	58	F
4	50	52	57	F
5	42	37	41	M
6	66	65	61	M

Gender- and age-adjusted subjects were grouped as listed in table 1. Western blot experiments were performed with samples from each group as described in the text.

Electrophoresis and immunoblotting: Because quantification of the 20S proteasome by functional activity measurements is difficult and inaccurate [20], we have chosen Western blot analysis to correlate mRNA expression, shown by us earlier [18], with protein expression. Sample extracts were diluted with a buffer containing 30 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol, heat denatured at 95 °C for 5 min and cooled down on ice prior to loading. Equal amounts of total protein (50 μg per lane) were subjected to electrophoresis on 12% SDS-polyarcylamide gels according to the method described by Laemmli [21]. In addition, a commercially available HeLa cell lysate (Bioreba AG, Reinach, Switzerland) was used as positive control for 20S proteasome α-subunit expression [22] (Figure 1). Protein migration was assessed using protein standards (Bio-Rad, Munich, FRG). Transfer to nitrocellulose membranes were performed overnight at 30 volts using a wet transfer system. The membranes were incubated in 5% non-fat milk in Tris-buffered saline with 0.03% Tween-20 (TBS-T) for 1 h in order to block non-specific protein binding sites on the membrane. Immunoblotting was performed with a polyclonal rabbit antibody recognizing the ι-chain of the α-subunit of human 20S proteasomes (molecular mass about 29,000; Alexis Biochemicals, Lausen, Switzerland) at a dilution of 1:1000 in a non-fat milk TBS-T, and subsequently probed with a secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) at a dilution of 1:6000 (Bioreba AG, Reinach, Switzerland). After each incubation step membranes were washed in TBS-T for 3 times at 15 min each time. The development of the membranes was performed with a chemiluminiscence kit (Pierce/Socochim SA, Luzern, Switzerland) according to the manufacturer's protocol. The membranes were then exposed to a X-ray film (Kodak/Sigma-Aldrich Chemie, Buchs, Switzerland) which was subsequently developed. The optimal exposure time was at 5 min. In order to control equal loading of the samples, the same membranes used for the detection of the α-subunit of human 20S proteasomes were reused to detect the expression of β-actin as an internal standard [23]. For this, the antibodies from the first western blotting procedure were erased from the membranes with stripping buffer (0.5 N acetic acid, 0.5 N NaCl) for 40 min. at RT. After washing in TBS-T for 2 times at 15 min each, membranes were blocked again in TBS-T containing 5% non-fat milk for 1 h. After extensive washing, membranes were reprobed with a monoclonal murine antibody to

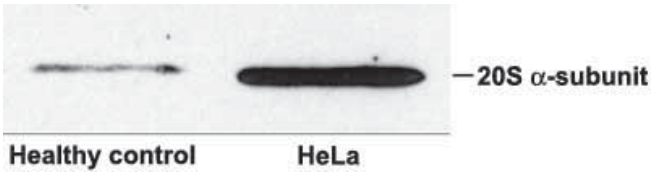

Figure 1. 20S proteasome α-subunit expression. Western blotting against 20S proteasome α-subunit in leucocytes of a healthy control in comparison to HeLa cells, which served as positive control.

human β-actin (1:6000; Sigma-Aldrich Chemie, Buchs, Switzerland) followed by extensive washing and incubation with a monoclonal anti-mouse IgG-HRP (Bioreba AG, Reinach, Switzerland). The development of the membranes was performed as described above. Semiquantitative analysis was performed by using the software program (NIH Image, version 1.62f, Bethesda, MD, USA). The experiment was repeated three times.

Statistical analysis: Statistical analyses were performed using the statistical package StatView 4.5 (Abacus Concepts, Berkley, CA, USA). Individual comparisons of significance of difference were performed using unpaired t-test. P values of less than 0.05 were considered statistically significant. Values are given in mean±standard deviation (SD).

RESULTS

Immunoblotting from three independent experiments gave similar results in each of the six sample groups (Figure 2). In all glaucoma patients (HTG and NTG) semiquantitative Western blot analysis showed a 3.4±0.47 ($p<0.0001$) fold increase in Arbitrary Densitometric Units of the α-subunit expression of human 20S proteasome when compared to healthy controls (Figure 3A). In addition, NTG patients revealed a more pronounced α-subunit expression ($p<0.05$) in comparison with HTG patients (Figure 3B).

DISCUSSION

Glaucoma is an optic neuropathy, where retinal ganglion cells (RGC) are lost. It is postulated that this is due to apoptosis, which is thought to be the most likely mechanism of cell death contributing to neurodegeneration. In glaucoma patients a dysregulation of the expression of various genes influencing cell survival like survivin, XPGC, and the 20S proteasome α-subunit can be observed [18]. The reason for this dysregulation is not yet known, but such results may point towards an imbalance between pro- and anti-apopoptotic processes. A possible mechanism initiating apoptosis could be the generation of oxygen free radicals after focal ischemia/reperfusion leading to oxidative stress and lipid peroxidation. In cultured cortical neurons apoptosis has been detected after a brief exposure to direct oxidative damage (H_2O_2) [24]. Apoptosis is a process that is regulated by several ubiquitous genes in many cells. Although a series of different stimuli can initiate apoptosis, events of programmed cell death occur in a fixed sequence independent of the cell type [25]. Eckert and coworkers demonstrated that oxidative stress factors induce apoptosis in mature human lymphocytes in a similar way as in neurons [26]. Here we demonstrate the dysregulation of 20S proteasomes manifested by an increased synthesis of their α-subunit in leukocytes of glaucoma patients. Although the group size was rather small (6 subjects in each group) we obtained a high similarity for the intra-assay results as well as for inter-assay results indicating a high specificity for glaucoma.

A common stress factor for both neurons and leukocytes might be Endothelin-1 (ET-1). Synthesis of this vasoconstrictor peptide is strongly induced by tumor necrosis factor α (TNF-α), which acts mainly via the transmembrane p55 TNF receptor [27]. ET-1 induces the activation of nuclear factor κB (NF-κB), a key transcriptional factor. In addition, ET-1 activates the degradation of I-κB-α, the cytosolic inhibitor of NF-κB [28], thereby leading to an increasing level of active NF-κB. This transcriptional factor is also responsible for a coordinated increase of the expression of many cytokines and

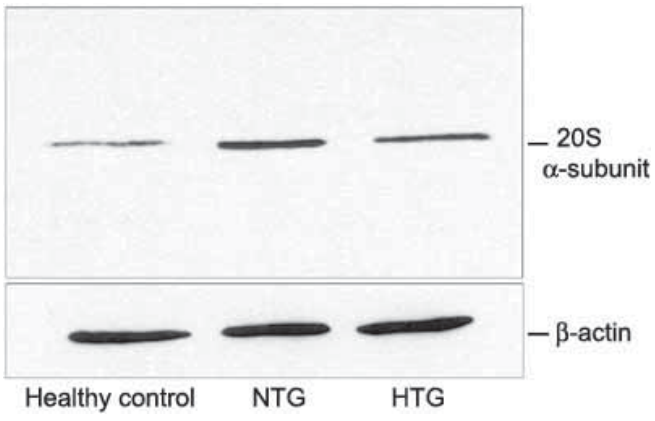

Figure 2. Comparison of 20S proteasome α-subunit protein levels. 20S proteasome α-subunit protein levels of patients with NTG, HTG, and a healthy control. Western blotting against β-actin of the same membrane was used as an internal control for loading.

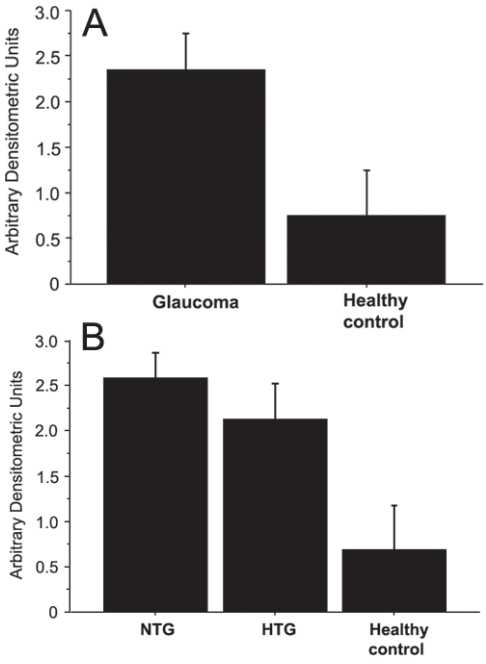

Figure 3. Quantification of 20S proteasome α-subunit levels. **A**: Densitometric quantification of the protein levels of the 20S proteasome α-subunit in leucocytes of glaucoma patients versus healthy controls. **B**: Comparison of the protein levels of the 20S proteasome α-subunit in leukocytes from patients with NTG, HTG, and healthy controls. Both diagrams represent the means and standard deviations of three independent experiments in Arbitrary Densitometric Units.

enzymes that play an important role in inflammation and proliferation [29]. The sequence of events that lead to I-κB-α degradation and activation of NF-κB involves phosphorylation and ubiqitination and subsequent degradation of I-κB by proteasomes [30].

At present we do not know why leukocytes of glaucoma patients enhance their synthesis of 20S proteasome α-subunit. It is known that leukocytes, especially lymphocytes, do respond to damage to the central nervous system, caused by both trauma and neurodegenerative processes like Alzheimer disease [26]. At the moment we can speculate, however, about the possibilities that may induce the described upregulation of the 20S proteasome α-subunit in glaucoma patients. In the glaucomatous optic nerve heads, the expression of both TNF-α and TNF-receptor is upregulated, primarily in glial fibrillary astrocytes, and appears to parallel the progression of optic nerve degeneration [31]. In addition, TNFα may also activate nitric oxide synthase-2, leading to the production of nitric oxide (NO) and retinal degeneration [32,33]. Elevated levels of ET-1, which have been demonstrated in glaucoma patients [34], may also be linked to the up-regulation of TNFα. To extend the hypothesis that apoptotic glaucomatous damage is provoked by events including ischemia/reperfusion, enhanced free oxygen radical generation and DNA damage [35], the increase of the 20S proteasome level presented in this study might be linked or involved in the complex pathway of TNFα-/ET-1/NF-κB-activation.

Several reports have shown that proteasome levels may change during pathological processes [13-15]. Although we can only demonstrate in an observational way an upregulation of the synthesis of the 20S proteasome α-subunit in leukocytes of glaucoma patients, this alteration, eventually accompanied with others, might be a diagnostic or eventually a prognostic marker for glaucomatous damage.

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