Elevated Plasma cfDNA May be Associated with Active Lupus Nephritis and Partially Attributed to Abnormal Regulation of Neutrophil Extracellular Traps (NETs) in Patients with Systemic Lupus Erythematosus

Sigong Zhang, Xin Lu, Xiaoming Shu, Xiaolan Tian, Hanbo Yang, Wenfang Yang, Yinli Zhang and Guochun Wang

Abstract

Objective The abnormal formation and insufficient clearance of neutrophil extracellular traps (NETs) has been reported to be involved in the pathogenesis of lupus nephritis (LN). The abnormal regulation of NETs may contribute to increases in the levels of circulating cell-free DNA (cfDNA). The present study tested the hypothesis that elevated plasma cfDNA levels are related to LN.

Methods Fifty-four systemic lupus erythematosus (SLE) patients and 43 control subjects were included in this study. The cfDNA concentrations were measured using the Picogreen Kit, the low-density granulocyte (LDG) percentage in peripheral blood mononuclear cells (PBMCs) was tested using a flow cytometer and the DNase I activity was measured according to the radial enzyme-diffusion method.

Results The mean cfDNA concentration in the SLE group was 236.66±40.09 ng/mL, which was significantly higher than that observed in the healthy control group (187.96±40.55 ng/mL, p<0.0001). Meanwhile, the mean cfDNA concentration in the patients with LN was significantly higher than that observed in the patients without LN (247.27±46.79 ng/mL vs. 213.56±31.34 ng/mL, p=0.0094), and the mean cfDNA concentration in the patients with active LN was significantly higher than that observed in the patients with inactive LN (254.22±50.16 ng/mL vs. 215.93±29.10 ng/mL, p=0.0349). In the SLE group, the cfDNA concentration was to positively correlate with the quantitative 24-hour urinary protein (r=0.350, p=0.013), LDG (r=0.6361, p=0.0019) and neutrophil (r=0.5990, p<0.0001) levels and inversely correlate with the albumin level (r=-0.500, p<0.0001) and endogenous creatinine clearance rate (r=-0.354, p=0.044). Compared to that observed in the control group, the SLE group exhibited a significantly increased percentage of LDGs in PBMCs and a significantly decreased DNase I activity.

Conclusion Our data indicate that elevated plasma cfDNA concentrations may be associated with active LN and partially attributed to the abnormal regulation of NETs in SLE patients, thus suggesting that NETs constitute an intrinsic link between cfDNA and active LN.

Key words: lupus nephritis, neutrophil extracellular traps, circulating cell-free DNA, low-density granulocytes, DNase I


Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease. Profound abnormalities in adaptive immunity triggered by genetic and environmental factors are thought to play an important role in the pathogenesis of SLE. However, as the first-line cell in innate immunity, neutrophils are less frequently involved. Current findings indicate that neutrophils, particularly neutrophil extracellular traps (NETs),
are closely associated with the pathogenesis of SLE (1-4).

Upon stimulation by microbes, neutrophils form NETs to trap and kill invading microbes by means of NETosis (5, 6), and thereby various intranuclear and intracellular materials are released. These materials generally include double-stranded DNA (dsDNA), citrullinated histones, myeloperoxidase (MPO), neutrophil elastase (NE) and antibiotic peptides, which are known to be potential autoantigens. Proinflammatory factors, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-17 and IL-8, can also induce neutrophils to form NETs (7). SLE patients have an abnormal cytokine environment (8), which results in enhanced NET formation in vivo. Furthermore, new findings indicate that low-density granulocytes (LDGs) in the peripheral blood mononuclear cells (PBMCs) of SLE patients can form NETs spontaneously and more fiercely than normal autologous neutrophils (2, 9). Abnormally increased LDG may form NETs excessively and abnormally.

DNase I is responsible for degrading NETs in vitro, and the insufficient clearance of NETs is related to the pathogenesis of lupus nephritis (LN) (3, 4). If NETs cannot be degraded completely by DNase I, NETs can form complexes with other proteins and may persist for abnormally long periods, resulting in prolonged antigenic stimulation (10) and deposition on the glomerular basement membrane, thus inducing nephritis (3, 9).

The excessive formation and insufficient clearance of NETs results in an increase in the levels of residual NETs, a main source of circulating cell-free DNA (cfDNA). However, whether or not some relationship exists between an elevated level of cfDNA and the incidence of LN remains unknown. In order to address this question, we tested the plasma cfDNA concentrations, plasma DNase I activity and percentage of LDGs in PBMCs and analyzed the relationships between these and other serological parameters.

Materials and Methods

Subjects and clinical data

Fifty-four patients from the department of rheumatology (March 2012 to October 2012) treated at the China-Japan Friendship Hospital fulfilling the American College of Rheumatology (ACR) criteria were recruited into this study (11). The study was approved by the Ethics Committee of China-Japan Friendship Hospital. Forty-three age- and sex-matched healthy Chinese volunteers were selected as the control group during the same period. All patients and controls provided their written informed consent. The SLE disease activity was evaluated using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (12). The diagnosis of active lupus nephritis (ALN) was made based on the fulfillment of any two of the following criteria: (1) a level of glomerular dsDNA of >0.5 g over a two- to three-month period; (2) evidence of one or more active lesions on a renal biopsy based on the classification criteria for LN established by the International Society of Nephrology/Renal Pathology Society (13, 14). Complete remission was defined as the absence of activity in the urinary sediment and a quantitative 24-hour urinary protein level of <0.5 g within the previous three months (14).

Ethylenediaminetetraacetic acid (EDTA) anticoagulant venous blood samples were separated via density gradient centrifugation at 400xg for 30 minutes. Divided PBMCs were used to test LDGs. The plasma was divided into six parts: one part was used to measure the DNase I activity, one was used to measure the cfDNA concentration and the others were stored at -80°C. Additional clinical and laboratory data for the patients were collected from the Electronic Medical Record system.

Reagents

Histopaque-1077, Calf thymus DNA and Sybr Green were purchased from Sigma-Aldrich, the Quant-iT PicoGreen dsDNA Reagent and Kit were purchased from Invitrogen. DNase I solution was purchased from Worthington and anti-CD15-fluorescein isothiocyanate (FITC), anti-CD14-PE and the corresponding isotypes were purchased from Biologend.

Measurement of the plasma cfDNA concentrations

The Quant-iT PicoGreen dsDNA Reagent and Kit were used to measure the plasma cfDNA concentrations (15). The plasma samples were centrifuged again at 300xg for 10 minutes before being used for cfDNA quantification. Lambda DNA of known concentration was serially diluted with Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to create standard DNA samples. Ninety microliters of TE buffer was added to each well in a 96-well black microplate, in which 10 ul of plasma samples was diluted. Then, 100 ul of the PicoGreen dye diluted 1:200 in TE buffer was added to the microplate in order to make a final volume of 200 ul. Each sample was assessed in triplicate. The SoftMax Pro 5.4.1 program was used to establish the standard curve and calculate the concentrations of the plasma samples. We also conducted an additional experiment to exclude the influence of centrifugation on the cfDNA measurements, as detailed in the supplementary materials.

PBMC isolation and LDG characterization

Blood was collected from the SLE patients and healthy individuals using EDTA vacutainers. PBMCs were isolated via density gradient centrifugation over Histopaque-1077 following the manufacturer’s instructions. The mononuclear fraction was resuspended in phosphate-buffered saline.
curve. Each sample was assessed in triplicate. The gel plate
jected into the gel plate in order to calculate the standard
dilutions of DNase I from 1.1 to 0.008 U/mL were also in-
each circle of the gel plate. Two microliters of eight double
croliters of plasma samples were injected into the center of
then poured into the cover of a 48/96 microplate. Two mi-
and mixed with the equivalent mixture. The mixed gel was
concentration of substrate DNA in the mixture was 500 ng/
strate and mixed with Sybr Green in DNase buffer. The end
the agar plate (16). Calf thymus DNA was used as a sub-
tify the activity of DNase I by measuring the dark area on
Measurement of the DNase I activity

Based on the hydrolysis of substrate DNA in a DNA-agar
plate, the radial enzyme-diffusion method was used to quan-
tify the activity of DNase I by measuring the dark area on
the agar plate (16). Calf thymus DNA was used as a sub-
strate and mixed with Sybr Green in DNase buffer. The end
concentration of substrate DNA in the mixture was 500 ng/
/mL, and 2% regular agarose was melted with distilled water
and mixed with the equivalent mixture. The mixed gel was
then poured into the cover of a 48/96 microplate. Two mi-
croliters of plasma samples were injected into the center of
each circle of the gel plate. Two microliters of eight double
dilutions of DNase I from 1.1 to 0.008 U/mL were also in-
jected into the gel plate in order to calculate the standard
curve. Each sample was assessed in triplicate. The gel plate
was incubated at 37°C in an airtight black box for 12 hours.
The areas of hydrolysed DNA were acquired using gel
documentation and an image analysis system (ChampGel
5500, SAGECREATION) and quantitated with the Image-
Pro Plus 6.0 program.

Table 2. Renal Biopsy Profiles of the Patients with LN

<table>
<thead>
<tr>
<th></th>
<th>Class III</th>
<th>Class IV</th>
<th>Class V</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALN (15)</td>
<td>A 4 A/C C</td>
<td>S-A G-A</td>
<td>S-A/C G-C</td>
</tr>
<tr>
<td>NLN (2)</td>
<td>1 1</td>
<td>3 3 2</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: A: active lesions, C: chronic lesions, G: global lesions, S: segmental lesions, ALN: SLE patients with active LN, LN: lupus nephritis, NLN: SLE patient without LN, RLN: SLE patients with inactive LN, SLE: systemic lupus erythematosus, SLEDAI: systemic lupus erythematosus disease activity index. The percentages reflect the rate of positive autoantibodies. Age was compared using Student’s t-test, and the anti-dsDNA and SLEDAI values were compared using the Mann-Whitney test. #, ALN vs. RLN, p<0.05; &, ALN vs. NLN, p<0.05

Statistical analysis

The GraphPad Prism 5 software program was used to per-
form comparisons between groups and draw figures. The calculations were based on a 95% confidence interval. A p
value of <0.05 was considered to be significant. Student’s t-
test was used to assess continuous variables fulfilling Gauss
distribution, while the Mann-Whitney test was used to
evaluate continuous variables that were not normally distrib-
uted. The SPSS17.0 software package was used to carry out
the linear correlation and regression analysis.

Results

SLE cohort characteristics

The SLE group included 37 patients with LN and 17 pa-
tients without LN (NLN). Among LN patients, twenty six
were in the active stage and 11 were in remission (RLN).
The general information for the patients can be found in Ta-
ble 1. The age of the ALN patients was significantly lower
than that of the RLN patients (p=0.0306), the SLEDAI val-
ues were significantly higher in the ALN group than in the
NLN group (p=0.0172) and the titer of anti-dsDNA was
slightly higher in the ALN group than in the RLN and NLN
groups, although the difference did not reach a significant
level. Fifteen of the 26 ALN patients and two of the 11
RLN patients had available renal biopsy results. The details
can be found in Table 2.

An elevated plasma cfDNA concentration is closely related to active LN in SLE patients

In order to guarantee the accuracy and stability of the
cfDNA measurements, we compared the cfDNA concentra-
tions in the plasma samples separated using different meth-
ods. The results indicated that centrifugation scarcely influ-
enced the plasma cfDNA measurements (Supplementary ma-
terial). The mean cfDNA concentration was 236.66±40.09
ng/mL in the SLE group, which was significantly higher
than that observed in the healthy control group (187.96±
Figure 1. Comparison of the plasma cfDNA concentrations in various groups. Each symbol represents one patient or control subject, and the bars within each group indicate the mean value. ALN: patients with active LN, LN: patients with LN, NLN: patients without LN, RLN: patients with inactive LN. Class III and Class IV indicate that the patients with ALN were divided into two subgroups according to the pathological classification of LN. All samples were tested in triplicate. The statistical analysis was performed with the two-tailed Student’s t-test. *: p<0.05, **: p<0.01, ***: p<0.0001

Table 3. Correlations between the cfDNA, DNase I Activity, LDG Levels and Serological Parameters in the SLE Patients

<table>
<thead>
<tr>
<th></th>
<th>Neut</th>
<th>LDGs</th>
<th>ESR</th>
<th>Alb</th>
<th>IgG</th>
<th>ANA</th>
<th>ADNA</th>
<th>C3</th>
<th>CRP</th>
<th>C4</th>
<th>Ccr</th>
<th>UP</th>
<th>DNase I</th>
<th>SLEDAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfDNA r</td>
<td>0.5990***</td>
<td>0.364**</td>
<td>-0.500***</td>
<td>-0.26</td>
<td>-0.134</td>
<td>0.369</td>
<td>0.042</td>
<td>0.291*</td>
<td>0.171</td>
<td>-0.354*</td>
<td>0.350*</td>
<td>0.083</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0019</td>
<td>0.007</td>
<td>-0.0001</td>
<td>0.061</td>
<td>0.333</td>
<td>0.053</td>
<td>0.763</td>
<td>0.032</td>
<td>0.221</td>
<td>0.044</td>
<td>0.013</td>
<td>0.549</td>
<td>0.885</td>
</tr>
<tr>
<td>n</td>
<td>54</td>
<td>21</td>
<td>54</td>
<td>54</td>
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<td>54</td>
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<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ADNA: Anti-dsDNA, Alb: Albumin, ANA: antinuclear antibody, Ccr: endogenous creatinine clearance rate, cfDNA: circulating cell-free DNA, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, LDGs: low-density granulocytes, Neut: neutrophils, SLEDAI: systemic lupus erythematosus disease activity index, UP: quantitative 24-hour urinary protein. The Pearson correlation coefficient (two-tailed) was used for data fulfilling the Gauss distribution. #: The Spearman correlation coefficient was used for the correlation analysis. *: p<0.05. **: p<0.01. ***: p<0.0001

40.55 ng/mL, p<0.0001, Fig. 1A). Thirty-seven SLE patients were complicated with LN, and the cfDNA concentrations were significantly higher in the patients with LN than in those without LN (247.27±46.79 ng/mL vs. 213.56±31.34 ng/mL, p=0.0094, Fig. 1B). Among the 37 LN patients, 26 were complicated with active LN, and the cfDNA concentrations were significantly higher in the patients with active LN than in those with inactive LN (254.22±50.16 ng/mL vs. 215.93±29.10 ng/mL, p=0.0349, Fig. 1C). Among the ALN patients, six had LN class III disease and eight had LN class IV disease. We further compared these two subgroups and found the cfDNA concentrations in the class IV patients to be slightly higher than those observed in the class III patients, although the difference did not reach a significant level (255.20±42.13 ng/mL vs. 225.20±33.49 ng/mL, p=0.1774, Fig. 1D). In the SLE group, the cfDNA concentrations positively correlated with the quantitative 24-hour urinary protein (r=0.350, p=0.013), LDG (r=0.6361, p=0.0019) and neutrophil (r=0.5990, p<0.0001) levels and inversely correlated with the albumin level (r=-0.500, p<0.0001) and endogenous creatinine clearance rate (Ccr) (r=-0.354, p=0.044, Table 3). The quantitative 24-hour urinary protein and albumin levels were found to be closely related to the activity of LN, while the Ccr value was found to be related to the severity of LN. Taken together, these data suggest that an elevated plasma cfDNA concentration is closely related to the presence of active LN in SLE patients.
SLE patients with a higher percentage of LDGs tend to have a higher plasma cfDNA concentration

Because LDGs can form NETs spontaneously and more fiercely than normal autologous neutrophils, we assessed the percentage of LDGs in PBMCs among 21 SLE patients and 19 healthy controls in order to analyze the cause of the elevated cfDNA concentrations. The segregated LDG population was directly adjacent to the monocyte pool, according to the forward and scatter characteristics (Fig. 2A). The monocytes and LDGs were clearly distinguished based on the expression of the neutrophil marker CD15 and monocyte marker CD14. The monocytes were CD14+/CD15− or CD14+/CD15+, whereas the LDGs had a CD14−/CD15− or CD14−/CD15+ profile (Fig. 2B) (2). Compared with that observed in the healthy controls (1.275%±0.734% of total

Figure 2. Increased percentages of LDGs in the PBMCs of the SLE patients. The forward and side scatter profile was used to determine the relative levels of LDGs in PBMCs obtained from healthy controls and SLE patients, as confirmed according to the CD14/CD15 coexpression. Dot plots of the Forward (X-axis) and Scatter (Y-axis) plots show the characteristics of PBMCs obtained from a normal control (A1) and SLE patient (A2). Dot plots of CD15 staining (X-axis) versus CD14 staining (Y-axis) demonstrate the characteristics of PBMCs obtained from the same normal control (B1) and SLE patient (B2). In C, the dot diagram shows the results of the comparison of LDGs between the controls and SLE patients analyzed using the two-tailed Mann-Whitney test. The long line represents the cutoff value (mean±2SD) of LDGs in the control group. The SLE patients were divided into two subgroups according to the cutoff value (normal LDG group and increased LDG group). The DNase I activity, cfDNA and SLEDAI values were compared between these two subgroups (D, E and F). The statistical analysis was performed with the two-tailed Mann-Whitney test, *: p<0.05, **: p<0.001
The SLE patients exhibited a significantly higher percentage of LDGs (8.291%±12.86% of total PBMCs, p=0.0036, Fig. 2C).

We applied an LDG percentage of 2.742% (mean±2SD) in the control group as the cutoff value for identifying SLE patients with a normal LDG percentage and patients with an elevated LDG percentage (Fig. 2C). These two subgroups were compared with respect to the cfDNA concentrations, DNase I activity and SLEDAI values. Twelve patients belonged to the elevated LDG percentage group. As indicated in Fig. 2D, the cfDNA concentrations in the elevated LDG group were significantly higher than those observed in the normal LDG group (256.37±34.75 ng/mL vs. 220.03±25.09 ng/mL, p=0.0157). In addition, the DNase I activity was lower and the SLEDAI values were higher in the elevated LDG group, although the difference did not reach a significant level (Fig. 2E, F).

**LDGs and neutrophils may contribute to elevation of plasma cfDNA in SLE by forming NETs**

We found that the SLE patients with a high percentage of LDGs in PBMCs exhibited elevated levels of cfDNA. Therefore, we analyzed paired data for 21 patients in the SLE group and found that the cfDNA concentration positively correlated with the LDG percentage (r=0.6361, p=0.0019, Fig. 3A). We also analyzed paired data for 19 control subjects in the control group and found no significant correlations (r=0.1811, p=0.4581, Fig. 3C). This finding is likely due to the fact that the percentage of LDGs in PBMCs among healthy subjects is very low; therefore, the contribution of LDGs to the plasma cfDNA concentration is negligible in healthy subjects. Moreover, a significant correlation between the cfDNA concentration and neutrophil level was also observed in the SLE patients (r=0.5990, p<0.0001, Fig. 3B). These results suggest that both LDGs and normal neutrophils may contribute to elevation of the cfDNA concentrations in SLE patients by forming NETs. To further confirm this notion, a multiple regression analysis was performed. Regression model 1 identified the neutrophil level to be the most influencing factor. Meanwhile, regression model two, in which the levels of neutrophils and Ccr were included, indicated that both neutrophils and Ccr were influencing factors. These factors can explain 41.2% of the cfDNA elevation observed in SLE patients, although the neutrophil levels contributed more significantly than the Ccr values in the model (Table 4). This result further supports our proposition that NETs excessively released by normal neutrophils and LDGs are main source of cfDNA in SLE patients.

**SLE patients exhibit an impaired DNase I activity**

DNase I is responsible for degrading NETs; therefore, its activity was measured to determine whether it contributes to elevation of the cfDNA concentration. The results indicated that the DNase I activity in the SLE group was 0.25±0.12 U/mL, which was significantly lower than that observed in PBMCs, the SLE patients exhibited a significantly higher percentage of LDGs (8.291%±12.86% of total PBMCs, p=0.0036, Fig. 2C).

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**Table 4. Stepwise Regression of Factors Influencing the cfDNA Levels in the SLE Patients**

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized coefficients</th>
<th>Standardized coefficients</th>
<th>t</th>
<th>sig</th>
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<tbody>
<tr>
<td>1 (Constant)</td>
<td>193.517</td>
<td>17.483</td>
<td>11.069</td>
<td>0.000</td>
</tr>
<tr>
<td>neutrophil</td>
<td>9.826</td>
<td>3.022</td>
<td>0.524</td>
<td>3.251</td>
</tr>
<tr>
<td>2 (Constant)</td>
<td>243.524</td>
<td>25.550</td>
<td>0.682</td>
<td>2.513</td>
</tr>
<tr>
<td>neutrophil</td>
<td>10.499</td>
<td>2.783</td>
<td>0.559</td>
<td>3.772</td>
</tr>
<tr>
<td>Ccr</td>
<td>-6.82</td>
<td>2.71</td>
<td>-0.373</td>
<td>-2.513</td>
</tr>
</tbody>
</table>

In the linear correlation analysis, the albumin, neutrophil, Ccr and quantitative 24-hour urinary protein levels were found to significantly correlate with the cfDNA concentration and were thus included in stepwise multiple regression models in order to further analyze the factors influencing the cfDNA concentration. LDGs were not included due to the insufficient number of cases. Ccr: endogenous creatinine clearance rate, t: t test value, sig: significance.
We also analyzed the cause of the elevated cfDNA concentration is closely related to the presence of active LN. These results suggest that an elevated plasma cfDNA concentration, Ccr and quantitative 24-hour urinary protein levels.

NETs are composed primarily of a backbone of dsDNA and nuclear histones and decorated with various cytoplasmic proteins and antimicrobial peptides contained in neutrophil granules (5, 6).

Lupus neutrophils exhibit an enhanced capability to form NETs in vitro compared to that of healthy control neutrophils (21). Furthermore, LDGs can form NETs spontaneously and more fiercely than normal autologous neutrophils in lupus patients (9). In the present study, abnormally increased LDGs were found in the majority of SLE patients. The LDG and normal neutrophil levels significantly correlate with the cfDNA concentration, which indicates that the overproduction of NETs by normal neutrophils and LDGs is a main cause of cfDNA elevation. The multiple linear regression analysis indicated that neutrophils are the most influential factor with respect to elevated cfDNA levels, which further supports our notion.

As a stable source of autoantigens, the timely removal of NETs is crucial for internal environment homeostasis in order to avoid presentation of self-antigens. The clearance of NETs largely depends on DNase I (3, 4). In the present study, an impaired DNase I activity was found in the SLE patients. However, no significant correlation between DNase I and cfDNA was found. The possible reason for this is that other factors also influence the cfDNA level and that the DNase I activity measured in vitro may not accurately represent the capability of DNase I to degrade NETs in vivo. It has been established that anti-NET antibodies can prevent DNase I from degrading NETs (3, 4). Although the DNase I activity is normal in vitro, it may function abnormally in vivo, as anti-NET antibodies are able to prevent DNase I from binding and degrading NETs, which is one possible explanation for the lack of detection of a significant correlation between the cfDNA and DNase I values in our study.

The insufficient clearance of NETs has been reported to be involved in the pathogenesis of LN. If NETs cannot be degraded normally, they may efficiently activate pDCs to release IFN-α in a TLR9-dependent fashion and activate au-

**Discussion**

In current research, SLE patients, especially those with active LN, have been reported to have significantly elevated cfDNA levels. Further analyses indicate that the cfDNA concentration exhibits a significant correlation with the albumin, Ccr and quantitative 24-hour urinary protein levels. These results suggest that an elevated plasma cfDNA concentration is closely related to the presence of active LN. We also analyzed the cause of the elevated cfDNA concentrations and found significantly increased LDG percentages and decreased DNase I activity levels in the SLE patients. We also found that the cfDNA concentration positively correlated with the LDG and neutrophil levels. These results suggest that the excessive release of NETs by LDGs and neutrophils and incomplete clearance by DNase I are primary causes of elevated cfDNA levels in SLE patients.

The level of cfDNA depends on the dynamic balance of production and clearance (17, 18). The accumulation of apoptotic debris and necrotic cell remnants used to be considered a main source of cfDNA (19, 20). Recently, however, there has been a gradual change in that several researchers have proposed that living cells actively release DNA (18). The findings of NETosis further support this standpoint. During NETosis, neutrophils actively release de-polymerized nuclei to form netlike structures (NETs) (5, 6).

**Figure 4.** Comparison of the plasma DNase I activity levels in various groups. A indicates the results of the comparison of the DNase I activity levels between the control and SLE groups. B shows the results of the comparison of the DNase I activity levels between the patients with and without LN. Each symbol represents one patient or control, and the bar within each group indicates the mean value. All samples were tested in triplicate. The statistical analysis was performed with the two-tailed Student’s t-test. ***: p<0.0001.
toreactive B cells to synthesize autoantibodies (1). In the present study, we also observed higher levels of anti-dsDNA antibodies in the ALN group. Anti-NET antibodies can bind to NETs to form immune complexes, which may be deposited on the glomerular basement membrane, thus inducing nephritis (1). Indeed, NET infiltration has been identified in the glomeruli in SLE patients. Furthermore, SLE patients with type IV LN exhibit a higher percentage of NETs infiltrating the glomeruli than those with type III LN (3, 9), consistent with our findings that the cfDNA concentrations were slightly higher in the patients with class IV LN than in those with class III LN. These findings should help to explain why an elevated plasma cfDNA level is associated with the presence of active LN.

In addition to their enhanced capacity to form NETs, lupus LDGs exhibit proinflammatory and pathogenic features (2). LDGs are able to secrete increased amounts of type I IFNs, TNF-α, and IFN-γ, thus inducing vascular endothelial cell damage in SLE patients (2, 9). Furthermore, the gene array profiles of LDGs are different from those of autologous normal-density neutrophils (9). Therefore, LDGs differ from autologous normal-density neutrophils, although they express neutrophil surface markers. In the present study, significantly increased LDG percentages were found in the SLE patients. Whether LDGs directly participate in the pathogenesis of LN is a question that deserves further investigation.

Two limitations of our study should be mentioned. First, testing for LDGs was not performed in all participants; therefore, the LDG level was not included in the multivariate regression analysis due to the insufficient number of cases. A second limitation is that no anterior-posterior comparison of the cfDNA, LDG and DNase I levels was conducted. As a result, the influence of immunosuppressive therapy on these parameters could not be identified.

In conclusion, our results indicate that the elevated plasma cfDNA concentrations may be associated with active LN and the abnormal regulation of NETs in patients with SLE may be a primary cause of the elevated plasma cfDNA concentration. Although the plasma cfDNA concentration was not identified to be a good biomarker for active LN, our findings indicate a novel link between active LN and NET-related markers, suggesting that biomarkers more specific to NETs may be used as clinical markers for active LN.

The authors state that they have no Conflict of Interest (COI).

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