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BAFF Expression is Increased in Patients with Lupus Nephritis and Associated with Antinucleosome Antibodies, C1 Inhibitor, A-1-Acid-Glycoprotein and Endothelial Activation Markers

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Abstract

Objectives: B cell activating factor (BAFF) inhibitor therapy has recently been approved for non-renal Systemic Lupus Erythematosus (SLE). While BAFF plays a role in experimental lupus nephritis (LN), its role human LN is not well studied.

Methods: Case control study in 102 SLE patients, 30 with LN (+LN) and 72 without LN (-LN) and 31 healthy controls. We analysed BAFF mRNA expression in PBMCs (BAFF-RQ) and serum BAFF (s-BAFF) levels and investigated their relation with clinical, histological- and additional acute phase proteins.

Results: s-BAFF and BAFF-RQ were increased in +LN patients compared to controls, but their expression did not correlate with ISN/RPS class, Activity- or Chronicity index on biopsy. s-BAFF correlated with levels of anti-nucleosome antibodies, C1 inhibitor and α-1-acid-glycoprotein (AGP), while BAFF-RQ correlated inversely with Factor VIII.

Conclusions: s-BAFF and BAFF mRNA levels are increased in LN patients, but do not reflect histological disease severity. The association of increased BAFF expression with both pro- and anti-inflammatory markers and reduced endothelial activation suggest that BAFF inhibition in LN may have diverse effects.

Keywords: B-lymphocyte activating factor; Systemic Lupus Erythematosus; Lupus Nephritis; acute phase reactants; C1 inhibitor; α-1-acid-glycoprotein

Abbreviations: SLE: Systemic Lupus Erythematosus; LN: Lupus Nephritis; BAFF: B Cell Activating Factor, BAFF-RQ; BAFF gene expression; PBMCs: Peripheral Blood Mononuclear Cells, Ag: Antigen; vWF: Von Willebrand Factor; PTH: Para Thyreoid Hormone, AGP: α-1-Acid-Glyco Protein; LDH: Lactate De Hydrogenase

Introduction

Systemic Lupus Erythematosus (SLE) is the prototype of non-organ specific autoimmune diseases with renal disease as one of its most serious complications. Lupus nephritis (LN) occurs in 25% to 70% in patients with SLE at some point in the disease course [1,2]. A complex and incompletely understood interplay between renal infiltrating leukocytes, cytokines, autoantibodies and complement factors underlies the classical immune complex-mediated glomerulonephritis in LN [3]. Additionally, thrombotic and inflammatory vascular lesions can affect intrarenal or systemic haemodynamics and thus contribute to disease severity [4]. Autoantibodies produced by differentiated B cells are hallmarks of SLE, and B-cell activating factor (BAFF, TNFSF13 or BLyS) is a central cytokine in SLE pathogenesis. BAFF belongs to the tumor necrosis factor (TNF) superfamily [5] and is produced by a range of immune cells [6] with IL-2 and INF-γ as known stimulants of BAFF [7,8]. Binding of BAFF to one of three receptors on mature B cells (BAFF-R, TACI and BCMA), induces either Ig class switching, cell proliferation or increased survival of B cells including autoreactive B cells [9]. Transgenic (Tg) mice that overexpress BAFF develop an autoimmune disorder, that resembles SLE including immunecomplex mediated glomerulonephritis [10-12]. In general, experimental studies suggest that overexpression of BAFF promotes LN, but evidence from human studies is lacking.

s-BAFF levels are frequently elevated in SLE patients and associated with disease activity, anti-dsDNA antibodies (Ab) levels and acute-phase reactants [13-15], while increased BAFF mRNA levels have been found to correlate with disease activity and anti-dsDNA Ab levels in some studies [15,16]. This suggests that BAFF may play a role in the development of LN through its effect on nephritogenic autoantibody production, although the exact mechanism remains unclear [9,17].

Given the scarcity of human data on BAFF expression in LN, we investigated if any associations exist in vivo, between BAFF mRNA levels, s-BAFF levels and histological as well as biochemical findings in patients with LN.

Materials and Methods

Study participants

A total of 102 adult SLE patients (> 15 years) were recruited for this cross-sectional study and attended at an extended clinic visit, where biological materials were collected. They were predominantly of Caucasian descent (98%), of female gender (88%) with a median age of 49 years and disease duration of 10 years. All patients fulfilled the revised and/or updated American College of Rheumatology (ACR) criteria for the classification of SLE [18,19]. Healthy volunteers (all...
Caucasian, 71% females with age at 48 years) were used as controls for serological investigations and gene expression. The study protocol was approved by the local ethical committee, the national privacy agency and the Ministry of Health (ref. no 12420) and all participants gave written informed consent for the anonymous use of their data in compliance with the Helsinki Declaration.

Clinical categorization of patients

Lupus Nephritis (LN) was defined as sustained proteinuria (>0.5 g/24 hours) and/or the presence of >5 red blood cells and/or >5 white blood cells and/or heme-granular or red blood cells casts (20). Renal tissue obtained through percutaneous biopsies was reevaluated independently by two pathologists for the following features: International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN [17,21] National Institutes of Health (NIH) Activity index (AI) and Chronicity index (CI) [22] and the presence of vasculitis/vascular thrombi. Histological scores represent the mean of the two observer scores and discrepancies in the classification were resolved by mutual discussion.

Serology

Serum BAFF levels were measured in duplex using a Quantikine Human BAFF/BlyS/TNFSF13B Immunoassay (R&D Systems, Minneapolis, USA) and results were averaged. Anti-nuclear antibodies (ANA) positive sera were routinely tested by enzyme immunoassays Elia (VarELISA Phadia, Freiburg, Germany) for the presence of IgG subclass antibodies (Ab) against double stranded DNA (anti-dsDNA), Ro (anti-SSA), La (anti-SSB), Smith (anti-Sm) anti-U1 small nuclear ribonucleoparticle (anti-U1-snRNP) and cardioliopin (aCL-G and aCL-M; normal levels <16 IU/mL). Anti-nucleosome Ab was analysed by ELISA (Orgentec, Mainz, Germany). Lupus anticoagulant was tested in a phospholipid-dependent coagulation assay [23]. Routine laboratory investigations were performed in the accredited Department of Laboratory Medicine and Immunology at the University Hospital of Northern Norway.

BAFF gene expression

Primers and probes were designed using the BAFF encoding gene TNFSF13B (NC_000013.10) and B2M gene encoding β2-microglobulin (β2M) (NC_000015.9) as templates. Primers were designed using Enhanced Avian HS RT-PCR software (Sigma-Aldrich, Saint Quentin Fallavier, France) and probes were selected using Universal ProbeLibrary Human Gene Assay (Roche Diagnostics, Mannheim, Germany) [13].

Total RNA from frozen PBMCs was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), following manufacturer’s instructions. RNA was DNase treated (RNA-free), and stored at -80°C. Total RNA (2µg) was used to synthesize cDNA in a total volume of 20µL using SuperScript VILO cDNA Synthesis Kit (Invitrogen, CA, USA) with regard of investigation full-length BAFF mRNA, according to the protocol. The quality of cDNA was then confirmed by PCR using cDNA (0.5µl), adenine phosphoribosyltransferase gene (APRT) primers (5pM) and Jumpstart ready mix Red Taq DNA polymerase (Sigma-Aldrich, Saint Quentin Fallavier, France). Thermal conditions were denaturation at 94°C for 20sec., 35 cycles at 94°C for 10sec. 65°C for 10sec., and 72°C for 15sec. and a final extension at 72°C for 5min. Agarose gel electrophoresis were run to ensure quality of cDNA, which was stored at −20°C.

Real-time PCR analysis was performed to determine the levels of BAFF mRNA in PBMCs using an ABI PRISM 7900HT, (version 2.3, Applied Biosystems, CA, USA). PCR reaction mixture contained 4µl cDNA, 10µl TaqMan MasterMix (TaqMan® Fast Universal PCR Master Mix (2x), No AmpErase® UNG, (Applied Biosystems), 5µM of each primer and probe in a final volume of 20µl. Each analysis was performed with initial incubation at 95°C for 20sec., followed by 40 cycles at 95°C for 1sec. and 60°C for 20sec. PCR reactions were done in triplicates. A range of primer concentrations was tested to ensure optimal amplification efficiency. Relative concentrations of cDNA present during the exponential phase of the reaction were determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background was determined. The BAFF transcript quantification was standardized using β2M as internal control. BAFF-RQ values are calculated as the ratios of BAFF mRNA to β2M mRNA using the following formula: 2 exp (Ctβ2M – CtBAFF). Cut-off levels were determined by the geometric mean +2 S.D. for healthy controls (n=31). The amplification was verified by melting curve analysis and crossing point. No amplification of non-specific products was observed.

Statistics

Due to relatively small numbers in the cohorts in addition to the fact that most data had a skewed distribution, nonparametric tests were used. Continuous data were analysed by Mann-Whitney U test, and categorical data by Poisson distribution contingency tables or Fishers’ exact test in case of low numbers. Linear regression analyses were performed and linear correlations were analysed by Spearman rank correlation coefficients. Statistical analyses were performed with SPSS v19.0.

Results

Study participants

Among the 102 SLE patients, 30 (29%) were presented with clinical evidence of LN during the disease course. There was no difference in gender (83% female vs. 88%, p=0.5), age at SLE diagnosis, (27.5 years vs. 37, p=0.7) (Table 1) or disease duration at study inclusion (12 years vs. 10, p=0.5) between +LN and –LN cohorts. Renal biopsy, performed on 17/30 (57%) of the +LN patients after a median lag time of 2.5 years, showed an average AI of 7 and CI 2. The majority 12/17 (70%) of the biopsies were classified as ISN / RPS class III or IV, with 2 (12%) in class II, 2 (12%) in class V and 1 (6%) as class VI (Table 1).

Serum-BAFF and association with BAFF gene expression

S-BAFF levels were higher in the +LN patients compared with controls (mean values; 2.09 vs 0.97ng/µl; p<0.001, (Figure 1, Table 3). Similarly, BAFF mRNA levels were increased in +LN patients (RQ 1.88, and 1.12, p<0.001), (Figure 2). However, the differences between +LN and -LN patients were not statistically significant. In +LN patients there was no correlation between s-BAFF and full-length BAFF mRNA levels in PBMCs (R²; 0.29, p=0.1) (Table 4).

Renal biopsy findings and association with serum-BAFF and BAFF gene expression

There was no significant difference in s-BAFF or BAFF-RQ levels between the different ISN/RPS classes (I-VI) (data not shown), while s-BAFF or BAFF-RQ did not correlate with AI (R²; -0.29, p=0.3 vs. R²; 0.32, p=0.2), nor CI (R²; 0.28, p=0.3 vs. R²; 0.32, p=0.2).

Autoantibodies

Compared with -LN patients, +LN patients more frequently had...
analysis between +LN patients, - LN patients and controls (Table 3).

In +LN patients significant trends were seen with regard to low C3 (0.83 vs. 1.01 and 1.19 g/L, p<0.001), increased coagulation Factor VIII (148 vs. 134 and 120 %, p=0.002) and increased PTH (9.6 vs. 5.2 and 4.5 pmol/L, p=0.026) (Table 3). Additionally, significant trends were seen for lower NK cells, CD4 cells, C4, albumen and hemoglobin levels, with significant trends seen for increased C1 inhibitor, fibrinogen, von Willebrand Factor (vWF), vWF antigen, homocystein and LDH (Table 3). No trend was seen for numbers of B- and CD8 cells, nor for levels of Ig, CRP, ferritin, cholesterol, triglycerides, apolipoproteine A1 and B, liver enzymes, FT4, TSH, vitamine B12, folat, HbA1c, IGF-1, IGFBP-3, alpha 1-antitrypsine, ceruloplasmine, α-1-acid-glycoprotein (AGP) and alpha1-phoetoprotein (data not shown).

To examine the relation between these biomarkers and BAFF more closely, Spearman rank correlation analyses were performed. In +LN patients, s-BAFF concentrations had a strong and independent positive correlation with levels for C1 inhibitor (R²; 0.68, p<0.001), AGP (R²; 0.57, p=0.002), haptoglobin (R²; 0.43, p=0.024) and inversely with IgA (R²; -0.49, p=0.009), albumin (R²; -0.48, p=0.008), apolipoprotein A1 (R²; -0.62, p=0.001) (Table 4). BAFF-RQ in LN+ patients correlated inversely with factor VIII levels, but not with immune cells counts (B, NK, CD4 and CD8), complement levels (C3 and C4), IgG, IgM, CRP, ferritin, creatinine, fibrinogen, vWF, vWF Ag, transferrin, alpha-foetoprotein, vitamin B12, IGF-1, HbA1c ionized calcium, alpha1-antitrypsine, ceruloplasmine were not associated with s-BAFF or BAFF-RQ levels (data not shown).

Immune cells and other laboratory findings

Additional inflammatory biomarkers were measured in all freshly collected samples and the data were analyzed using a regression trend analysis between +LN patients, - LN patients and controls (Table 3). In +LN patients significant trends were seen with regard to low C3 (0.83 vs. 1.01 and 1.19 g/L, p<0.001), increased coagulation Factor VIII (148 vs. 134 and 120 %, p=0.002) and increased PTH (9.6 vs. 5.2 and 4.5 pmol/L, p=0.026) (Table 3). Additionally, significant trends were seen for lower NK cells, CD4 cells, C4, albumen and hemoglobin levels, with significant trends seen for increased C1 inhibitor, fibrinogen, von Willebrand Factor (vWF), vWF antigen, homocystein and LDH (Table 3). No trend was seen for numbers of B- and CD8 cells, nor for levels of Ig, CRP, ferritin, cholesterol, triglycerides, apolipoproteine A1 and B, liver enzymes, FT4, TSH, vitamine B12, folat, HbA1c, IGF-1, IGFBP-3, alpha 1-antitrypsine, ceruloplasmine, α-1-acid-glycoprotein (AGP) and alpha1-phoetoprotein (data not shown).

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Immune cells and other laboratory findings

Additional inflammatory biomarkers were measured in all freshly collected samples and the data were analyzed using a regression trend
In contrast, s-BAFF levels in -LN patients were correlated with CRP ($R^2; 0.36, p=0.002$), fibrinogen ($R^2; 0.25, p=0.049$) and IgM ($R^2; -0.26, p=0.039$). Similar as the +LN cohort, in the -LN group s-BAFF levels correlated with AGP ($R^2; 0.30, p=0.014$), although not as strong.

In the -LN cohort, BAFF-RQ correlated only with markers not found in the +LN group (inverse correlation with CD4 cell counts ($R^2; -0.28, p=0.024$) and IgG ($R^2; -0.30, p=0.018$).
Discussion

This cross sectional study investigated several aspects of BAFF to increase our understanding of the contribution of BAFF in human LN. We show that both BAFF gene expression and s-BAFF levels were increased in LN patients, but there is no linear correlation between them. Also, while s-BAFF was associated to anti-nucleosome Ab levels neither s-BAFF levels nor BAFF gene expression was associated with the histological severity of LN; however s-BAFF was strongly associated with levels of the anti-inflammatory C1 inhibitor protein.

A recent study from Switzerland showed increased mRNA expression of BAFF in glomeruli and tubulointerstitial tissue of patients with proliferative LN as compared with living donors [24]. BAFF gene expression in the PBMCs of LN patients in this study was unrelated to the histological findings, suggesting that BAFF gene expression occurring in the inflamed renal tissues may be of greater importance. Our study design did not allow gene expression studies in tissues or other cell types, which would have been of interest to determine whether the source of renal BAFF expression is intrinsic renal cells or infiltrating leukocytes. The fact that BAFF mRNA expression in PBMCs did not correlate with s-BAFF suggest a limited role of PBMCs in this process [13]. Furthermore, no correlation between BAFF gene expression and the nephritogenic antibodies against nucleosome and ds-DNA were observed in LN patients, which is in accordance with the lack of association between BAFF expression and renal disease observed in lupus prone mice, that strongly express anti-nucleosome and anti-dsDNA Ab [25]. This raises questions, whether there is a direct link between BAFF expression by PBMCs and pathogenic autoantibody production.

In line with other studies, anti-nucleosome Ab and anti-ds-DNA Ab were more prevalent in LN patients compared with SLE patients without renal involvement [4,26]. Even though we observed a strong correlation between autoantibodies that are typically considered pathogenic in LN (Table 4), anti-nucleosome Ab was the only which correlated with s-BAFF. This correlation has not yet been described in the literature, but would suggest a role for BAFF in anti-nucleosome production in LN. Immune deposits which consist of oligo-nucleosomes, are located in the basement membrane in the renal glomeruli [27] in LN and are considered the result of ineffective clearance of apoptotic material [28,29]; this may then trigger antigen presenting cells to produce BAFF, that subsequently is involved in the production of anti-nucleosome Ab.

We observed an inverse correlation between BAFF gene expression and, coagulation factor VIII and apolipoprotein B in LN patients. These findings have not been described in the literature before, but the increased factor VIII and vWF indicate a state of endothelial activation in LN patients as plasma vWF binds to collagen in the subendothelial connective tissue. Collagen binding appears to induce a conformational change within the factor VIII–binding motif of vWF that lowers the affinity for factor VIII. Consequently, released factor VIII may locally support fibrin clot formation, and thus contribute to renal thrombotic vascular lesions [30]. Finally, we found increased PTH levels in LN patients while ionized calcium levels were found to be normal, which likely indicates a predisposition to osteopenia in patients with LN.

Several other proteins were studied for their association with BAFF in LN. s-BAFF was strongly correlated with AGP, which is an acute phase reactant that act as a carrier of basic and neutrally charged lipophilic compounds and has been suggested to be a useful biomarker for LN [31]. In active LN, elevated AGP is observed both in plasma and urine, where urinary levels of AGP is increased 3 months prior to the clinical diagnosis of worsening LN activity [32]. High levels of s-BAFF were also associated with low levels of apolipoprotein A1, which is a major component of the high-density lipoprotein complex (HDL). Lipid abnormalities like reduced HDL levels and apolipoprotein A1 are likely to be contributing causes to cardiovascular disease risk which is increased in patients with SLE [37]. HDL is an atheroprotective lipoprotein that contributes to reverse cholesterol transport from the atherosclerotic vessels back to the liver. While HDL-associated apolipoprotein A1 is a specific inhibitor of cytokine production in monocyte-macrophages upon contact with stimulated T cells, this complex might have important anti-inflammatory properties by inhibiting endothelial cells [38]. In this way, BAFF may protect against endothelial activation and vascular damage.

C1 inhibitor can prevent activation of the complement system by blocking the classical and lectin activation pathways at the protease step [33]. C1 inhibitor behaves as an acute-phase reactant, and has additional anti-inflammatory functions such as reduced rolling and transmigration of leukocytes across the endothelium and interacts with extracellular matrix components to concentrate C1 inhibitor at sites of inflammation [33]. The fact that s-BAFF was strongly associated with C1-inhibitor, but only in LN+ patients could indicate that BAFF expression coincides with the acute phase response. The observed corresponding associations of BAFF with AGP and fibrinogen levels would support this.

The limitations of this study need mentioning. SLE is characterised by intermittent disease activity and laboratory findings. The data from this cross-sectional analysis cannot allow any conclusions about BAFF expression levels over time, despite the fact that variation in s-BAFF levels appear to be limited [15]. The study design also limits the strength of the correlation between serological and histological findings. Finally, the small number of LN patients increases the possibility of type II error, and similar studies that include more patients are required before a final conclusion can be made.

In summary, our results show that both s-BAFF levels and PBMCs BAFF gene expression are increased in patients with LN. While s-BAFF levels correlated with nephritogenic autoantibodies, they were also correlated with the anti-inflammatory markers C1 inhibitor and AGP, as well as reduced endothelial activation. Based on these findings, targeted treatments to inhibit BAFF might be relevant in patients with LN, but also has the potential for unwanted effects on BAFF related anti-inflammatory mechanisms.

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