Preliminary Clinical Measurement of the Expression of B-Cell Activating Factor in Chinese Systemic Lupus Erythematosus Patients

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It has recently been found that excessive serum B-cell activating factor (BAFF) production triggers severe autoimmune disorders in mice resembling systemic lupus erythematosus (SLE). Whether such dysregulation in circulating levels of BAFF results from overexpression of BAFF gene in clinical patients with SLE is still unclear. In this study, we investigated the relationship between the expression of BAFF and SLE. Here, circulating levels of BAFF were measured in 59 Chinese patients with SLE using enzyme-linked immunosorbent assay (ELISA). In addition, we have quantified the specific mRNA levels of BAFF in unstimulated peripheral-blood mononuclear cells (PBMCs) using real-time polymerase chain reaction (PCR). Serum samples of all SLE patients were also assayed for quantitative immunoglobulins (IgG, IgA, IgM) and

anti-double-stranded DNA (anti-dsDNA) antibody levels, and compared with samples from 40 healthy control subjects. Serum BAFF levels in all SLE patients were significantly elevated ($P < 0.001$) and correlated with the levels of IgG and the titers of anti-dsDNA antibody ($r = 0.442$, $r = 0.85$, $P < 0.00$). More importantly, 66% (39/59) of these SLE patients had significantly higher levels of blood BAFF mRNA, closely paralleling with serum BAFF levels ($r = 0.652$, $P < 0.001$). Dysregulation of BAFF is relatively common in Chinese patients with SLE. Excessive serum BAFF production may result from overexpression of the BAFF gene. BAFF also plays an important role in activating specific autoreactive B cells and modulating the production of autoantibodies. *J. Clin. Lab. Anal.* 21:183–187, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

B-cell activating factor (BAFF), also known as B-lymphocyte stimulator (BlyS), TALL-1 (tumor necrosis factor [TNF] and apoptosis ligand-related leukocyte-expressed ligand 1), zTNF4, THANK (a TNF homolog that activated apoptosis NF- κ B, NH₂-terminal kinase), has emerged as one of the critical factors controlling B cell maturation, tolerance, and malignancy since 1999. It is a novel member of the TNF ligand superfamily, and is primarily expressed by monocytes, macrophages, and various cell types of myeloid origin. BAFF binds to three known receptors, transmembrane activator and calcium

signal modulating cyclophilin ligand (CAML) interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFF-R or BR3), to induce B cell proliferation,

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differentiation, and immune modulation. Several lines of evidence indicate that these ligand-receptor systems play an important role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) (1–5). In the present study, the enzyme linked immunosorbent assay (ELISA) and real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) were used to measure the serum level of BAFF protein and BAFF gene expression in peripheral blood in Chinese patients with SLE. The relationships between serum BAFF concentration and concentration of immunoglobulins, autoantibody titers, and the amount of BAFF mRNA in peripheral blood were analyzed, to explore the role of BAFF expression in the pathogenesis of SLE.

MATERIALS AND METHODS

Subjects

A total of 59 SLE patients (including both inpatients and outpatients) admitted to our hospital between October 2001 and March 2003 were included as study subjects. A total of 51 patients were female and eight were male and their ages ranged from 35 to 55 years. All met the American College of Rheumatology (ACR) diagnostic criteria for SLE, with an antinuclear antibody (ANA) titer $>1:1,000$, presence of anti-double-stranded DNA (anti-dsDNA), and a score of 1 to 3 on the Llwowitz Lupus Activity Index. A group of 40 healthy volunteers (30 females and 10 males, aged 35–56 years) who attended a health checkup clinic at our hospital were included as controls. In these subjects, biochemical parameters were normal and both hepatitis viruses and autoantibodies were absent. Five milliliters of anticoagulated venous blood samples obtained from each participating subject was centrifuged to remove cells and the serum was stored at -20°C until used. To prepare samples of peripheral blood mononuclear cells (PBMCs), cells of the remaining blood sample were separated by sedimentation of a Ficoll-Hypaque gradient and stored at -80°C for later use.

Detection of Serum BAFF Protein Using ELISA Assay

A sandwich ELISA was used to quantify serum BAFF protein as previously described with modification assay (6,7). Briefly, 96-well Nunc-Immuno ELISA plates (Nunc, Roskilde, Denmark) coated $2\mu\text{g/mL}$ mouse monoclonal anti-Human BAFF antibody (PeproTech, Rocky Hill, NJ) were incubated overnight at 4°C . The plates were washed three times with 0.02% Tween-20 (Euroimmune, Lübeck, Germany) in phosphate buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA; Hua Mei Biotech, Beijing, China) overnight at 4°C . Serum

samples were diluted 1:10, added in duplicate and incubated for 4 hr at 37°C . After three washes, plates were incubated with $0.2\mu\text{g/mL}$ of biotinylated anti-human BAFF polyclonal antibody for 1 hr at 37°C . Streptavidin horseradish peroxidase (HRP) (Serotec, Raleigh, NC) was added for 1 hr at 37°C . Color development achieved in the dark using a substrate reagent pack (R&D Systems, Minneapolis, MN) was stopped with $1\text{ M H}_2\text{SO}_4$ at room temperature (RT). Plates were read at 450 nm absorbance using a Model 550 microplate reader system for ELISA (BioRad, Hercules, CA). A standard curve was created for each plate using recombinant human BAFF (PeproTech). Serum BAFF protein levels were calculated by means of logistic model using Ascent software (Shanghai, China). The intraassay variability was 4.6% and the interassay variability, assessed by measuring a small number of samples in three independent experiments, was 12.5%.

Measurements of Total Serum Immunoglobulin and Anti-dsDNA Antibodies

One milliliter of each serum sample was also examined nephelometrically to measure serum IgG, IgA, and IgM concentrations using a BN ProSpec Analyzer (DADE Behring, Deerfield, IL) with total IgG, IgA, and IgM detection kit (AoBo BioTech, Chengdu, China). Assay was performed according to the manufacturer's instructions. In addition, each serum sample of SLE patients was diluted 1:10, and $25\mu\text{L}$ of the diluted serum was tested for anti-dsDNA antibody. Indirect immunofluorescence kit for anti-dsDNA was purchased from Euroimmune Diagnostics (Lübeck, Germany). Assay was also performed according to the manufacturer's instructions using a Leica-DMLB fluorescence microscope (Leica Microsystems, Ernst-Leitz-Strasse, Germany). Positive serum samples then underwent multiple dilutions for antibody detection, as outlined in the manufacturer's instructions. A titer below 10 was considered to be negative for anti-dsDNA antibodies.

Preparation of Standard Plasmid and Quantitative Real-Time PCR

Total RNA was extracted and isolated from PBMCs using a Qiagen Rneasy minikit (Qiagen, Hilden, Germany). The concentration and purity of the extracted RNA were determined by the optical density $\text{OD}_{260}/\text{OD}_{280}$ ratio using ultraviolet (UV) light spectrophotometry (DU640; Beckman, Shanghai, China). Real-time PCR for BAFF was performed using predesigned assays (Applied Biosystems, Foster City, CA). Its primer pair and TaqMan probe for a gene-specific 205-bp PCR product (position 444 to 649) were synthesized by Jikang Life Technology (Shanghai, China). Human housekeeping gene: glyceraldehydes-3-phosphate-dehydrogenase

(GAPDH) served as an internal control. After purification, the PCR product was inserted into a pMD 18-T vector plasmid (TakaRa Biotech, Dalian, Japan) to construct a prokaryotic expressing vector pMD18-T-BAFF. Following bacterial transformation, this standard plasmid was identified with PCR and DNA sequencing. The concentration of the plasmid standard was determined by spectrometry at 260 nm. Single-stock solutions of serial dilutions from 10^9 to 10^3 copies were prepared and stored at -20°C .

The extracted RNA obtained from patients and controls were amplified in duplicate using ABI PRISM 7000 Sequence Detection Systems (PE Biosystems, Foster City, CA). A standard curve was created for each plate using serial standard dilutions (8). With the help of the standard curve, threshold cycle (Ct) values, indicating the cycle at which amplification enters the exponential phase of PCR, were used to determine the corresponding mRNA quantities in each sample. Samples with a Ct value greater than 40 were excluded from the analysis. Analysis was performed using ABI PRISM 7000 SDS Software (PE Biosystems). Results were normalized for GAPDH gene expression. The intraassay variability was less than 5% and the interassay variability was lower than 20%.

Statistical Analysis

Data were analyzed by the paired or unpaired *t*-test, analysis of variance (ANOVA) and linear regression using SAS 6.12 software (Shanghai, China). Probability values lower than 0.001 were considered significant.

RESULTS

BAFF Protein in Plasma of SLE Patients

Soluble BAFF (sBAFF) protein in plasma was detected 59 relatively stable SLE patients and 40 healthy controls. For all samples and controls, two spectrophotometric readings were obtained and averaged. Their serum concentrations were listed as follows: for 59 patients with SLE, the mean value was $8.42\mu\text{g/L}$ (standard

deviation [SD], 1.55). In 40 healthy individuals, the mean value of sBAFF was $3.19\mu\text{g/L}$ (SD, 0.88). sBAFF protein levels in plasma differed significantly between SLE patients and healthy controls ($P < 0.001$).

Relation Between sBAFF Protein Levels and Serum Immunoglobulin, Anti-ds-DNA Antibody Levels

The serum levels of IgG, IgA, and IgM were significantly higher in the SLE patient group than those in the healthy controls ($P < 0.001$) (Table 1). In the SLE group, serum BAFF protein levels were positively correlated with IgG levels in plasma ($r = 0.442$, $P = 0.0005$) (Fig. 1), but not with serum IgA or IgM concentrations ($r = 0.056$, $P = 0.674$; and $r = 0.086$, $P = 0.519$, respectively). Correlation analysis to assess the relationship between anti-dsDNA titers and quantity of sBAFF protein in plasma revealed a close relationship between the two parameters ($r = 0.850$, $P < 0.001$) (Fig. 2).

Relation Between Serum BAFF Protein Levels and BAFF mRNA Levels in PBMNCs

BAFF mRNA levels in unstimulated PBMNCs from the 59 Chinese patients with SLE and 40 healthy individuals were quantified. The BAFF mRNA level of all 40 controls was $7.06 \pm 0.88 \times 10^4$ copies/ μg RNA. Compared to the healthy control group, 39 of the 59 SLE patients (66%) displayed significantly elevated BAFF mRNA levels ($4.19 \pm 7.14 \times 10^7$ copies/ μg RNA, $P < 0.001$). Finally, serum BAFF protein levels and peripheral PBMNCs BAFF mRNA levels were examined by linear correlation analysis. A significant correlation was evident ($r = 0.652$, $P < 0.001$) (Fig. 3).

DISCUSSION

Recent studies support the suggestion that, in autoimmune diseases such as SLE, autoreactive B cells not only produce autoantibodies but also act as antigen presenting cells and participate in the abnormal autoimmune processes (9). However, the mechanisms of loss of B cell immune tolerance during the pathological process, native antigen-mediated activation of autoreactive B cell production, and autoantibody production by abnormally activated B cells are unclear. BAFF, which is an important regulatory factor for B cell proliferation, may play a crucial role throughout this process (10,11).

The relationship between BAFF and autoimmune diseases has become a popular interest of research. Studies on serum protein levels indicate that abnormal BAFF expression is closely associated with the immunopathology of SLE (12–14). There have been no

TABLE 1. Total serum immunoglobulin levels in SLE patients and healthy controls (g/L)[†]

	IgG	IgA	IgM
SLE group	$18.11 \pm 3.82^*$	$3.47 \pm 1.48^*$	$2.03 \pm 0.61^*$
Normal control	11.24 ± 1.69	1.63 ± 0.38	1.36 ± 0.39

[†]A total of 59 relatively stable SLE patients and 40 healthy controls were included in this study. Total IgG, IgA, and IgM were measured by nephelometric assay.

*Indicates that when analyzing the differences between SLE and the normal control groups, P was < 0.001 . The P value was determined by *t*-test.

Fig. 1. Correlation between serum BAFF level and IgG concentration in 59 SLE patients. A total of 59 relatively stable SLE patients and 40 healthy controls were included in this study. Serum levels of BAFF were measured by ELISA. Levels of IgG were determined in the same serum samples as were BlyS levels. The x-axis values are serum BlyS levels, and the y-axis values are IgG concentrations. The slide line is the regression line.

Fig. 3. Correlation between BlyS mRNA expression and serum BlyS level of peripheral blood cell in 59 SLE patients. A total of 59 relatively stable SLE patients and 40 healthy controls were included in this study. Serum levels of BAFF were measured by ELISA. Levels of BlyS mRNA were determined in the same SLE patients as were serum BlyS levels. The x-axis values are serum BlyS levels, and the y-axis values are BlyS mRNA expression.

Fig. 2. Correlation between serum BlyS level and anti-dsDNA titer in 59 SLE patients. A total of 59 relatively stable SLE patients and 40 healthy controls were included in this study. Serum levels of BAFF were measured by ELISA. Serum anti-dsDNA levels were determined in 59 SLE patients within 1 year of serum collection for BlyS determination. The y-axis values are reciprocal titers.

reported clinical studies on the relationship between BAFF and SLE in Chinese patients. We were thus prompted to investigate serum protein levels and gene expressions in unstimulated peripheral blood cells to explore the influence of BAFF on the pathogenesis of SLE.

In this study of 59 patients with relatively stable SLE, the average serum BAFF protein level was $8.42 \pm 1.55 \mu\text{g/L}$, significantly higher than the healthy control group. This finding is consistent with international studies (15–17), and suggests that high serum BAFF protein level is a characteristic finding in the immunopathology of SLE. To further elucidate the relationship between high serum BAFF level and the production of autoantibodies, correlation analysis was conducted between serum BAFF concentration of the SLE subjects, their immunoglobulin levels, and autoantibody titers. The observation that serum BAFF protein level was clearly positively correlated with IgG concentration and anti-dsDNA titer may reflect the participation of BAFF in promoting B cell proliferation (including autoreactive B cells). Under continuous stimulation by endogenous autoantigens, patients with high serum BAFF levels preferentially trigger autoreactive B cell activities, interrupting the immune tolerance, and enhancing their proliferation, differentiation, and production of high levels of IgG. BAFF protein

also concomitantly and selectively accelerates dsDNA antigen-mediated loss of B cell tolerance, inducing autoreactive B cell activation and production of specific autoantibodies. The impact of high level BAFF protein on autoreactive B cell activation and the production of specific autoantibodies demonstrates the important regulatory role of BAFF in the disease onset of SLE.

Protein expression is regulated mainly by upstream mRNA level. Abnormal mRNA often results in aberrant downstream protein expression. Thus, the cause of the elevated serum BAFF protein might well be uncovered by back-tracking and studying the mRNA levels. Real-time quantitative RT-PCR was used to measure BAFF mRNA level, which was compared with the serum BAFF protein level. Results showed that BAFF gene expression in PBMCs of 66% of SLE patients was significantly elevated, and positively correlated with serum BAFF protein level ($r = 0.652$). The results were similar to those reported by Stohl et al. (13) and Collins et al. (17). This finding illustrates that, in SLE patients with the predisposition to highly express the BAFF gene, the BAFF mRNA level in their PBMCs is also upregulated, resulting in an elevated level of downstream serum BAFF protein and supporting the claim that a high serum BAFF level results from higher BAFF gene expression. However, we observed that in a small proportion of SLE patients, the PBMCs BAFF mRNA levels were relatively normal. The difference in genetic predisposition in SLE patients therefore cannot solely influence serum BAFF level.

The SLE subjects selected for this study were patients with relatively stable disease. However, the small sample size is an acknowledged limitation. Further research with larger samples is needed to understand the effects of BAFF on the natural course, prognosis, and responsiveness for medical treatment of SLE.

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