Circulating 20S Proteasome Levels in Mixed Connective Tissue Disease and Systemic Lupus Erythematosus

Running title: Circulating 20S proteasomes in MCTD and SLE

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Abstract:

The associations of circulating 20S proteasomes (c20S) with clinical and serologic disease indices in systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) are unknown. We present the initial report that c20S are elevated in MCTD and correlate with clinically relevant changes in disease activity in SLE and MCTD.
Proteasomes are important proteolytic machineries in all eukaryotic cells and a major source of antigenic peptides displayed on MHC-class I molecules [3,8,10]. 20S proteasomes are also detectable in normal serum and plasma, and elevated circulating 20S proteasome (c20S) concentrations have been described in several diseases, such as hematologic malignancies, sepsis, trauma and autoimmune diseases including systemic lupus erythematosus (SLE) [5,12,14,15]. Although evidence for a functional role of c20S has not yet been provided, its systemic concentrations are thought to reflect cellular damage and immunological activity. Measurements of c20S in a single patient with SLE suggested that its concentrations may parallel disease activity [5]. However, the association of c20S with clinical disease indices and serologic parameters in patients with SLE are unknown and c20S have not been studied in mixed connective tissue disease (MCTD). Thus, we evaluated in a prospective observational study whether c20S are also elevated in MCTD and correlate with disease activity and damage measures in SLE and MCTD patients.

Patients were recruited from the Division of Rheumatology and Immunology at the University of Miami following an IRB approved protocol upon the presence of either anti-RNP or anti-Sm antibodies. Fifty-six patients (53 female/3 male; age (mean±SD): 34±10 years) were clinically diagnosed as SLE and 35 (all females; age (mean±SD): 44±14 years) as MCTD. SLE patients met 4+ American College of Rheumatology criteria for classification of SLE; MCTD patients met the classification criteria of Alarcon-Segovia and Cardiel [1,13]. Twenty-two healthy blood donors served as controls (all females, age (mean±SD): 37±14 years).
Disease activity was assessed using the SLE Disease Activity Index (SLEDAI) [4] and disease damage using the Systemic Lupus International Collaborating Clinics (SLICC) damage index scores [7]. The SLEDAI has been used successfully as a validated measure of disease activity in MCTD in recent studies [9].

Complete blood count, urinalysis, routine blood chemistry, and antinuclear antibody (ANA) testing were done on all patients. Serologic testing for reactivity with specific ANA was performed by ELISA and immunoblotting, as described [9]. C3 and C4 were measured by nephelometry. Anti-RNP and anti-Sm reactivity were scored as positive based upon the presence of an EU result at least 4 standard deviations above the mean for healthy blood donors when tested in RNP-specific or Sm-specific ELISA and/or immunoblotting reactivity with sera compared to well characterized positive and negative control sera, as described [9]. Serum concentrations of L-selectin (sCD62L) were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). Serum concentrations of c20S were measured by ELISA as described [11], with the investigators blinded to the patient-related data.

If not otherwise mentioned, data are described as median with interquartile range. Data were analyzed with the Komolgorov-Smirnov test to assess normal distribution and differences in c20S concentrations between groups were analyzed with the Mann-Whitney-U test, the Kruskal-Wallis-H test with Dunn’s multiple comparison test for multiple testing and correlations were assessed with the Spearman’s correlation coefficients using the SPSS program (SPSS Inc., Chicago, IL). Linear regression analyses were calculated with the GraphPad Prism4 program (GraphPad, San Diego, CA). Differences were considered significant on a two-tailed p<0.05 level.
The determined c20S levels in sera from healthy blood donors in the present study (mean±SD: 454±274 ng/mL, normal range (mean±2xSD): 0-1002 ng/mL) were comparable to the normal concentrations described by Egerer et al. (mean±SD: 221±73 ng/mL) and Wada et al. (mean±SD: 359.6±88 ng/mL) [5,14].

As compared with healthy volunteers, c20S serum concentrations were significantly elevated in SLE and MCTD patients (Fig. 1; ctrl (n=22): 445.5(242–679) ng/mL; SLE (n=56): 889(558–2014) ng/mL; MCTD (n=35): 831(507–1159) ng/mL; p<0.001 for SLE and MCTD vs. ctrl.). These results are consistent with previously described alterations in SLE and other autoimmune diseases, such as primary Sjögren's syndrome, rheumatoid arthritis and polymyositis [5]. Twenty-three of 56 SLE patients (41%) and 13 of 35 MCTD patients (37%) of the present study presented with c20S levels above the normal range. This proportion of SLE patients was lower than previously reported [5]. However, a direct comparison is difficult since information on disease activity or damage has not been reported previously.

The correlations of c20S serum concentrations with the clinical disease indices and serologic measurements are shown in Table 1. Levels of c20S correlated significantly positive with the SLEDAI, but not with the SLICC. Linear regression analyses showed that c20S concentrations increased linearly with increases in SLEDAI values in SLE and MCTD patients (Fig. 2A). As shown in Fig. 2B, c20S levels increased from 781(420–948) ng/mL in patients with SLEDAI of 0-5 (n=26) to 842(434–1477) ng/mL and 1159(751–2705) ng/mL with SLEDAI of 6-10 (n=33) and above 10 (n=35), respectively (p = 0.014). The proportion of patients with c20S levels above the normal range increased from 19% (5/26) with SLEDAI 0-5 to 39% (13/33) and 54% (19/35) with
SLEDAI of 6-10 and 11+, respectively. Although the number of patients was not high enough to reach statistical significance for the subgroups of SLE and MCTD patients alone, the correlation coefficients for SLE, MCTD and the combined patient population were identical (Tab. 1) and linear regression analyses showed a significant increase in c20S with increased disease activity in SLE and MCTD. Comparison of c20S levels between patients with and without a specific disease manifestation revealed that pulmonary and renal manifestation was accompanied by higher c20S concentrations (Fig. 2C). Among the serological parameters, there were significant positive associations with the anti-dsDNA antibody titers and CRP concentrations (Table 1, Fig. 3A/B). c20S levels were negatively associated (Tab. 1) and correlated significantly linear with C3 and C4 concentrations (Fig. 3C/D).

The origin of c20S is currently unclear. Our finding that c20S serum levels were similar in patients with and without hematological disease manifestation further strengthens the assumption that blood cells are probably not its source [15]. Since the lung and kidney are organs with high proteasome content [16], the significantly higher c20S serum levels in patients with disease manifestation in these organs point towards release of c20S from damaged tissues. Previous studies showed that c20S are elevated in a variety of autoimmune diseases [5] and also in non-autoimmune diseases, such as sepsis, trauma [12] or burns (unpublished observation). Thus, c20S is not a specific biomarker for autoimmune diseases and its serum concentrations appear to reflect cell damage independent of the underlying etiology.

The findings that c20S levels correlate with the clinical presence of acute disease activity along with its negative association with complement concentrations and its
positive association with CRP concentrations support the concept that c20S levels primarily reflect ongoing inflammatory processes associated with cell damage, consistent with other biomarker and serologic measurements.

Antigens released during tissue injury appear to play a central role in the pathogenesis of SLE and MCTD. Along with previous observations that proteasome subunits are primary targets of autoantibodies in SLE and other systemic autoimmune diseases [2,6], our findings are similarly consistent with the release of self-antigens during tissue injury which serve as biomarkers of disease activity and which may have a more direct role in pathogenesis.

The present study suggests that changes in c20S level correlate with clinically meaningful changes in disease activity and thus, implies that measurement of c20S may assist in monitoring and directing therapy. In addition, we present the first report that c20S is a novel measure of disease activity in MCTD when compared with other established serologic and clinical measures of disease activity. However, the finding that only 54% of patients with the highest disease activities showed c20S levels above the normal range shows that its sensitivity to detect SLE/MCTD is rather low and that single time point measurements will have limited clinical relevance. Nevertheless, based on these data future longitudinal studies to confirm the value of serial measurements of c20S as a biomarker of disease activity in MCTD and SLE are justified.
References


Figure Legends:

**Figure 1:** c20S serum concentrations (ng/mL) are elevated in SLE and MCTD. The horizontal line shows the median, error bars show the interquartile range. ○: Volunteers (n = 22). □: SLE (n = 56). ●: MCTD (n = 35). *: p < 0.05 vs. volunteers.

**Figure 2:**  
A. c20S serum concentrations correlate linear with SLEDAI in SLE and MCTD. ■: SLE (n = 56). ●: MCTD (n = 35). —: Linear regression line for the combined patient population ($r^2$: 0.122, p = 0.0007; SLE: $r^2$: 0.112, p = 0.011; MCTD: $r^2$: 0.12, p = 0.047).  
B. c20S serum concentrations in patients grouped according to their SLEDAI. The horizontal line shows the median, error bars show the interquartile range. *: p < 0.05.  
C. c20S serum concentrations in SLE and MCTD patients grouped according to specific disease manifestations (Yes: ○ / No: □). Skin: Any skin involvement, such as scleroderma, alopecia, malar/discoid rash, photosensitivity, calcinosis, Raynaud syndrome (Yes: n=82; No: n=7). Muscle: Any muscle involvement, such as swelling, weakness, morning stiffness, myalgia, myositis, rheumatoid nodules (Yes: n=84, No: n=7). Joint: Arthritis (Yes: n=16, No: 23). Blood: Any hematological symptoms, such as anemia, leukopenia, thrombocytopenia or thrombocytosis (Yes: n=59, No: n=32). GE: Gastroesophageal reflux (Yes: n=39, No: n=50). Lung: Pulmonary fibrosis or hypertension (Yes: n=19, No: n=47). Kidney: Serum creatinin >1.1 mg/dL or proteinuria or hematuria (Yes: n=34, No: n=48). Serosa: Pleuritis or myocarditis (Yes: n=3, No: n=49). CNS: Any neurological or psychiatric symptoms, such as seizures, psychosis, neuropathy (Yes: n=44, No: n=47). *: p < 0.05.
**Figure 3:** A.-B. c20S serum concentrations in patients grouped according to their anti-dsDNA titer (A.) and CRP concentration (B.). C.-D. c20S serum concentrations correlate linear with C3 (C.) and C4 (D.) concentrations in SLE and MCTD. □: SLE (C3: n = 55; C4: n = 54). ●: MCTD (C3: n = 35; C4: n = 34). —: Linear regression line for the combined patient population ($r^2$: 0.11, $p = 0.0014$; SLE: $r^2$: 0.06, $p = 0.07$; MCTD: $r^2$: 0.24, $p = 0.003$).
**Table 1:** Correlation of c20S with clinical and serological disease parameters.

<table>
<thead>
<tr>
<th>c20S (ng/mL) vs.</th>
<th>All ( r_s (p) / n )</th>
<th>SLE ( r_s (p) / n )</th>
<th>MCTD ( r_s (p) / n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>-0.119 (.201) / 91</td>
<td>-0.181 (.183) / 56</td>
<td>-0.011 (.951) / 35</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>-0.073 (.491) / 91</td>
<td>-0.141 (.314) / 56</td>
<td>-0.086 (.628) / 35</td>
</tr>
<tr>
<td>SLICC</td>
<td>0.102 (.322) / 91</td>
<td>0.011 (.934) / 56</td>
<td>0.009 (.601) / 35</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>0.288 (.005) / 91</td>
<td>0.263 (.050) / 56</td>
<td>0.258 (.140) / 35</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>0.047 (.663) / 85</td>
<td>-0.068 (.641) / 50</td>
<td>0.119 (.366) / 35</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.529 (&lt;0.001) / 45</td>
<td>0.466 (.012) / 28</td>
<td>0.608 (.009) / 17</td>
</tr>
<tr>
<td>Anti-Cardiolipin</td>
<td></td>
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<tr>
<td>IgG (EU/mL)</td>
<td>0.140 (.259) / 64</td>
<td>0.008 (.956) / 47</td>
<td>0.373 (.141) / 17</td>
</tr>
<tr>
<td>IgM (EU/mL)</td>
<td>0.199 (.116) / 61</td>
<td>0.132 (.382) / 46</td>
<td>0.532 (.053) / 15</td>
</tr>
<tr>
<td>Anti-dsDNA antibody titer (EU/mL)</td>
<td>0.280 (.029) / 61</td>
<td>0.288 (.061) / 43</td>
<td>0.352 (.152) / 18</td>
</tr>
<tr>
<td>ANA titer (EU/mL)</td>
<td>0.055 (.624) / 77</td>
<td>0.020 (.893) / 47</td>
<td>-0.028 (.884) / 30</td>
</tr>
<tr>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 (mg/dL)</td>
<td>-0.289 (.005) / 90</td>
<td>-0.162 (.239) / 55</td>
<td>-0.394 (.019) / 35</td>
</tr>
<tr>
<td>C4 (mg/dL)</td>
<td>-0.256 (.014) / 88</td>
<td>-0.109 (.432) / 54</td>
<td>-0.386 (.024) / 34</td>
</tr>
<tr>
<td>sCD62L (ng/mL)</td>
<td>0.019 (.870) / 76</td>
<td>-0.32 (.835) / 45</td>
<td>0.105 (.575) / 31</td>
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\( r_s \): Spearman correlation coefficient. \( p \): level of statistical significance. \( n \): number of observations. ESR: Erythrocyte sedimentation rate. Significant correlations are highlighted (bold font).
Fig. 1
Fig. 3

A

anti-dsDNA titer

c20S (ng/mL)
< 1:30 1:30 - 1:100 > 1:100

B

CRP (mg/L)
< 0.7 > 0.7

c20S (ng/mL)

C

C3 (mg/dL)

c20S (ng/mL)

D

C4 (mg/dL)

c20S (ng/mL)