Immunoproteasome subunit LMP2 expression is deregulated in Sjögren’s syndrome but not in other autoimmune disorders

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Background: The proteasome system has a pivotal role in the control of the immune response, which suggests that it might be involved in the pathogenesis of autoimmune disorders.

Objective: To investigate the expression profile of selected proteasomal genes in human peripheral blood mononuclear cells in patients with a variety of autoimmune diseases compared with healthy subjects.

Methods: Real-time quantitative RT-PCR was used to analyse the mRNA expression pattern of the proteasome activator subunits PA28α and PA28β and of constitutive proteasome and interferon-γ-inducible immunoproteasome subunits in peripheral blood mononuclear cells. Simultaneously, protein expression of selected proteasome subunits was quantified by immunoblotting.

Results: Under systemic inflammatory conditions the proteasome subunits LMP2 (β1i), LMP7 (β5i), MECL1 (β2i), and PA28α were expressed abundantly at the protein level in the vast majority of systemic autoimmune disorders. However, simultaneous mRNA and protein quantification showed a characteristic proteasome expression signature in primary Sjögren’s syndrome. At the transcript level, the interferon-γ-responsive subunits LMP2 (β1i), MECL1 (β2i), and the proteasome activator subunit PA28α were markedly upregulated. In contrast, LMP2 (β1i) deficiency was evident at the protein level, indicating deregulation of proteasome expression in Sjögren’s syndrome.

Conclusions: These data provide evidence for a regulatory defect in the proteasome system in human autoimmune disorders, pointing to a unique role for LMP2 (β1i) in the pathogenesis of primary Sjögren’s syndrome.

The first evidence for the potential involvement of the proteasome in autoimmune disorders was provided by the detection of autoantibodies directed against different proteasome components. Autoantibodies against the proteasome activator subunit PA28α were also found in systemic lupus erythematosus (SLE) and Sjögren’s syndrome. The proteasome was identified as a major autoantigen in multiple sclerosis and in young patients with type 1 diabetes mellitus. Although the presence of autoantibodies does not necessarily imply the involvement of the targeted antigen in the disease process and might represent a bystander phenomenon, circulating proteasomal components may be markers of cell damage and immunological activity in autoimmune diseases.

Deregulation and aberration of proteasomal pathways contribute to the pathogenesis of human diseases such as malignant, degenerative, metabolic, and autoimmune disorders. Regarding autoimmunity, the proteasome has a pivotal role in the MHC class I presentation pathway of endogenous antigens. However, detailed proteasomal gene and protein expression in autoimmune patients in comparison with healthy subjects has not been simultaneously monitored yet. In the present study, we have focused on the expression profile of selected proteasomal genes in healthy and autoimmune conditions. The analysis at the
transcriptional level as well as the protein expression level allowed us to identify distinct gene regulation mechanisms in individual autoimmune disorders.

PATIENTS AND METHODS

Patients

The study included 45 patients who were diagnosed at the Department of Rheumatology and Clinical Immunology, Charité-University Medicine Berlin from 1997 to 2000 with the following autoimmune disorders: 26 patients with SLE (23 female, 3 male, mean age at time of analysis 45.5 years, age range 36–72 years) meeting the revised American College of Rheumatology criteria, two patients with primary Sjögren’s syndrome (pSS) (female, mean age 56.3 years, age range 31–64 years) diagnosed according to the criteria of the European Study Group on Diagnostic Criteria for Sjögren’s Syndrome, 23 four patients with systemic sclerosis (SSc) (three female, one male, mean age 57.5 years, age range 41–53 years) meeting the criteria according to Bohan and Peter, 25,26 three patients with rheumatoid arthritis (RA) (one female, two male, mean age 61.0 years, age range 45–69 years) diagnosed according to the revised American College of Rheumatology criteria, and two patients with ANCA associated primary vasculitis (female, mean age 49 years, 42 and 56 years) fulfilling the diagnostic criteria according to Jennette et al. 27

At the time of blood sample collection, 15 patients with SLE received an immunosuppressive drug (azathioprine n = 1, hydroxychloroquine n = 3) in addition to corticoids (n = 10, cyclophosphamide n = 1, mycophenolate mofetil n = 1, low dose prednisolone n = 1, methylprednisolone 750 mg/day). The remaining 11 patients with SLE as well as all patients with pSS were treated with low dose corticoids. Three patients with SSc obtained low dose corticoids, one of them in combination with azathioprine. One patient with SSc had no immunosuppressant drugs. Two patients with PM received immunosuppressive treatment (one with azathioprine and one with cyclophosphamide) in addition to low dose prednisolone. All patients with RA received low dose corticosteroids. Patients with vasculitis were treated with corticoids and cyclophosphamide. Informed consent was obtained from all donors before peripheral blood was taken. The study was approved by the ethics committee of the Charité-University Medicine, Humboldt University, Berlin, Germany.

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated immediately after taking heparinised blood by density gradient centrifugation using Ficoll Separating Solution (Biochrom, Berlin, Germany). PBMCs from 22 healthy blood donors (Institute for Transfusion Medicine, Charité-University Medicine Berlin) were used as age and sex matched controls.

Cell culture and IFNγ stimulation

JOSK-M cells derived from human PBMCs from a patient with chronic myeloid leukaemia were obtained from the German Collection of Micro-organisms and Cell Cultures, DSMZ, Braunschweig. For IFNγ treatment (Sigma, Deisenhofen, Germany), the medium was supplemented with 20 and 600 U/ml IFNγ respectively, for 72 hours.
cycles at 95 °C for 15 seconds and 60 °C for 1 minute, with

The TaqMan PCR conditions for all amplifications were 40

neighbouring exons, which are indicated by a vertical bar (|).

designed using the PrimerExpress software (PE Applied

primers (F, forward, R, reverse) and probes (P), which were

contained TaqMan Universal PCR Master Mix (PE Applied

Biosystems), 50–900 nM forward and reverse primers in

amplification reactions

Prism 7700 Sequence Detection System (PE Applied

Switzerland) and reverse transcribed using Moloney murine

leukaemia virus reverse transcriptase (Gibco). Real time

quantitative RT-PCR analysis was performed using the ABI

treatment with IFNγ for 72 hours (20 U/ml, white bars; 600 U/ml grey bars). Untreated JOSK-M cells served as controls (black bars). The non-

proteasomal housekeeping gene HPRT was used as a reference. Relative expression values were calculated based on the PCR amplification efficiencies

E of the individual amplicons. The values (E[ΔCt]) are given as relative mRNA expression. Values are given as means (SEM) Significance was assessed by
two sided Student’s t test (*p<0.05, **p<0.01).

Real time quantitative reverse transcriptase-
polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from PBMCs and JOSK-M cells by

the TRIzol method (TRIzol reagent, PEQLAB, Erlangen, Germany). RNA (1 μg) was DNase-I digested (Roche, Basel, Switzerland) and reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Gibco). Real time

quantitative RT-PCR analysis was performed using the ABI

Prism 7700 Sequence Detection System (PE Applied Biosystems, Weiterstadt, Germany). Amplification reactions contained TaqMan Universal PCR Master Mix (PE Applied Biosystems), 50–900 nM forward and reverse primers in experimentally established optimal ratios, 200 nM 5′-FAM-3′-TAMRA labelled probe (BioSource Europe, Nivelles, Belgium), and about 250 ng of cDNA. Table 1 lists the primers (F, forward, R, reverse) and probes (P), which were
designed using the PrimerExpress software (PE Applied Biosystems). Probes were chosen to span exon junctions of neighbouring exons, which are indicated by a vertical bar (|). The TaqMan PCR conditions for all amplifications were 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, with

initial steps of 2 minutes at 50°C and 10 minutes at 95°C.
Non-template controls for individual amplicons were

included in each run. Specificity and quality of the PCR

products were confirmed by agarose gel electrophoresis.

Standard curves were generated for each gene measured using cDNA from healthy controls. Expression of the target
gene relative to the reference gene hypoxanthine phosphoribosyl transferase (HPRT) was calculated as the difference between threshold cycles of these two genes (2ΔΔCt) using the comparative Ct (threshold cycle) method.29 Differences between duplicate Ct values were always <0.5. The relative mRNA expression in autoimmune patients versus the

expression in healthy control subjects was calculated according to the 2−ΔΔCt method.29

Western blot analysis

PBMCs were lysed on ice in 1/100 volume 10% Triton X-100
(final concentration 0.1%). Total protein (30 μg) in each lane was fractionated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to

nitrocellulose, which was incubated with the following primary antibodies (1:1000) for 1 hour at room temperature: polyclonal rabbit antiserum against the proteasome subunits C3 (α2), C9 (α3), LMP2 (β1i), LMP7 (β5i), MECL1 (β2i), and PA28α.30 31 The secondary antibody was a horseradish peroxidase conjugated, affinity purified goat antirabbit IgG Fc fragment (Dianova, Hamburg, Germany, 1: 20 000). Protein immunoreactivity was visualised by enhanced electrochemiluminescence (ECL; Amersham, Freiburg, Germany). Specific bands were quantified with the NIH image 1.61 software tool (http://rsb.info.nih.gov/nih-image/, accessed 18 May 2006) with integrated density scanning and normalised to the constitutive subunit C3 (α2).

Evaluation of gene expression and statistical analysis

The relative expression software tool REST was used for
group-wise comparison and statistical analysis of the real
time quantitative RT-PCR results. REST analysis included an
efficiency correction step and Ct values normalised to HPRT

in order to compensate for variation between RT-PCR

analyses or variation from sample to sample. For the analysis of

protein expression levels, significance was assessed by a
two sided Student’s t test or Mann-Whitney U test where appropriate (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

Real time RT-PCR analysis of selected
immunoproteasome and proteasome activator subunit expression after IFNγ up regulation in vitro

A real time quantitative RT-PCR assay was established in order to investigate the gene expression of selected proteasome

and proteasome activator subunits. Similar PCR
amplification efficiencies of control and target genes were

confirmed using serial dilutions of cDNA from human control

PBMCs. These experiments were conducted to obtain

standard curves as the prerequisite for the 2−ΔΔCt method.29

When the quantitative RT-PCR system was established, the

assay was verified in an in vitro IFNγ stimulation experiment.

JOSK-M cells from a human chronic myeloid leukaemia cell

line were stimulated for 72 hours with 20 and 600 U/ml
Untreated JOSK-M cells served as controls for basal expression levels. The mRNA expression of LMP2, LMP7, MECL1, C3, C9, PA28α, and PA28β was determined. The expression of HPRT was measured as a control gene unrelated to the proteasome system.

After IFNγ stimulation of JOSK-M cells, the relative mRNA expression of the inducible immunoproteasome subunits and the expression of the activator subunits PA28α and PA28β were increased significantly compared with untreated control cells (fig 1). The expression of the constitutive subunits C3 and C9 and the housekeeping gene HPRT remained stable. Normalisation to HPRT, C3 or C9 demonstrated significant up regulation of the immunoproteasome.

Figure 2  Proteasomal gene expression profiling in autoimmune patients. Gene expression of IFNγ-inducible immunoproteasome subunits (A, B, C), proteasome activator subunits (D, E), and two selected constitutive proteasome subunits (F, G) was measured by real time quantitative RT-PCR in PBMCs from patients with SLE (n = 26), pSS (n = 6), PM (n = 4), SSc (n = 4), RA (n = 3), and primary vasculitis (Vasc; n = 2) showing clinically active disease. Healthy blood donors served as age and sex matched controls (C; n = 22). Only significant relative up regulation >1.6-fold was discussed. Patients with vasculitis and RA were not included in the statistical evaluations owing to the small number of cases. Values are given as the mean (SEM) relative mRNA expression. If applicable, a two sided Mann-Whitney U test was used to verify statistical significance (*p<0.05, **p<0.01, ***p<0.001).

Figure 3  Expression profile of LMP2 (β1i) in autoimmune disorders by immunoblot analysis of PBMC lysates. Representative data are shown for SLE (n = 7, lanes 1–7), pSS (n = 6, lanes 8–13), SSc (n = 4, lanes 14–17), PM (n = 4, lanes 18–21), and healthy controls (n = 6, lanes 22–27). Cell lysates were normalised for protein (30 µg total protein per lane) and analysed by immunoblotting with antibodies against LMP2 (β1i) and the constitutive human proteasome subunits C3 (α2). Note that lanes 8–13 were slightly overexposed in order to demonstrate reduced LMP2-specific signals in most patients with pSS.
controls (for numbers of cases see fig 2) were subjected to immunoblotting and densitometry. Mean protein expression is shown for LMP2 (β1; A), LMP7 (β5i; B), MECL1 (β2i; C), and PA28β (D). Values are normalised to C3 (r2) and are given as relative protein expression in %C3 (r2) plotted on a logarithmic scale. If applicable, a two sided Mann-Whitney U test was used to assess significance (⁎p<0.05, **p<0.01, ***p<0.001).

Figure 4 Measurement of relative proteasomal protein expression in autoimmune disorders. PBMC lysates of all autoimmune patients and healthy controls (n = 22). Relative mRNA expression of each diagnosis group.

The present study provides evidence for a disturbed expression of components in the proteasome system at both the mRNA and the protein level in human autoimmune disorders. Interestingly, we found characteristic expression profiles for distinct autoimmune diseases. We substantiated the strong induction of IFNγ-inducible proteasomal subunits as well as of the proteasome activator in systemic autoimmune diseases at the mRNA level. However, increased...
tissues at high levels. Previously, it was demonstrated that IFN system are induced by proinflammatory cytokines such as autoimmune conditions components of the proteasomal in the periphery may be changed both qualitatively and population in PBMCs of patients with pSS. As a consequence, not been provided up to now, decreased LMP2 protein levels the IFN regulatory factor IRF-1. 33 there may be a concerted regulation of both LMP2 and autoimmune patients C3 underlies differential induction systemic vasculitis. Therefore, we suggest that in expression by nuclear factor-kappaB (NF-\kappaB). These data imply an alternative induction mechanism of C3 mRNA because the vast majority of the autoimmune patients in this study obtained steroid immunosuppression. In this study, no difference was found between the respective immunosuppressive treatment and the expression of any of the investigated proteasome subunits at the mRNA level in patients with SLE.

Surprisingly, in contrast with the other autoimmune disorders, our data disclosed a significant up regulation of the IFNγ-inducible subunits LMP2, MECL1, and PA28\gamma at the mRNA level in patients with pSS. On the other hand, the protein level of LMP2 was very low in most patients with pSS compared with healthy controls and other related autoimmune disorders. This intriguing finding was obtained in a small, but homogenous pSS study group. Increased mRNA levels of LMP2 may reflect (a) increased mRNA steady state levels, (b) increased mRNA stability, or (c) an increased transcription rate. Whereas increased mRNA levels in other autoimmune diseases correspond with the respective protein expression levels, this correlation is reversed in pSS. The relative lack of the subunit LMP2 might be due to raised C3 levels. However, the protein expression of C3 was not markedly increased in pSS. A decrease of LMP2 protein might result from (a) a post-transcriptional defect of the LMP2 mRNA, (b) an impaired translation process, or (c) reduced protein stability of LMP2. The proteasome system is important for T cell education and antigen presentation by MHC class I molecules. Although experimental evidence has not been provided up to now, decreased LMP2 protein levels may contribute to an altered composition of the proteasome population in PBMCs of patients with pSS. As a consequence, the MHC class I peptide repertoire of antigen presenting cells in the periphery may be changed both qualitatively and quantitatively and might promote autoimmune pathogenesis. A specific proteasome defect has been identified in non-obese diabetic (NOD) mice, an animal model of autoimmune diabetes, in lymphocytic and monocytic lineages, resulting in the down regulation of LMP2 expression. It has been suggested that the molecular defect resides within the promoter-enhancer region of LMP2, which results in decreased mRNA levels of LMP2. This defect may prevent proteolytic proteasomal processing of NF-\kappaB, affecting immune and inflammatory responses, as well as susceptibility to apoptosis induced by tumour necrosis factor α. Interestingly, NOD mice provide a suitable model of Sjögren's syndrome, in particular autoimmune sialadenitis exhibiting exocrine gland lymphocytic infiltration typical of the Sjögren's syndrome-like disease. 39

Our findings cannot simply be explained by a transcriptional defect in the LMP2 gene in patients with pSS according to the NOD mouse model, because LMP2 mRNA was abundant in PBMCs. Previously, it had been shown that proteasomal subunits are up regulated at the transcriptional level upon treatment with proteasome inhibitors. In addition, there is evidence for a regulatory feedback loop not only in yeast but also in the mammalian proteasomal system. Based on these results, we suggest that decreased protein levels of LMP2 in PBMCs in pSS may be followed by reactive up regulation of mRNA in the proteasomal system. This situation appears to be unique for pSS, because in SLE, SSC, PM, and RA transcript as well as protein levels of the respective subunits were regulated accordingly.

For the first time, expression patterns in the proteasome system at both the transcriptional and translational level were analysed simultaneously in active systemic rheumatoid disorders. Our observations showed that autoimmune diseases have distinct proteasomal gene and protein expression signatures. In this study, we have provided new clues for the pathogenic role of LMP2 in the pathogenesis of pSS, suggesting a regulatory molecular defect of LMP2 in pSS. Further genetic and biochemical studies should extend our understanding of the pathophysiology of the proteasome system, leading to new diagnostic and therapeutic strategies in human autoimmune disorders.

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The authors declare no competing financial interests.

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