B Lymphocyte Stimulator (BLyS) Overexpression in Patients with Systemic Lupus Erythematosus

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Abstract

Members of the tumour necrosis factor (TNF) family induce pleiotropic biological responses, including cell growth, differentiation and even death. B lymphocyte stimulator (BLyS), a member of the tumour necrosis factor (TNF) superfamily, is a cytokine that induces B-cell survival, expansion, and differentiation both in vitro and in vivo. Considerable evidence points to a role for (BLyS) overproduction in murine and human Systemic Lupus Erythematosus (SLE). Increased levels of BLyS mRNA correlates well with biologic and clinical sequelae of BLyS overexpression.

Aim of Work: To assess the level of mRNA BLyS in systemic lupus erythematosus patients and correlate it to clinical and laboratory features of the disease.

Material and Methods: Thirty SLE patients and ten age- and sex-matched healthy individuals were enrolled in the study. Blood samples from patients and controls were subjected to the following: Detection of BLyS mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) method, followed by semiquantitation of their levels by comparing levels of BLyS mRNA to β-actin mRNA levels using densitometric analysis.

Results: The mean intensity of full-length BLyS mRNA expression was significantly higher in SLE patients when compared to the control group (p < 0.001). Elevated BLyS mRNA in SLE patients are significantly associated with disease activity as they are statistically correlated with SLE disease activity index (SLEDAI) score. BLyS mRNA levels were closely associated with serum anti-dsDNA levels and are not correlated to percentage of leucocyte lineages in the patients’ peripheral blood samples, denoting that among SLE patients, the elevated levels of BLyS mRNA are not related to percentage of myeloid lineage, they are expressed from.

Conclusion: BLyS mRNA levels may be a helpful biomarker in the clinical monitoring of SLE patients. These findings also reinforce the rationale underlying clinical trials with BLyS antagonists in SLE.

Key Words: SLE – BLyS mRNA – Inflammation.

Introduction

B LYMPHOCYTE stimulator (BLyS, also known as TALL-1, THANK, BAFF and zTNF4), a member of the tumour necrosis factor (TNF) superfamily of cytokines, induces B-cell proliferation and immunoglobulin secretion and is a key regulator of peripheral B-cell population in vivo [1].

BLyS protein is a 285-amino acid member of the TNF ligand superfamily. Members of the TNF cytokine family are critically involved in the regulation of inflammation, of the immune response to infections and of tissue homeostasis. As with other members of the TNF ligand family, BLyS is a type II membrane protein that is cleaved at the cell surface, forming soluble protein. Although members of the TNF ligand family show significant sequence diversity, they are structurally related. Upregulation of TNF-related ligands on T-cells is important for the activation and stimulation of neighbouring cells [2].

Expression of BLyS protein is highly restricted to myeloid lineage cells, including monocytes, macrophages and dendritic cells. The cellular targets of BLyS protein are also highly restricted. The three known receptors for BLyS protein are BCMA; TACI and BAFF-R. mRNA expression of each of these receptors is restricted to B cells and tissues that contain B cells, although activated T cells may express some TACI mRNA. TACI and BCMA bind both BLyS and APRIL, another TNF superfamily member and their roles are more controversial. The agonist effects of BLyS on B cells are mediated mainly via BAFF-R. BLyS protein binds strongly to B cells and weakly to T cells and not at all to NK cells or to monocytes [1].

BLyS was mapped to chromosome 13q32-34. Membrane-bound BLyS was processed and secreted through the action of a protease whose specificity matches that of the furin family of paraprotein convertases, resulting in the release of a soluble
**B Lymphocyte Stimulator (BLyS) Overexpression in Patients**

17-kDa protein. BLyS protein is an effective costimulator of B cell expansion and function. Soluble recombinant BLyS (rBLyS) protein costimulates in vitro B cell proliferation and immunoglobulin (Ig) production and in vivo administration of rBLyS protein to mice induces B lymphocytosis and polyclonal hypergammaglobulinemia [3]. A causal relation between constitutive overproduction of BLyS and development of systemic lupus erythematosus (SLE)-like illness has incontrovertibly been established in mice. BLyS-transgenic mice often develop SLE-like features as they age [4-6] and SLE-prone mice respond clinically to treatment with BLyS antagonists (decreased disease progression and improved survival) [4,7].

Considerable inferential evidence points to a role for BLyS overproduction in human SLE as well. Cross-sectional studies have demonstrated elevated circulating levels of BLyS in 20-30% of human SLE patients tested at a single point in time [8,9]. Moreover, a 12-month longitudinal study documented persistently elevated serum BLyS levels in about 25% of SLE patients and intermittently elevated serum BLyS levels in an additional 25% of patients [10]. Remarkably, circulating BLyS levels did not correlate with disease activity (measured using the SLE Disease Activity Index [SLEDAI]) in these cross-sectional or longitudinal studies [8-10]. Although a statistically significant correlation between circulating BLyS levels and SLEDAI has been observed in a more recent 24-month longitudinal study of 245 SLE patients (with >1,700 plasma samples analyzed) [11], the correlation remains weak.

The limited correlation between circulating BLyS protein levels and disease activity in previous studies [8-10] may have exposed an inadequacy of the former to reflect faithfully endogenous BLyS overproduction. In addition to the rate of BLyS protein production, several other factors (for example, utilization and excretion) can affect circulating BLyS protein levels. Although there are no practicable means of directly measuring in vivo BLyS production per se in humans, the level of BLyS mRNA may serve as a better surrogate marker of in vivo BLyS production than does the level of BLyS protein. Candidate BLyS mRNA isoforms include the full-length BLyS mRNA isoform, which encodes the full-length protein and the alternatively spliced ΔBLyS mRNA isoform, which encodes a protein with a small peptide deletion [12]. ΔBLyS does not bind to cells expressing BLyS receptors and therefore it has no agonistic activity. Moreover, ΔBLyS can form heterotrimers with full-length BLyS, thereby actually functioning as a dominant-negative antagonist of BLyS activity.

**Patients and Methods**

Thirty patients who met at least four of the American College of Rheumatology (ACR) criteria for SLE and ten normal control subjects, age- and sex-matched, were enrolled in this study. Patients were selected from Rheumatology and Rehabilitation department, Kasr El-Eini Teaching Hospital, Cairo University. Controls are volunteers from our working staff. All patients and controls were subjected to careful history taking and thorough clinical examination (age, sex, disease duration, malar rash, discoid lesions, photosensitivity, oral ulcers, arthritis and renal, neurological and hematological disorder).

Lupus disease activities were assessed using SLE disease activity index (SLEDAI) score which defines SLE activity according to 24 clinical and laboratory parameters, with final calculated score possible being 105. Patients were divided into 2 groups:

- **Group I:** 10 healthy volunteers.
- **Group II:** Included 30 SLE cases; 27 females (90%) and 3 males (10%).

**Blood BLyS mRNA determination:**

The whole blood was added to RNAlaterTM (Ambion, Austin, TX, USA) at a 1:4 vol/vol ratio after RNA stabilization, stored at -70°C, and assayed for full-length BLyS mRNA. Total RNA was purified from samples using RNAeasy miniprep kits (Qiagen, Valencia, CA, USA) and contaminating genomic DNA was removed by DNase-I digestion. RNA quantity was assessed by determination of the optical density at 260 and 280nm using spectrophotometer. One-tenth volume of total RNA was used as template in the first-strand cDNA reaction using oligo-dT and the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Duplicated samples of cDNA were amplified with primers against β-actin and full-length BLyS: β-actin sense 5′-CGAGAAGATGCCCATGCT-3′; β-actin anti-sense 5′-GGCATA CCCCTCTATGATGG-3′; full-length BLyS sense 5′-GCAGACAGTGAAACACCAACTATAC-3′ and full-length BLyS anti-sense 5′-TGCCAGCTG AATAGCAGGAATTAT-3′.

A 165bp amplicon for β-actin was PCR-amplified using the following PCR cycling conditions: 3min at 94°C and 35 cycles of 1min at 94°C, 1min at 65°C and 1min at 72°C and a final extension step of 7min at 72°C. A 296bp amplicon for full-length BLyS was PCR-amplified under the same PCR-cycling conditions with the exception of annealing at 64°C. The amplified products were detected by 1.5% agarose gel electrophoresis, after ethidium bromide staining.
Semi-quantitation of BLyS mRNA levels:

Semi-quantitation of the amplified products was performed by densitometric analysis. The absorbance values were measured for each band by densitometry (model DS670 image densitometer, Bio-Rad Hemel-Hempstead, Hertfordshire, UK), using the Molecular Analyst PC software for Bio-Rad’s Image Analysis systems. Levels are expressed as optical density units (DU), after normalization with β-actin band intensity.

Results

The current study was conducted on 30 patients with systemic lupus erythematosus disease compared to 10 age and sex matched healthy volunteers representing the control group.

The studied individuals were divided into 2 groups:

Group I (Controls): Comprised 10 healthy volunteers not suffering from any rheumatic diseases. They were 7 females (70%) and 3 males (30%) with age range from 17 to 36 years with a mean of 24.9±6.4 years.

Group II (SLE): Included 30 subjects suffering from SLE, 27 females (90%) and 3 males (10%) their ages ranged between 16 and 50 years with a mean of 25.6±9.3 years.

Clinical and immunological laboratory data:

The main presenting manifestations were arthritis in 24 cases (80%), malar rash, photosensitivity and oral ulcers in 18 cases (60%), alopecia in 16 cases (53%) and lupus nephritis in 15 cases (50%).

Other manifestations included: Respiratory infection in 10 cases (33%) fever in 9 cases (30%), neurological manifestations in 6 cases (20%), DVT in 4 cases (13%), hepatomegally in 5 cases (17%), vasculitis in 4 cases (13%) and 2 APLS in 2 cases (7%).

Laboratory data of SLE patients are summarized in Table (1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Cases (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>WBCs (x10^9 /l)</td>
<td>1.8-16.2</td>
</tr>
<tr>
<td>Hb g/dl</td>
<td>5.3-13.6</td>
</tr>
<tr>
<td>Platelet (x10^9 /l)</td>
<td>94-529</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>22-150</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>13-187</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.38-3</td>
</tr>
<tr>
<td>C3 (g/l)</td>
<td>0.47-1.5</td>
</tr>
<tr>
<td>C4 (g/l)</td>
<td>0.008-0.32</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>6-48</td>
</tr>
<tr>
<td>sCTLA4 mRNA intensity</td>
<td>113.58-244.37</td>
</tr>
<tr>
<td>(DU)</td>
<td></td>
</tr>
</tbody>
</table>

Table (2): ANA pattern among SLE patients (n=30).

<table>
<thead>
<tr>
<th>Item</th>
<th>No. of cases</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenous</td>
<td>19</td>
<td>64</td>
</tr>
<tr>
<td>Rim</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Speckled</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Homogenous &amp; rim</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

Results of autoantibodies showed positive ANA in all patients: Nineteen patients (64%) showed homogenous pattern, four (13%) showed homogenous and rim patterns and one (3%) showed only rim, while six (20%) showed speckled pattern (Table 2).

All patients had positive anti-DNA using Immunofluorescence Technique (I.F.).

Results of full-length BLyS mRNA expression in PBCs by RT-PCR:

The bands of BLyS mRNA at 296bp could be readily found in all samples of SLE patients (Fig. 2). The amplified product of β-actin are found in all samples as bands at 165bp (Fig. 1).
Overexpression of BLyS mRNA in SLE patients can be seen from the picture, when comparing the intensity of SLE patients’ bands to those of controls. Semiquantitation of the BLyS mRNA was established by measuring its levels after being normalized to β-actin mRNA levels in peripheral blood leukocytes. The mean of the full-length BLyS mRNA among the SLE patients was significantly greater than those among normal control individuals. The intensity level by the densitometric analysis among SLE patients ranged from 113.58 x10^3 to 244.37 x10^3 densitometric unit (DU) with a mean value of (201.1 ± 13.8 x10^3). The bands intensity among controls ranged from 98.37 x10^3 to 120.12 x10^3 densitometric unit with a mean value of (103.1 ± 6.8 x10^3). The mean intensity of BLyS mRNA expression was statistically significantly higher in SLE patients when compared to the control group (p<0.01) (Table 3).

Table (3): Comparison between SLE patients and control group regarding BLyS mRNA expression.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases (n=30)</th>
<th>Control (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLyS mRNA x10^3 (DU)</td>
<td>201.1 ± 13.8</td>
<td>103.1 ± 6.8</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Among these SLE patients, none of the measured BLyS parameters correlated with patient age, sex, race, or daily dose of corticosteroids (data not shown).

Correlations between BLyS parameters and disease activity:

A significant correlation between SLEDAI and full-length BLyS mRNA levels was found. Fig. (3) shows that there is a good positive correlation (r=0.676).

Lack of correlation between levels of BLyS mRNA and percentages of individual leukocyte cell types:

Among cells in peripheral blood, BLyS is predominantly expressed by cells of the myeloid lineage (monocytes and neutrophils) [13-16]. Accordingly, a shift in the differential leukocyte count away from lymphocytes to monocytes and/or neutrophils could substantially alter BLyS mRNA results. Because of the limited amount of blood we were permitted to obtain from the SLE patients (due to the high prevalence of anemia among these patients), we were unable to purify the individual leukocyte populations for BLyS mRNA analysis. Nevertheless, to demonstrate that the elevated BLyS mRNA levels in SLE did not simply reflect a shift in differential leukocyte count, we assessed the correlations between the individual BLyS parameters on one hand and the percentages of blood neutrophils, monocytes and lymphocytes on the other hand. No correlations were observed.

BLyS mRNA levels and Anti-dsDNA antibody titres among SLE patients:

Although the levels of anti-ds DNA antibodies among patients with elevated BLyS is higher than those reported among patients with lower levels of BLyS mRNA, yet it didn’t show statistically significant difference.

Statistics:

Data management and analysis were performed using Statistical Analysis Systems. Numerical data were summarized using means and standard deviations, categorical as percentages. Comparisons between the groups were done using Mann-Witney test. The association between individual percentages of blood leucocytes as well as SLEDAI score and BLyS mRNA value was calculated using the Pearson’s correlation coefficient [17].

All p-values are two-sided. p-values ≤ 0.05 were considered significant.

Discussion

Elevated blood levels of BLyS protein and mRNA are well described features of human SLE [8-10]. We confirmed these observations in our study by documenting statistically significant increase in levels of full-length BLyS mRNA among SLE patients. The relationship between circulating BLyS protein levels and disease activity was addressed in several previous studies, but significant correlations between the two measures did not emerge [8-10]. In the largest study to date, a 2-year longitudinal study of 245 patients in which more than 1,700 plasma samples were analyzed,
a significant but weak correlation between the two was appreciated [11]. In the present study, a statistically significant correlation between BLyS mRNA levels and disease activity was documented.

There is a clear-cut association in BLyS transgenic mice between BLyS overexpression and development of SLE-like features [4-6] and treatment of SLE-prone mice with BLyS antagonists retards the progression of disease and improves survival [47]. Moreover, development of precocious glomerular pathology in autoimmune-prone mice correlates strongly with circulating BLyS levels [19]. On the other hand a study reported a weak correlation between circulating plasma BLyS and disease activity. To circumvent these confounding processes, they used BLyS mRNA levels as a surrogate marker of endogenous BLyS production. Overall, the correlations between disease activity and full-length BLyS mRNA levels were much stronger than that between disease activity and BLyS protein levels [18]. In 2006, a study declared that the expression of BLyS mRNA in patients with active SLE is higher than that reported in patients with inactive SLE. A statistically significant difference between both groups was documented (p<0.001) [20]. These results correlate with our results.

No correlations between expression of BLyS mRNA levels and any specific organ system involvement could be recorded in our study. This is in concordance with another study who reported that serum BLyS did not associate with specific organ system involvement [21]. On the contrary, in 2007 a new study documented an abnormal production of BAFF in T cells from SLE especially in patients with kidney involvement [22].

Although the level of anti-ds DNA antibodies among patients with elevated BLyS mRNA is higher than those reported among patients with lower levels of BLyS mRNA, yet it didn’t show statistically significant difference (p>0.05). In 2001, a study reported that increased level of BLyS in SLE patients was associated with an increased production of anti-dsDNA antibody, which may participate in disease pathogenesis, but was not associated with the production of other antinuclear protein antibodies [23]. In concordance with these findings, suppression of anti-dsDNA antibodies’ production from cultured lymphocytes with TACI-Ig in SLE patients after harvesting the culture supernatants and determining anti-dsDNA titres using an ELISA kit revealed a suppression in the anti-dsDNA levels examined and clarified that TACI-Ig significantly suppressed in vitro T cell-dependent anti-dsDNA antibodies production by B cells [22]. Together with our findings, these results strongly suggest that BLyS plays an important role in T-cell-dependent anti-dsDNA antibodies production in SLE patients through TACI (BLyS-R).

Although the associations between full-length BLyS mRNA levels and disease activity in SLE were usually strong when the SLE cohort was analyzed in aggregate, there were several SLE patients in whom BLyS mRNA levels were quite high despite little objective ongoing disease activity, and there were several SLE patients in whom BLyS mRNA levels were low despite considerable ongoing disease activity. One must recognize that the bulk of the pathogenic autoimmune responses probably takes place in the spleen and lymph nodes, rather than in the peripheral blood, where myeloid lineage cells produce BLyS and support B cell survival and expansion [24]. Local BLyS production in the secondary lymphoid tissues will be more important to the development and maintenance of an autoimmune response than will remote BLyS levels in the circulation. Because at least some autoreactive B cells may be more sensitive to BLyS-mediated survival signals than non–autoreactive B cells [25,26], local increases in BLyS production could preferentially promote expansion of autoreactive B cells. These cells, in turn, could activate autoreactive T cells by presenting autoantigen to them and some of the autoreactive B cells would respond to T cell derived signals and mature into (pathogenic) autoantibody secreting plasma cells. In contrast to murine studies, in which investigators can readily harvest and analyze lymphoid and myeloid lineage cells from any site (for example, spleen, bone marrow), such is not the case for human studies. Peripheral blood is the only site readily accessible for human studies and it is possible that, at least in some patients, BLyS mRNA levels in circulating leukocytes do not reflect local BLyS production in the secondary lymphoid tissues.

One must also recognize that disease activity in SLE is not solely driven by B cells. Systemic inflammation and SLE flares can be triggered via B cell independent means. Not all SLE patients treated with a B cell depleting course of rituximab experience clinical remission [27], strongly pointing to the importance of non-B cells in disease pathogenesis/maintenance. Conversely, not all pathogenic B cells necessarily require high levels of BLyS to effect their pathogenicity. Murine studies have unequivocally documented B cell subpopulations that do not depend upon BLyS for their survival [28-30]. Although mice completely devoid of BLyS have reduced numbers of mature B cells and harbor
reduced levels of immunoglobulin, these reductions are incomplete. Thus, it is possible that some SLE patients harbor pathogenic B cells that are relatively insensitive to BLyS and could drive considerable disease activity even in the context of low endogenous BLyS production. Conversely, patients with high BLyS mRNA levels may be those patients whose disease is strongly driven by BLyS and may be especially helped by BLyS antagonist therapy. Future clinical trials should be able to establish whether the BLyS mRNA levels are good predictors of response to such agents.

Conclusion:

BLyS protein very likely plays an important contributory role in SLE pathogenesis in at least a subset of patients. BLyS mRNA overexpression not only promotes development of disease but also actively contributes to the ongoing maintenance of disease in such SLE patients. Cross-sectional studies, with single measurements at single time points for individual patients, may be rather insensitive to subtle changes in disease activity. Longitudinal studies that monitor changes in disease activity in individual patients with their corresponding changes in BLyS protein levels are more likely to yield true positive results.

Several studies suggest a promising therapeutic approach for SLE by blocking BLyS. Results from a phase I clinical trial with a BLyS antagonist in human SLE have shown the antagonist to be biologically active and safe. These features collectively point to BLyS as an attractive therapeutic agent in human disease. Much additional investigations are necessary and BLyS protein antagonists will find an important niche in the armamentarium of the rheumatologist within the next several years.

References
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