Serum BLC/CXCL13 Concentrations and Renal Expression of CXCL13/CXCR5 in Patients with Systemic Lupus Erythematosus and Lupus Nephritis

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ABSTRACT. Objective. Systemic lupus erythematosus (SLE) is a prototype of systemic autoimmune disease in which cytokines such as B lymphocyte chemoattractant (BLC, or CXC motif ligand 13, CXCL13) may play important roles in pathogenesis. We investigated the implications of CXCL13 in SLE and lupus nephritis.

Methods. Serum samples from 425 patients with SLE and 106 healthy control individuals were analyzed for the concentration of CXCL13 by ELISA. Tissue expression of CXCL13 and its corresponding receptor CXCR5 were observed in lupus kidney. The CXCR5-bearing B cells in SLE patients were analyzed by flow cytometry.

Results. Serum levels of CXCL13 were higher in SLE patients compared to controls. SLE patients with lupus nephritis or positive anti-dsDNA antibodies had significantly higher serum CXCL13 levels. The peripheral venous blood B cells that bear CXCR5 were more abundant in SLE patients as detected by flow cytometry. CXCR5 and CXCL13 were highly expressed in the renal cortex from patients with lupus nephritis.

Conclusions. Our results suggest that BLC/CXCL13 as well as its corresponding receptor, CXCR5, may play important roles in the pathogenesis of SLE and in lupus nephritis. (First Release Dec 1 2009; J Rheumatol 2010;37:45–52; doi:10.3899/jrheum.090450)

Key Indexing Terms: B LYMPHOCYTE CHEMOATTRACTANT ANTI-dsDNA ANTIBODIES SYSTEMIC LUPUS ERYTHEMATOSUS CXC CHEMOKINE LUPUS NEPHRITIS

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by production of a variety of phlogistic autoantibodies and massive immune complex deposition in the target tissues such as renal glomeruli. The massive autoantibody production may result from the breakdown of immune tolerance. A marked mononuclear cell (MNC) infiltration in the kidneys, lungs, or vessel walls has been demonstrated in animal lupus models as well as in human SLE. Accumulating evidence has indicated that chemokines or chemokine receptors produced by these cells may play a pivotal role in the autoantibody production and target tissue damage. Some authors have shown that in New Zealand Black x New Zealand White F1 mice (BWF1) there is an aberrant B cell trafficking to the target organs due to ectopic expression of B cell chemokines.

B1 cells are a specialized subset of B cells that are distinct from the majority of recirculating conventional B2 cells. These cells have been found to be increased in patients with primary Sjögren’s syndrome and rheumatoid arthritis. B lymphocyte chemoattractant or CXC motif ligand 13 (BLC/CXCL13), a major stimulant of B1 cells, has been found ectopically and highly expressed in the thymus and kidney of aged BWF1 mice. This ectopic expression was attributed to mature myeloid dendritic cell (mDC) infiltration in the target organs. mDC were also shown to be increased in the peripheral blood of aged BWF1 mice and differentiated into BLC/CXCL13-producing DC in the presence of granulocyte-macrophage colony stimulating factor, tumor necrosis factor-α (TNF-α), or interleukin 1β (IL-1β). B1 cells expressing high levels of CXCR5...
have been described migrating towards cells that produce CXCL13 much better than B2 cells and thus preferentially recruiting to the target organs, including the kidney, in BWF1 mice, resulting in lupus nephritis. With the help of IL-2, TNF-α, IL-1β, and other cytokines, these B1 cells can also activate autologous CD4+ T cells and stimulate themselves in an autocrine manner to produce large amounts of destructive autoantibodies. It is unknown whether these mechanisms of aberrant B cell trafficking as shown in experimental animals are also attributable to human lupus. We demonstrate that these mechanisms may be present in human lupus, and that the level of serum CXCL13 might fluctuate in parallel to the disease activity of SLE.

MATERIALS AND METHODS

Sample collection. Four hundred twenty-five patients (399 female, 26 male, mean age 38.2 ± 12.5 yrs, range 14–76 yrs) fulfilling the updated American College of Rheumatology criteria for diagnosis of SLE were recruited from the Division of Allergy, Immunology and Rheumatology at Taipei Veterans General Hospital (VGH). One hundred sixty healthy individuals (97 female, 9 male, mean age 42.1 ± 12.9 yrs, range 23–61 yrs) including volunteer blood donors and hospital staff served as the controls. Informed consent was obtained from each participant under protocols approved by the Institutional Review Board of the Taipei VGH.

Clinical and laboratory profiles including anti-dsDNA antibody titer, complement components C3 and C4, and complete blood count, urinalysis and daily urine protein excretion were recorded if available. Lupus disease activity in all patients was evaluated serially by the SLE Disease Activity Index (SLEDAI) score system. Autopsy renal tissue specimens from 2 patients who died from lupus nephritis were also obtained with the permission of patients’ family members.

Enzyme-linked immunosorbent assay (ELISA) for CXCL13. The concentrations of CXCL13 in sera and urine from patients and controls were determined by sandwich ELISA. Samples with 1:5 dilution were added to a 96-well microtiter plate and incubated 90 min at room temperature according to the manufacturer’s instructions (RayBiotech, Norcross, GA, USA; detection limit, 1.5 pg/ml). Optical absorbance was measured at 450 nm using a SpectraMax Plus device (Molecular Devices, Sunnyvale, CA, USA). The standard curve was generated with a log-log graph using SoftMax Pro software.

Immunohistochemical study. Immunohistochemical staining was as described with modifications. Renal tissues obtained from autopsy with 200 µl fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD19 monoclonal antibody (R&D Systems) and 200 µl of phycoerythrin (PE)-conjugated mouse anti-human CXCR5 monoclonal antibody (R&D Systems) at room temperature for 45 min, incubated with lysing buffer (to disrupt erythrocytes) for 30 min, and washed with PBS twice before fixation with paraformaldehyde and subjected to flow cytometric analysis (FACScan, Becton-Dickinson, Los Amos, CA, USA). PE-conjugated mouse IgG1 (R&D Systems) was used as the isotype control for detection of CXCR5 on B cells. Cells were gated for mononuclear cells under forward and side scatter and the acquisition dot plots were sketched automatically. A similar procedure was used to detect the CD3+ T cells bearing CXCR5 using PE-conjugated mouse anti-CXCR5 and FITC-conjugated mouse anti-CD3 (R&D Systems) in place of the monoclonal antibodies used in the B cell experiments. The percentage of cells bearing CXCR5 was calculated as:

% CXCR5-bearing cells = % CXCR5 (+) cells — % isotype control.

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed by Student’s t-test, chi-square test, Pearson correlation, or Spearman correlation as required using SPSS 15 for Windows software. A value of p < 0.05 was considered significant.

RESULTS

Demographic features of patients with SLE. The 425 SLE patients were a mean age of 30.3 ± 11.2 years at disease onset, and had disease duration of 7.8 ± 6.6 years. The mean disease activity evaluated by SLEDAI was 5.6 ± 4.1. Their predominant clinical manifestation was lupus nephritis, with a prevalence of up to 44.2% (188/425). Most patients with lupus nephritis had received induction therapy previously (methylprednisolone and/or cyclophosphamide pulse therapy) and were under maintenance therapy with immunosuppressive agents (azathioprine, mycophenolate mofetil, or cyclosporin A). Daily urine protein excretion varied with their different disease stages and responses to therapies, and some patients (42/188, 22.34%) with lupus nephritis were in the remission status with minimal proteinuria. Neuro-psychiatric or other manifestations were much less frequent in our patients.

Serum CXCL13 concentrations. As shown in Table 1, compared with the healthy controls, patients with SLE had significantly higher serum concentrations of CXCL13 (168.49 ± 14.49 pg/ml vs 107.40 ± 8.80 pg/ml; p < 0.001, 2-sample t-test). In SLE patients, serum CXCL13 concentration was correlated with SLEDAI score (r = 0.48, p < 0.001, Spearman correlation). Correlation between serum CXCL13 concentration and serum level of complement C3 or C4 was not significant (Pearson correlation, data not shown). On the other hand, there was no significant gender difference of serum CXCL13 levels between controls and SLE patients (data not shown). We also tested urinary CXCL13 levels in 26 lupus nephritis patients and 27 controls, but failed to detect this chemokine in all the 53 samples.

CXCL13 level in SLE patients with and without lupus nephritis. As shown in Figure 1, patients with lupus nephritis (188/425) had significantly higher serum concentrations of CXCL13 than those without lupus nephritis (239.28 ± 28.69 pg/ml vs 112.34 ± 11.38 pg/ml; p < 0.001, 2-sample

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Among patients with lupus nephritis, 6/188 (3.19%) were in end-stage renal disease (ESRD) receiving maintenance hemodialysis therapy and another 6 patients were in pre-ESRD status (chronic kidney disease, stage V). Serum CXCL13 concentrations in these 12 patients varied from 40 to 2175 pg/ml and 50% of them had anti-dsDNA antibodies. Serum CXCL13 concentrations in SLE patients with and without anti-dsDNA antibodies. Anti-dsDNA antibodies were measured from all 425 SLE patients and controls during blood sampling. Among them, 218 (51.29%) patients had a titer ≥ 50 IU/ml and 207 (48.71%) patients had a titer < 50 IU/ml, which was regarded as negative. In contrast, anti-dsDNA antibodies were not detected in any of the controls. As shown in Figure 2, serum concentrations of CXCL13 were significantly higher in SLE patients who were anti-dsDNA antibody-positive than in patients who did not present with anti-dsDNA antibodies (209.75 ± 24.27 pg/ml vs 125.05 ± 14.72 pg/ml; p = 0.005, 2-sample t-test). All the antibody titers from patients with anti-dsDNA antibodies were analyzed for correlation to the CXCL13 concentration in serum. There was no significantly positive or negative correlation of CXCL13 concentrations with anti-dsDNA antibody titers (data not shown). The reason for this absence of correlation was not clear. However, it may indicate that there are additional confounding factors that contribute to the production of anti-dsDNA antibodies other than B cells chemoattracted by CXCL13.

SLE patients with very high serum CXCL13 concentrations. The patients (33/425, 7.76%) with a CXCL13 titer ≥ 500 pg/ml were defined as a group with very high CXCL13. They were all female, of mean age 34.4 ± 11.7 years, age at disease onset of 27.5 ± 11.0 years, and disease duration of 6.4 ± 5.7 years. The mean SLEDAI score was 8.91 ± 4.59, higher than for those with concentration < 500 pg/ml (5.38 ± 3.92; p < 0.05, Mann-Whitney U test; Figure 3). Among them, 23 patients (23/33, 69.7%) also had anti-dsDNA antibodies in serum. There were also no significant correlations between serum CXCL13 level, disease duration, or anti-dsDNA antibody titers in this group with very high-titer CXCL13. These patients had various clinical manifestations, among which lupus nephritis was the most frequent; other presentations included 4 with complicated antiphospholipid syndrome, 5 with thrombocytopenia (3 of them had undergone splenectomy), 5 with neuropsychiatric manifested.

**Table 1.** Demographic features and serum CXCL13 levels of SLE patients and controls.

<table>
<thead>
<tr>
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<th>SLE, N=425</th>
<th>Control, N=106</th>
<th>p</th>
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<tr>
<td>Age, yrs, mean ± SD</td>
<td>38.2±12.5</td>
<td>42.13±12.9</td>
<td>0.058</td>
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<tr>
<td>Disease onset, yrs of age</td>
<td>30.3±11.2</td>
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<tr>
<td>Disease duration, yrs</td>
<td>7.8±6.6</td>
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<tr>
<td>Female/male</td>
<td>15.3 (399/26)</td>
<td>10.8 (97/9)</td>
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<td>Lupus nephritis</td>
<td>44.23% (188/425)</td>
<td>0% (0/106)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Anti-dsDNA (+) antibody</td>
<td>51.29% (218/425)</td>
<td>0% (0/106)</td>
<td></td>
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<tr>
<td>Serum CXCL13, pg/ml, mean ± SEM</td>
<td>168.49 ± 14.49</td>
<td>107.4 ± 8.80</td>
<td>&lt; 0.001</td>
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<tr>
<td>Minimum</td>
<td>Minimum 5</td>
<td>Minimum 16</td>
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<tr>
<td>Maximum</td>
<td>Maximum 3605</td>
<td>Maximum 438</td>
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**Figure 1.** Comparison of serum CXCL13 concentrations (pg/ml) in SLE patients with (n = 188) or without lupus nephritis (n = 237). p < 0.001, 2-sample t-test.

**Figure 2.** Comparison of serum CXCL13 concentrations (pg/ml) in SLE patients with presence of anti-dsDNA antibodies (≥ 50 IU/ml, n = 218) and absence of anti-dsDNA antibodies (< 50 IU/ml, n = 207). p = 0.005, 2-sample t-test.
In-situ localization of CXCL13 in renal tissues from patients with active lupus proliferative glomerulonephritis. Two patients who died from complicated proliferative lupus nephritis underwent autopsy (with informed consent from their family). Compared to subjects with normal renal tissue, the lupus renal glomeruli and tubules revealed significantly higher signal of CXCL13 and CXCR5 on immunohistochemical stains (Figure 4).

Flow cytometric measurement of CXCR5-bearing CD19+ B cells in SLE patients. Peripheral venous blood samples from several patients with lupus nephritis and several controls (with informed consent) were double-stained with FITC-conjugated monoclonal anti-CD19 antibody and PE-conjugated anti-CXCR5 polyclonal antibody. As shown in Figure 5 and Table 3, the values of CXCR5-positive B cells were higher in the lupus nephritis patients than in controls. Although the difference was not statistically obvious, there was a tendency that patients with recent-onset lupus nephritis who were not treated for a lengthy period exhibited a higher percentage of CXCR5+ CD19 B cells. In similar experiments, CXCR5-bearing CD3+ T cells were also found in higher percentages in the lupus nephritis patients (Table 3).

DISCUSSION
Chemokines and chemokine receptors have been found to be implicated in the pathogenesis of experimental models of
systemic autoimmune diseases such as SLE\textsuperscript{4-7}. We hypothesized that BLC/CXCL13 might contribute to the pathogenesis of human SLE, especially in the subset with lupus nephritis. We investigated the expression of BLC/CXCL13 in lupus patients to determine if its serum concentration correlated with the clinical manifestations or with other established serum markers for lupus. Previous investigations demonstrated a high and aberrant expression of BLC/CXCL13 by CD11b+CD11c+ dendritic cells (DC) in the thymus and kidneys of lupus-prone mice associated with abnormal aggregation of CXCR5-bearing B1 cells. It is believed that aggregation of CXCR5-bearing B1 cells results from the chemoattraction by BLC/CXCL13. We observed significantly increased serum levels of CXCL13 in patients with SLE, compared to healthy controls. In addition, when we compared these results in terms of the presence or absence of lupus nephritis and anti-dsDNA antibodies, the average CXCL13 level was significantly higher in patients with lupus nephritis or with the presence of autoantibodies (Figure 1 and 2). These results indicate that CXCL13 may contribute somewhat to the production of anti-dsDNA antibodies and pathological development of lupus nephritis.

Animal experiments showed that B1 cells are important contributors to the production of various autoantibodies in autoimmune diseases\textsuperscript{14-19} because these cells that bear CXCR5 can be chemoattracted by the CXCL13 to the target-tissue milieu. We found that patients with higher titers of anti-dsDNA antibodies were not necessarily exhibiting high CXCL13 concentration, although an average elevation of CXCL13 was definitely demonstrated in patients with the anti-dsDNA antibodies. This may suggest that in addition to B1 cells bearing CXCR5 that are chemoattracted by CXCL13, other contributing factors can influence anti-dsDNA antibody production. Alternatively, anti-dsDNA antibodies are probably not the only pathogenic autoantibodies causing renal damage that are derived from CXCR5-CXCL13 interaction and activation. Further, the finding that there is a tendency of increased CXCR5-bearing B cells in the early stage of lupus nephritis has suggested that additional factors other than B1 cell production of the pathogenic autoantibodies may be present in later stages of lupus disease. This requires further in-depth investigation.

Another interesting finding in our investigation was an indirect demonstration by immunohistochemistry of CXCL13 and CXCR5 expression in the renal tissue of patients with devastating lupus nephritis (Figure 4). In addition, we found that in those patients with extremely high levels of CXCL13 ($\geq 500$ pg/ml), there was high prevalence of lupus nephritis (72.7%). One possibility is that interaction between CXCL13, a free ligand in serum, and CXCR5, a surface receptor of B cells, is important for the development of lupus renal inflammation. Although we did not directly observe B1 cells in the inflamed renal tissue, these results might indicate that chemoattraction of B cells with subsequent excretion of large amounts of autoantibodies could happen \textit{in situ}, resulting in extensive target-tissue damage.

\textbf{Figure 4.} Immunohistochemical staining of normal and lupus kidney tissues. A. Normal kidney stained with PBS; B. Normal kidney stained with anti-CXCL13; C. Normal kidney stained with anti-CXCR5. D. Kidney from a patient with severe lupus nephritis stained with PBS; E. Kidney from the same patient stained with anti-CXCL13; F. Kidney from the same patient stained with anti-CXCR5. Results show increased expression of CXCL13 and CXCR5 (brown color) in the glomeruli as well as tubules. Original magnification 200x; counterstained with hematoxylin.
Figure 5. Typical flow cytometric analysis of CD19 B cells that bear CXCR5 in peripheral venous blood of patients with active lupus nephritis and in healthy individuals. Whole blood was stained with fluorescent isothiocyanate conjugated mouse monoclonal anti-CD19 antibody (CD19-FITC) and phycoerythrin conjugated mouse monoclonal anti-CXCR5 antibody (anti-CXCR5-PE). For isotype control experiments, anti-CXCR5-PE was replaced with PE conjugated mouse IgG1 (IgG1-PE). A and B, from a 19-year-old woman with lupus nephritis; C and D, from a healthy 40-year-old woman.

Table 3. The percentage of CXCR5-bearing B cells (CD19+) and T cells (CD3+) in peripheral venous blood of patients with SLE and controls.

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<th>Control, n = 9</th>
<th>SLE, n = 9</th>
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<tr>
<td>CD19+CXCR5+</td>
<td>9.58 ± 2.39% (10.55%)*</td>
<td>14.35 ± 1.65% (14.72%)*</td>
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<tr>
<td>p</td>
<td>0.120</td>
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<tr>
<td>CD3–CXCR5+</td>
<td>4.84 ± 1.04% (3.92%)*</td>
<td>8.23 ± 1.94% (6.39%)*</td>
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<tr>
<td>p</td>
<td>0.146</td>
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* Data in parentheses indicate median value; p value calculated by Student t test.
On the other hand, serum levels of both CXCL13 and anti-dsDNA antibodies were not necessarily higher in patients with lupus nephritis-associated chronic kidney failure. These findings suggested that in ESRD with compromise of the immune function, the importance of B cell chemotraction as well as phlogistic autoantibody destruction might attenuate, although some of these patients might still have residual lupus activity despite renal failure.

The production of anti-dsDNA antibodies is important in the pathogenesis of SLE. These antibodies participate in the formation of circulating immune complexes and may also exert direct damage to organs such as the kidney. It is possible that production of CXCL13 by immune-activated cells may lead to recruitment of the antibody-producing B cells into the target organs, where destructive autoantibodies such as anti-dsDNA are released in large amounts, leading to significant tissue damage. We observed that SLE patients with anti-dsDNA antibodies did have higher levels of CXCL13 in serum. Although this could not directly prove the effect of CXCL13 on the production of anti-dsDNA, it sheds light on a possible role of CXCL13-CXCR5 interaction in the pathogenesis of these destructive antibodies. Because of the heterogeneity of the anti-dsDNA-positive SLE patients, with protein clinical manifestations other than renal damage, whether BLC/CXCL13 also plays a role in other organ damage in SLE could not be determined from our results. In addition, our results suggest existence of other complex confounding factors, leading to difficulty determining the pathogenesis of renal damage.

A drawback of our investigation was that many SLE patients were in quiescent disease status with low disease activity and with longterm disease duration. In addition, many patients with lupus nephritis were in remission status under maintenance immunosuppressants long after the induction therapy. Further, in the long period of sample collection, fluctuations of anti-dsDNA titers occurred with evolution of various clinical conditions or titration of therapeutic doses. These factors might have decreased the power of the investigation to achieve definite conclusions. However, we still see a tendency of increased B cell chemotraction and potential importance of CXCL13-CXCR5 interaction in the pathogenesis of lupus nephritis.

The B cells that bear CXCR5 in the peripheral venous blood were also quantified in some patient samples. Our results showed that, compared to healthy controls, patients with SLE had a tendency to exhibit more CXCR5+CD19+ cells in the peripheral circulation, especially patients in the early stages of development of nephritis (Table 3). We hypothesize that in the presence of high concentration of CXCL13 (a free ligand) in the serum and target tissue, the CXCR5-bearing B cells may migrate rapidly to their final destination through peripheral circulation, resulting in transient or sustained elevation of the number of CXCR5+ B cells in peripheral blood. These speculations require verification.

Implications of chemokines in the autoimmune inflammatory process have been proved in murine lupus models. As well, the therapeutic potential of several chemokines and/or chemokine receptor antagonists has been discussed in models of lupus nephritis and other human renal diseases. Our findings are in agreement with previous reports on murine lupus models that showed highly expressed BLC/CXCL13 and expansion of B1 cells bearing CXCR5 in target organs, possibly contributing to autoimmunity. These findings may be useful in development of new therapeutic modalities for lupus nephritis that target chemokine-chemokine receptor networks.

Our investigation has provided evidence that CXCL13-CXCR5 interaction may be important in the pathogenesis of SLE not only in mice but also in humans. An association of high CXCL13 levels with the development of lupus nephritis, even in low disease activity state, has been demonstrated. Further studies are needed into longterm followup of CXCL13 fluctuations in the disease course (before/after treatments for nephritis) and evaluation of its role in organ damage.

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