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

Matthias Majetschak, Magdalena Perez, Luis T. Sorell, Janet Lam, Marcos E. Maldonado, and Robert W. Hoffman

The associations of circulating 20S proteasomes (c20S) with clinical and serologic disease indices in patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) are unknown. We present the initial report that c20S levels are elevated in MCTD and correlate with clinically relevant changes in disease activity in SLE and MCTD.

Proteasomes are important proteolytic machinery in all eukaryotic cells and a major source of antigenic peptides displayed on major histocompatibility complex class I molecules (3, 8, 10). 20S proteasomes are also detectable in healthy 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, 13, 15, 16). Although evidence for the functional role of c20S has not yet been provided, systemic concentrations of c20S 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 concentrations with clinical disease indices and serologic parameters in patients with SLE is unknown, and c20S have not been studied in mixed connective tissue disease (MCTD). Thus, we determined whether c20S levels are also elevated in patients with MCTD and whether levels 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 institutional review board-approved protocol, based upon the presence of either anti-RNP or anti-Sm antibodies. Fifty-six patients (53 female, 3 male; mean age ± standard deviation [SD], 34 ± 10 years) were clinically diagnosed as having SLE, and 35 patients (all females; mean age ± SD, 44 ± 14 years) as having MCTD. SLE patients met more than four American College of Rheumatology criteria for the classification of SLE; MCTD patients met the classification criteria of Alarcon-Segovia and Cardiel and Tan et al. (1, 14). Twenty-two healthy blood donors served as controls (all females; mean age ± SD, 37 ± 14 years).

Disease activity was assessed using the SLE Disease Activity Index (SLEDAI) (4), and disease damage was assessed using the Systemic Lupus International Collaborating Clinics (SLICC) damage index score (7). The SLEDAI has been used successfully as a validated measure of disease activity in patients with MCTD in recent studies (9).

Complete blood count, urinalysis, routine blood chemistry, and antinuclear antibody tests were performed for all patients. Serologic testing for reactivity with specific antinuclear antibody was performed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting, as described previously (9). Complements C3 and C4 were measured by nephelometry. Anti-RNP and anti-Sm reactivity levels were scored as positive based upon the presence of an ELISA unit result at least 4 SD above the mean for healthy blood donors when they were tested with RNP-specific or Sm-specific ELISA and/or an immunoblotting reactivity with sera compared to that of well-characterized positive and negative control sera, as described previously (9). Serum concentrations of t-selectin (also known as soluble cluster of differentiation CD62 ligand [sCD62L]) were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). Serum concentrations of c20S were measured by ELISA as described previously (11), with the investigators blinded to the patient-related data.

If not otherwise mentioned, data are described as median values with interquartile ranges. Data were analyzed with the Komolgorov-Smirnov test to assess normal distributions. Differences in c20S concentrations between groups were analyzed with the Mann-Whitney U test and the Kruskal-Wallis H test with Dunn’s multiple comparison test for multiple testing, and correlations were assessed with Spearman’s correlation coefficient, using SPSS software (SPSS Inc., Chicago, IL). Linear regression analyses were calculated with GraphPad Prism4 (GraphPad, San Diego, CA) software. Differences were considered significant on a two-tailed P value of <0.05.

The c20S levels determined in sera from healthy blood donors in the present study (mean ± SD, 454 ± 274 ng/ml; range, 0 to 1,002 ng/ml) were comparable to the normal concentra-
tions described by Egerer et al. (mean ± SD, 221 ± 73 ng/ml) and Wada et al. (mean ± SD, 359.6 ± 88 ng/ml) (5, 15).

Compared with serum concentrations in healthy volunteers, c20S serum concentrations were significantly elevated in SLE and MCTD patients (Fig. 1) as shown by the following values: control (n = 22), 445.5 (range, 242 to 679) ng/ml; SLE (n = 56) patients, 889 (range, 558 to 2,014) ng/ml; and MCTD (n = 35) patients, 831 (range, 507 to 1,159) ng/ml (P < 0.001 for SLE and MCTD versus control). These results are consistent with those previously described for 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%) in the present study presented with c20S levels above the normal range. This proportion of SLE patients was lower than that reported previously (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. Correlation of c20S levels was significantly positive with the SLEDAI but not with the SLICC score. 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 (range, 420 to 948) ng/ml in patients assessed with a SLEDAI range of 0 to 5 (n = 26) to 842 (range, 434 to 1,477) ng/ml and 1,159 (range, 751 to 2,705) ng/ml in patients assessed with a SLEDAI range of 6 to 10 (n = 33) and above 10 (n = 35; P = 0.014), respectively. The proportion of patients with c20S levels above the normal range increased from 19% (5/26) with a SLEDAI range of 0 to 5 to 39% (13/33) and 54% (19/35) with a SLEDAI range of 6 to 10 and 11 or more, respectively. Although the number of patients was not high enough to reach statistical significance for the SLE and MCTD subgroup patients alone, the correlation coefficients for the SLE population, MCTD population, and combined patient population were identical (Table 1), and linear regression analyses showed a significant increase in c20S levels with increased disease activity in SLE and MCTD patients. Comparison of c20S levels in patients with specific disease manifestations with those of the patients without such manifestations revealed that pulmonary and renal manifestations were accompanied by higher c20S concentrations (Fig. 2C). Among the serological parameters, there were significantly positive associations with the anti-double-stranded DNA (dsDNA) antibody titers and C-reactive protein (CRP) concentrations (Table 1; Fig. 3A and B). c20S levels were negatively associated (Table 1) and correlated

![FIG. 1. c20S serum concentrations (ng/ml) are elevated in patients with SLE and MCTD. The horizontal line shows the median, and error bars show the interquartile range. Volunteers, n = 22; SLE, n = 56; MCTD, n = 35. *, P < 0.05 versus volunteers.](image-url)

### TABLE 1. Correlation of c20S with clinical and serological disease parameters

| Patient parameter | All study patients | SLE | MCTD |
|-------------------|--------------------|-----|------|
|                   | rs | n  | P value | rs | n  | P value | rs | n  | P value |
| Age (yr)          | −0.119 | 91 | 0.201 | −0.181 | 56 | 0.183 | −0.011 | 35 | 0.951 |
| Disease duration (mo) | −0.073 | 91 | 0.491 | −0.141 | 56 | 0.314 | −0.086 | 35 | 0.628 |
| SLICC score       | 0.102 | 91 | 0.322 | 0.011 | 56 | 0.934 | 0.093 | 35 | 0.601 |
| SLEDAI            | 0.288 | 91 | **0.005** | 0.263 | 56 | 0.050 | 0.258 | 35 | 0.140 |
| ESR (mm/h)        | 0.047 | 85 | 0.663 | −0.068 | 50 | 0.641 | 0.158 | 35 | 0.366 |
| CRP (mg/liter)    | 0.529 | 45 | **<0.001** | 0.466 | 28 | **0.012** | 0.609 | 17 | **0.009** |
| Anti-cardiolipin (EU/ml) | | | | | | | | | |
| IgG               | 0.140 | 64 | 0.259 | 0.008 | 47 | 0.956 | 0.527 | 17 | 0.141 |
| IgM               | 0.199 | 61 | 0.116 | 0.132 | 46 | 0.382 | 0.509 | 15 | 0.053 |
| Anti-dsDNA antibody titer (EU/ml) | 0.280 | 61 | **0.029** | 0.288 | 43 | 0.061 | 0.352 | 18 | 0.152 |
| ANA titer (EU/ml) | 0.055 | 77 | 0.624 | 0.020 | 47 | 0.893 | −0.028 | 30 | 0.884 |
| Complement (mg/dl) | | | | | | | | | |
| C3                | −0.289 | 90 | **0.005** | −0.162 | 55 | 0.239 | −0.394 | 35 | **0.019** |
| C4                | −0.256 | 88 | **0.014** | −0.109 | 54 | 0.432 | −0.386 | 34 | **0.024** |
| sCD62L (ng/ml)    | 0.019 | 76 | 0.870 | −0.32 | 45 | 0.835 | 0.105 | 31 | 0.575 |

*a* ESR, erythrocyte sedimentation rate; IgG/M, immunoglobulin G/M; EU, ELISA unit; ANA, antinuclear antibody.

*b* Statistical values: rs, Spearman correlation coefficient; P, level of statistical significance; n, number of observations. Significant correlations are in bold.
significantly linearly with C3 and C4 concentrations (Fig. 3C and D).

The origin of c20S is currently unclear. Our finding that c20S serum levels were similar in patients with and without hematological disease manifestations further strengthens the assumption that blood cells are probably not its source (16).

Since the lungs and kidneys are organs with high proteasome contents (12), the significantly higher c20S serum levels in patients with disease manifestations in these organs point toward release of c20S from damaged tissues. Previous studies showed that c20S levels are elevated in a variety of autoimmune diseases (5) and also in nonautoimmune diseases, such

FIG. 2. (A) c20S serum concentrations correlate linearly with the SLEDAI in SLE and MCTD patients. □, SLE (n = 56); ●, MCTD (n = 35). Solid line, 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’s 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, $n = 23$). 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 creatinine value of $>1.1$ mg/dL, or proteinuria or hematuria (yes, $n = 34$; no, $n = 48$). Serosa, pleuritis or myocarditis (yes, $n = 3$; no, $n = 49$). CNS, central nervous system, any neurological or psychiatric symptoms, such as seizures, psychosis, or neuropathy (yes, $n = 44$; no, $n = 47$). *, $P < 0.05$. 

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as sepsis, trauma (13), 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 indicates c20S as a novel measure of disease activity in MCTD patients, according to comparisons 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 patients with MCTD and SLE are justified.

FIG. 3. (A and B) c20S serum concentrations in patients grouped according to their anti-dsDNA titer (A) and CRP concentration (B). (C and D) c20S serum concentrations correlate linearly with C3 (C) and C4 (D) concentrations in patients with SLE and MCTD. SLE (C3 n = 55; C4, n = 54); MCTD (C3, n = 35; C4, n = 34). Solid line, 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]).
REFERENCES

1. Alarcon-Segovia, D., and M. H. Cardiel. 1989. Comparison between 3 diagnostic criteria for mixed connective tissue disease. Study of 593 patients. J. Rheumatol. 16:328–334.

2. Arribas, J., M. Luz Rodriguez, R. Alvarez-Do Forno, and J. G. Castaño. 1991. Autoantibodies against the multicatalytic proteinase in patients with systemic lupus erythematosus. J. Exp. Med. 173:423–427.

3. Baumeister, W., J. Walz, F. Zühl, and E. Seemüller. 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell 92:367–380.

4. Bombardier, C., D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang. 1992. Derivation of the SLEDAI. A disease activity index for lupus patients. Arthritis Rheum. 35:630–640.

5. Egerer, K., U. Kuckelkorn, P. E. Rudolph, J. C. Rückert, T. Dörner, G. R. Burmester, P. M. Kloetzl, and E. Feist. 2002. Circulating proteasomes are markers of cell damage and immunologic activity in autoimmune diseases. J. Rheumatol. 29:2045–2052.

6. Feist, E., T. Dörner, U. Kuckelkorn, G. Schmidtk, B. Micheel, F. Hiepe, G. R. Burmester, and P. M. Kloetzl. 1996. Proteasome alpha-type subunit C9 is a primary target of autoantibodies in sera of patients with myositis and systemic lupus erythematosus. J. Exp. Med. 184:1313–1318.

7. Gladman, D., E. Ginzler, C. Goldsmith, P. Fortin, M. Liang, M. Urowitz, P. Bacon, S. Bombardieri, J. Hanly, E. Hay, D. Isenberg, J. Jones, K. Kalunian, P. Maddison, O. Nived, M. Petri, M. Richter, J. Sanchez-Guerrero, M. Snith, G. Sturfelt, D. Symmons, and A. Zoma. 1996. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. Arthritis Rheum. 39:363–369.

8. Goldberg, A. L., P. Cascio, T. Satic, and K. L. Rock. 2002. The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. Mol. Immunol. 39:147–164.

9. Greidinger, E. L., and R. W. Hoffman. 2001. The appearance of U1 RNP antibody specificities in sequential autoimmune human antisera follows a characteristic order that implicates the U1-70 kd and B/B proteins as predominant U1 RNP immunogens. Arthritis Rheum. 44:368–375.

10. Hershko, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425–479.

11. Majetschak, M., and L. T. Sorell. 2008. Immunological methods to quantify and characterize proteasome complexes: development and application. J. Immunol. Methods 334:91–103.

12. Patel, M. B., and M. Majetschak. 2007. Distribution and interrelationship of ubiquitin proteasome pathway component activities and ubiquitin pools in various porcine tissues. Physiol. Res. 56:341–350.

13. Roth, A., B. Moser, C. Krenn, F. Roth-Walter, H. Hetz, S. Richter, M. Brunner, E. Jensen-Jarolim, E. Wolner, K. Hoetzecker, G. Boltz-Nitulescu, and H. J. Ankersmit. 2005. Heightened levels of circulating 20S proteasome in critically ill patients. Eur. J. Clin. Investig. 35:399–403.

14. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 25:1271–1277.

15. Wada, M., M. Kosaka, S. Saito, T. Sano, K. Tanaka, and A. Ichihara. 1993. Serum concentration and localization in tumor cells of proteasomes in patients with hematologic malignancy and their pathophysiologic significance. J. Lab. Clin. Med. 121:215–223.

16. Zoeger, A., M. Blau, K. Egerer, E. Feist, and B. Dahlmann. 2006. Circulating proteasomes are functional and have a subtype pattern distinct from 20S proteasomes in major blood cells. Clin. Chem. 52:2079–2086.