SHORT REPORT

Concentrations of circulating matrix metalloproteinase 9 inversely correlate with autoimmune antibodies to double stranded DNA: implications for monitoring disease activity in systemic lupus erythematosus

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Aims: To compare circulating matrix metalloproteinase (MMP) concentrations with antibodies to single and double stranded DNA (ssDNA and dsDNA) to determine their relation in inflammatory arthritic diseases, such as systemic lupus erythematosus (SLE).
Methods: Fibroblast MMP-2 and neutrophil MMP-9 were resolved by gelatin zymography and measured by densitometry. Anti-ssDNA and anti-dsDNA were determined by enzyme immunoassay and samples grouped on antibody content as follows: low anti-ssDNA/low anti-dsDNA antibodies (group 1); high anti-ssDNA/low anti-dsDNA antibodies (group 2); and high anti-ssDNA/high anti-dsDNA antibodies (group 3).
Results: Group 3 samples contained significantly lower amounts of MMP-9 when compared with group 1 samples. Higher molecular weight MMP-9 forms (130 and 225 kDa) were virtually absent. Group 2 samples contained intermediate MMP-9 concentrations. Fibroblast MMP-2 was unchanged in all groups. Mean complement C3 and C4 concentrations showed a consistent, but variably significant, decrease with increasing anti-ssDNA and anti-dsDNA antibodies. The mean erythrocyte sedimentation rate was raised in all patient groups.
Conclusions: Neutrophil MMP-9, an inflammatory marker, inversely correlates with anti-dsDNA antibodies, which are a specific marker for SLE, and may be important in monitoring disease activity during antibody deposition in tissues.

Recent evidence has implicated the matrix metalloproteinases (MMPs) in a variety of inflammatory rheumatic diseases, including systemic lupus erythematosus (SLE).[1–4] Although one report implicated circulating concentrations of neutrophil MMP-9 in SLE, it could not specifically correlate MMP-9 with SLE disease activity index (SLEDAI) in women, the predominant sex affected by this disease.[3] Circulating concentrations of fibroblast MMP-2 were found to be unchanged. Another study reported increased MMP-3 (stromelysin) in SLE, but was also unable to correlate this finding with SLEDAI.[2]

The presence of autoimmune antibodies to double stranded DNA (anti-dsDNA) is one of the diagnostic criteria for SLE.[5 6] Although anti-dsDNA concentrations fluctuate in SLE, the correlation with disease activity is still controversial.[6–8] This issue has been complicated by differences in clinical assessment indices[8] and analytically with anti-dsDNA measurement.[6 8 9] Antibodies to single stranded DNA (anti-ssDNA) also occur in SLE (with and without anti-dsDNA), but their presence appears to be clinically unimportant.[6] Methodologies such as enzyme immunoassays (EIAs) that lack the ability to discriminate between anti-ssDNA and anti-dsDNA antibodies have contributed towards this problem.[6 9] Despite the use of international reference material, this problem has continued to date.[9]

> "Although anti-double stranded DNA concentrations fluctuate in systemic lupus erythematosus, the correlation with disease activity is still controversial"

In our study, we compared serological MMP-2 and MMP-9 concentrations in three groups of patient samples containing: (1) low anti-ssDNA/low anti-dsDNA antibodies; (2) high anti-ssDNA/low anti-dsDNA antibodies; and (3) high anti-ssDNA/high anti-dsDNA antibodies. Anti-ssDNA and anti-dsDNA antibodies were determined using EIAs with high

Abbreviations: dsDNA, double stranded DNA; EIA, enzyme immunoassay; ESR, erythrocyte sedimentation rate; IFA, indirect immunofluorescent antibody test; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase associated lipocalin; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; ssDNA, single stranded DNA; TIMP, tissue inhibitor of metalloproteinases

Table 1 Anti-ssDNA and anti-dsDNA antibody concentrations in the different patient groups

Group number	Age (years)	Anti-ssDNA1	Anti-dsDNA2
(M/F ratio)	Mean (SD; range)	Mean (SD; range)	Mean (SD; range)
1 (0/12)	44 (14; 20–65)	12 (6; 2–19)	3 (5; 0–17)
2 (2/10)	47 (13; 32–75)	245 (97; 142–479)	15 (6; 5–22)
3 (1/11)	52 (14; 37–87)	383 (86; 270–501)	297 (136; 152–524)

The antibodies were measured in units of IU/ml.
ds, double stranded; ss, single stranded.

analytical specificity. MMPs were evaluated by substrate gel electrophoresis (gelatin zymography), a technique that simultaneously resolves different molecular weight forms, discriminates between active and latent proforms, eliminates endogenous inhibitors (tissue inhibitors of metalloproteinases; TIMPs), and activates latent enzymes.[10 11] Our results indicate that the concentration of neutrophil MMP-9, an inflammatory marker composed of three molecular weight forms at 92 kDa (monomer), 130 kDa (heterodimer), and 225 kDa (homodimer),[12 13] was inversely proportional to the concentration of anti-dsDNA antibodies. In contrast, circulating fibroblast MMP-2, a constitutively present MMP,[14] was unchanged irrespective of antibody concentration. The relevance of these findings with respect to SLE inflammatory disease exacerbation is discussed.

MATERIALS AND METHODS

Patient samples

Samples (n = 36), submitted for anti-dsDNA antibody analysis to the rheumatology clinic, were collected in serum separator Vacutainer tubes (Becton-Dickinson, Franklin Lakes, New Jersey, USA). After clotting (30 minutes at 25°C), the samples were centrifuged (1600 ×*g* for 15 minutes). Serum was removed and stored at −30°C. To measure the erythrocyte sedimentation rate (ESR), whole blood was collected in dipotassium ethylenediaminetetracetic acid (K_2EDTA) Vacutainer tubes and tested immediately. The patient population used in our study was predominantly female (33 of 36), with most (32 of 36) having the diagnosis of SLE.

Enzyme immunoassay for ssDNA and dsDNA antibodies

The anti-ssDNA and anti-dsDNA EIAs were from Helix Diagnostics, Inc (West Sacramento, California, USA). Serum was diluted 1/100 in assay diluent and the EIAs were performed according to the manufacturers' directions. Absorbance measurements (at 450 nm) were performed on a Thermomax microplate reader (Molecular Devices, Menlo Park, California, USA). The analytical performances of the anti-ssDNA antibody and anti-dsDNA antibody EIAs were compared with indirect immunofluorescent antibody (IFA) testing using *Crithidia luciliae*, the gold standard for anti-DNA antibody testing,[8 15] on a total of 139 samples. Agreement between the anti-dsDNA and anti-ssDNA antibody EIAs and IFA was 94.1% and 94.2%, respectively.[16] The following guidelines were recommended by the manufacturers; anti-dsDNA: < 30 IU/ml, negative; 30–60 IU/ml, low positive; 60–200 IU/ml, positive; and > 200 IU/ml, strong positive; anti-ssDNA: < 20 U/ml, negative; 20–25 U/ml, borderline positive; and > 25 U/ml, positive. Specimens were grouped (12/group) based on the EIA results as follows: low anti-ssDNA/low anti-dsDNA antibodies (group 1); high anti-ssDNA/low anti-dsDNA antibodies (group 2); and high anti-ssDNA/high anti-dsDNA antibodies (group 3) (table 1).

Gelatin zymography

Gelatin zymography was performed as described previously.[10] Serum was diluted 1/20 in non-reducing sample buffer, 10 µl was loaded on to 7.5% sodium dodecyl sulfate-polyacrylamide gels containing copolymerised gelatin (1.5 mg/ml), and electrophoresis was performed at constant current (22 mA/gel for 1.5 hours). The amount of serum used (0.5 µl/lane) was within the calibration range for serum (0.25–1.0 µl) previously shown for gelatin zymograms prepared as described above by our laboratory.[10] After electrophoresis, the gels were processed identically in the same run. Zymograms were washed twice in 2.5% Triton X-100 (200 ml for 30 minutes) and incubated in 200 ml 50mM Tris/HCl, pH 7.6 containing 5mM $CaCl_2$ (18–20 hours at 37°C) in a thermostatically controlled (±1°C) water bath (Model 25, Precision Scientific Instruments, Inc, Chicago, Illinois, USA).

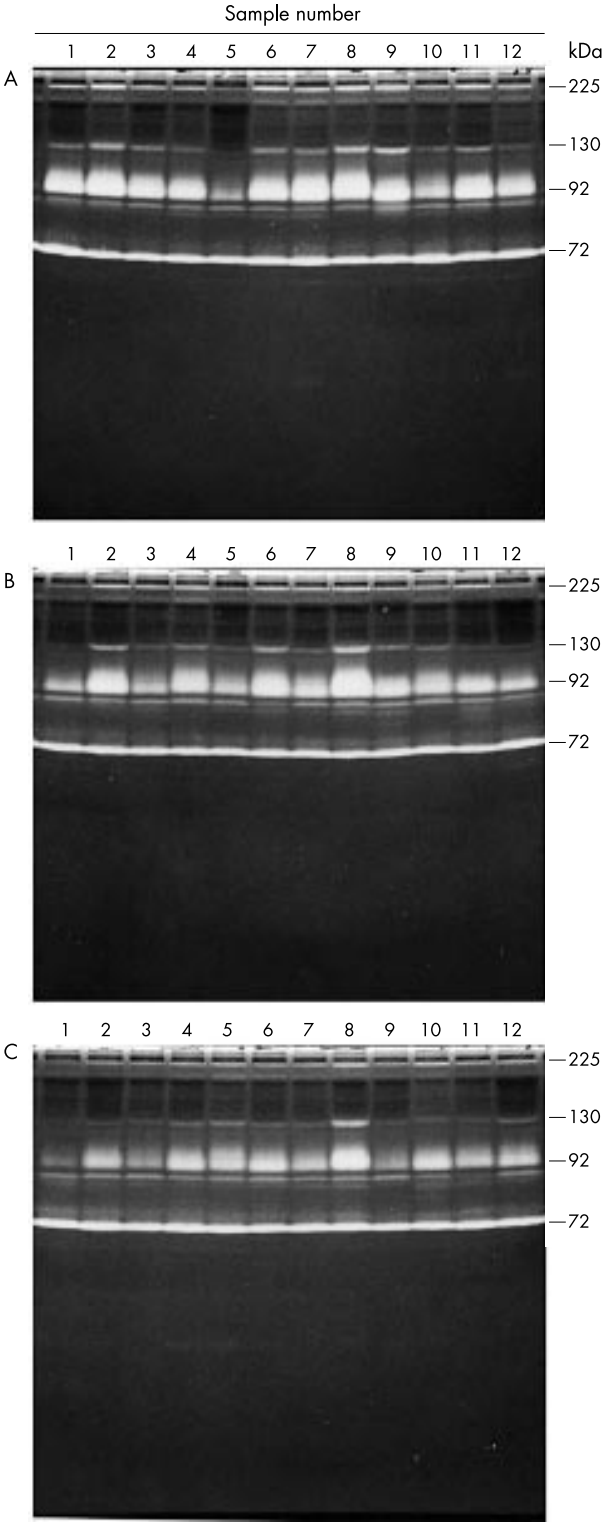

Figure 1 Gelatin zymography of matrix metalloproteinases (MMPs) in patient samples containing anti-DNA antibodies. (A) Low anti-ssDNA/low anti-dsDNA antibodies (group 1); (B) high anti-ssDNA/low anti-dsDNA antibodies (group 2); (C) high anti-ssDNA/high anti-dsDNA antibodies (group 3). Twelve samples/group were analysed. Sample number (1–12) are shown at the top. The position of the MMP molecular weight calibration markers is shown on the right.

To minimise differences in staining and destaining, zymograms were stained together with 0.2% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and destained together in

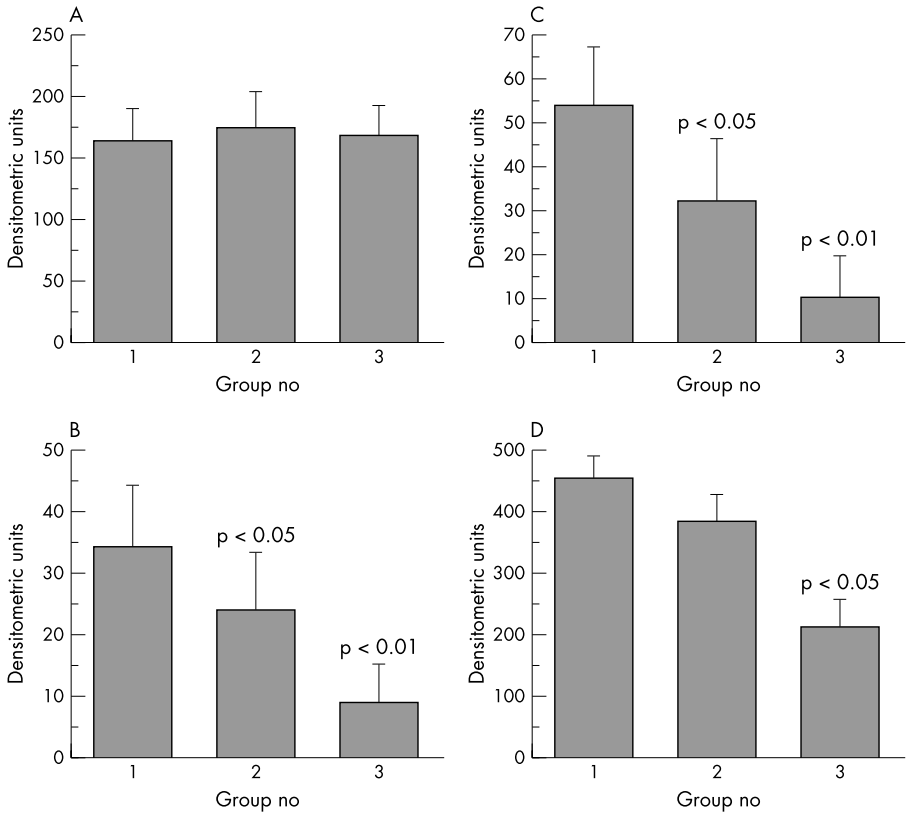

Figure 2 Densitometric analysis of matrix metalloproteinases (MMPs). (A) 72 kDa MMP-2; (B) 225 kDa MMP-9; (C) 130 kDa MMP-9; and (D) 92 kDa MMP-9. Mean arbitrary densitometric units shown. Error bars indicate SD and the p values versus group 1 are shown (p<0.05, significant).

40% methanol and 10% acetic acid. MMPs, identified as "cleared" (that is, degraded) regions against a dark background, were compared with calibration standards.[10] The zymograms were photographed wet with background lighting and dried between porous cellophane sheets with heating under vacuum. Densitometric scanning was performed on dried zymograms (Appraise, Beckman-Coulter, Brea, California, USA).

Complement and ESR determination

Serum complement C3 and C4 were measured by rate nephelometry (Model Array 360; Beckman-Coulter).[17] The ESR was determined in EDTA anticoagulated whole blood using the Westergren method (Dispette Westergren Method, Ulster Medical Products, Albequerque, New Mexico).[18]

Statistical analysis

Data are presented as mean (SD). Statistical differences between groups were evaluated using an unpaired Student's *t* test and results were considered significantly different at $p < 0.050$.

RESULTS

Gelatin zymography revealed substantial differences in the concentrations of neutrophil MMP-9 in its latent composite forms at 92 kDa (monomer), 130 kDa (heterodimer with neutrophil gelatinase associated lipocalin, NGAL), and 225 kDa (homodimer)[12 13] when compared with anti-ssDNA and anti-dsDNA antibodies (fig 1). In contrast, no observable differences were noted for fibroblast 72 kDa MMP-2, a constitutively expressed MMP in the circulation,[14] in the three patient groups. The proteolytic MMP profile obtained with group 1 specimens is similar to that obtained with normal control patients.[19] In samples that contained high amounts of both anti-ssDNA and anti-dsDNA antibodies (group 3), the 225 kDa (homodimer) and the 130 kDa (heterodimer) MMP-9 forms were found to be virtually absent, whereas the 92 kDa form (monomeric MMP-9) was substantially decreased. Samples that contained high anti-ssDNA, but negligible anti-dsDNA antibodies (group 2), had intermediate MMP-9 values. The highest concentrations of all three MMP-9 forms were present in samples with negligible anti-ssDNA or anti-dsDNA antibodies (group 1). No partially proteolysed "activated" MMP-2 or MMP-9 lower molecular weight forms were present in the patient samples. Densitometric analysis confirmed that group 3 samples contained significantly less neutrophil MMP-9 (of all three forms) when compared with group 1 samples (fig 2). Patient samples with only anti-ssDNA antibodies (group 2) also contained significantly lower concentrations of 225 kDa (homodimer) and 130 kDa (heterodimer) MMP-9. Although the concentration of 92 kDa MMP-9 was lower in group 2 samples, it was not significantly different from group 1 samples.

The samples from the three groups were also tested for other markers of inflammation; that is, complement (C3 and C4) and ESR (table 2). Results indicated a consistent, but variably significant, decrease in mean C3 and C4 concentrations with increasing anti-ssDNA and anti-dsDNA antibody values. Despite these decreases, it should be noted that the mean complement concentrations were usually within the normal ranges, except for the mean C3 concentrations in group 3 patients (high anti-ssDNA/high anti-dsDNA antibodies). In contrast, the ESR was higher than the normal reference interval in all three groups, but showed no correlation with anti-ssDNA or anti-dsDNA concentrations.

DISCUSSION

Although studies have indicated that concentrations of circulating MMPs and anti-dsDNA antibodies fluctuate in SLE,[2 4–8]

Table 2 Inflammatory markers in the three patient groups

Group number	C3 (mean (SD))	C4 (mean (SD))	ESR (mean (SD))
1	1220 (160)	200 (50)	32 (30)
2	880 (200)*	170 (90)	39 (38)
3	780 (180)†	120 (80)*	33 (22)

The normal ranges for C3, C4, and ESR are 670–1540 mg/l, 160–660 mg/l, and 0–20 mm/h, respectively.
*Significantly different *v* group 1 ($p<0.050$); †significantly different *v* group 1 ($p<0.010$).
ESR, erythrocyte sedimentation rate.

Take home messages

- Neutrophil matrix metalloproteinase 9 (MMP-9), an inflammatory marker, inversely correlates with anti-double stranded DNA (dsDNA) antibodies, which are a specific marker for systemic lupus erythematosus (SLE)
- MMP-9 could therefore be a useful marker for monitoring SLE disease activity during antibody deposition in tissues
- Fibroblast MMP-2 did not correlate with anti-dsDNA antibodies

the usefulness of these indicators for monitoring disease activity has not been studied. For this purpose, we specifically compared these two analytical variables in serum using an EIA that is highly specific for anti-ssDNA and anti-dsDNA antibodies and gelatin zymography, a useful technique that simultaneously resolves different MMP molecular weight forms, discriminates between active versus latent MMP proforms, eliminates endogenous inhibitors (TIMPs), and activates latent MMPs.[10 11] Although other studies have measured MMPs in autoimmune diseases,[1–4] these studies have relied on EIAs, which may be susceptible to variable reactivity with MMP proforms and/or MMPs complexed to inhibitors (TIMPs)[11] or other endogenous proteins (NGAL).[12 13]

> "Variably decreased concentrations of complement proteins C3 and C4 in patients with high concentrations of anti-single stranded DNA and anti-double stranded DNA antibodies probably reflect consumption"

In our study, we found that the concentrations of neutrophil MMP-9 were inversely correlated with anti-dsDNA antibodies, a diagnostic marker of SLE. In contrast, fibroblast MMP-2 was unchanged, irrespective of antibody values. Samples that contained only anti-ssDNA antibodies, which are a questionable marker for SLE,[6] also showed decreased MMP-9 concentrations, with significantly lower amounts of the higher molecular weight forms—that is, 130 kDa heterodimeric and 225 kDa homodimeric MMP-9. Variably decreased concentrations of complement proteins C3 and C4 in patients with high concentrations of anti-ssDNA and anti-dsDNA antibodies probably reflect consumption. Recent evidence has suggested that decreased anti-dsDNA antibodies result from tissue deposition during SLE flares.[8] Our evidence supports this finding because raised concentrations of serum MMP-9 probably reflect an increased inflammatory response.

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