Clinical and serological findings associated with the expression of ITGAL, PRF1, and CD70 in systemic lupus erythematosus

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ABSTRACT

We determined the expression of Integrin alpha L chain (ITGAL), Perforin 1 (PRF1), and CD70 and studied the associations with laboratory and clinical parameters. CD4+ T cells were isolated from 35 SLE patients and 30 healthy controls. The transcript levels of ITGAL, PRF1, and CD70 were quantified by real-time reverse-transcription polymerase chain reaction (RT-PCR). The SLE patients had significantly elevated transcript levels of ITGAL (18.61±22.17 vs. 7.33±9.17, p=0.042), PRF1 (21.67±26.34 vs. 10.67±11.65, p=0.039), and CD70 (1.45±1.63 vs. 0.67± 0.28, p=0.011). Patients with anti-microsomal and/or anti-thyroglobulin antibodies showed high levels of ITGAL (33.41±30.14 vs. 13.58±16.43, p=0.044; and 34.01±27.66 vs. 11.90±16.17, p=0.007, respectively). No association was seen either for the typical antibodies of SLE or for the disease activity. Although ITGAL, PRF1, and CD70 are overexpressed in SLE CD4+ T cells, their expression is not linked to the typical clinical and serological parameters associated with the disease. The role that ITGAL may play in autoimmune thyroiditis deserves further investigation.

Introduction

An abnormal expression of different genes has been found in CD4+ T cells from patients with systemic lupus erythematosus (SLE). One such gene is ITGAL, which encodes the integrin alpha L chain, a structural part of the lymphocyte function-associated antigen-1 (LFA-1). LFA-1 plays a central role in leukocyte intercellular adhesion and it also functions in lymphocyte co-stimulatory signalling. LFA-1 overexpression causes T-cell autoreactivity *in vitro* and a lupus-like disease *in vivo* (1, 2). Interestingly, DNA hypomethylation in SLE affects sequences flanking the ITGAL promoter (3) and an overexpression occurs on T cells from active SLE patients (4). Perforin 1 (PRF1) has also been identified as a gene whose transcription is increased in CD4+ T cells from active SLE patients and it seems to be due to demethylation of a conserved region located between the promoter and upstream enhancer (5). This aberrant overexpression of PRF1 in CD4+ T cells may contribute to the killing of autologous monocytes/macrophages by T cells observed in SLE (4).

The expression of some B-cell co-stimulatory molecules on CD4+ T cells is also affected in SLE. This is the case of CD70. The CD70 gene encodes a member of the tumour necrosis factor (TNF) which regulates B-cell activation and immunoglobulin synthesis. The overexpression of CD70 found on T cells from patients with active SLE has been shown to overstimulate the production of IgG (6).

In the present work, we carried out, for the first time, a study of the association of ITGAL, PRF1, and CD70 with the clinical features and laboratory parameters found in SLE patients.

Patients and methods

Patients

Data were collected from 35 Caucasian individuals (7 men and 28 women; mean age: 34.54 yrs, range: 20–64 yrs) who suffered from SLE. An ethnically matched random healthy control population (blood donors) was also included in the study (n=30, 16 men and 14 women; mean age: 36.93 yrs, range: 21–66 yrs). Subjects' written consent was obtained according to the Declaration of Helsinki (7), and the design of the work conformed to standards currently applied in Spain. All the SLE patients fulfilled at least four of the American College of Rheumatology criteria (8). Complete medical histories were obtained and physical examinations and laboratory tests were conducted for patients at the time of sample withdrawal. Laboratory parameters were evaluated as previously described (9). Clinical manifestations were defined according to the American Rheumatism Association glossary committee (10). A flare was defined as any clinical event directly attributable to disease activity that required a change in treatment. In fact, almost all our patients (n=31) were at flare at the time the blood sample was withdrawn. SLE activity was assessed by the SLE disease activity index (SLEDAI) (11) and those with a SLEDAI equal or above 6 were considered to

Table I. Number (and percentage) of patients with a particular serological (**A**) and clinical feature (**B**).

A. Serological findings[a]

Anti-dsDNA	Anti-RNP	Anti-Sm	Anti-SSA/Ro	Anti-SSB/La	aCL	Lupus anticoagulant	Anti-microsomal	Anti-thyroglobulin
30 (85.7%)	9 (27.3%)	8 (23.5%)	12 (35.3%)	4 (11.8%)	6 (18.2%)	3 (9.1%)	10 (34.5%)	11 (36.7%)

Low Lymphocyte count	Low C3 levels	Low C4 levels	Low CH_{50} activity	Thrombocytopenia
22 (62.9%)	25 (73.5%)	22 (64.7%)	24 (70.6%)	7 (20%)

B. Clinical features and diseases's status

Renal	Arthritis	Cutaneous	Raynaud's phenomenon	Pericarditis	Pleuritis	Haemolytic anaemia	Neurologic	Asymptomatic	SLEDAI ≥6
20 (57.1%)	31 (88.6%)	23 (65.7%)	10 (28.6%)	8 (22.9%)	11 (31.4%)	3 (8.6%)	5 (14.3%)	4 (11.4%)	24 (68.6%)

[a]High anti-dsDNA titers: >15 IU/ml; high anti-ribonucleoprotein (anti-RNP) titers: >12 U/mL; high anti-Smith (anti-Sm) titers: >12 U/mL ; high anti-SSA/Ro titers: >12 U/mL; high anti-SSB/La titers: >12 U/mL; high anti-cardiolipin (aCL) IgG titers: >15 GPL/mL ; high anti-microsomal titers: >35 IU/mL; high anti-thyroglobulin titers: >40 IU/mL; low lymphocyte count: <1.2 x 10^9 cells/L; low complement C3 count: <85 mg/dL; low complement C4 count: <10 mg/dL; low CH_{50} activity: <34 U/ml; thrombocytopenia: <150 x 10^9 platelets/L.

have active disease. All the serological variables and clinical features we were able to compile are detailed in Table I.

Methods

– Isolation of peripheral blood mononuclear cells (PBMCs) and CD4⁺ T cells

$CD4^+$ T cells were isolated by negative selection as previously described (12). The purity of the enriched $CD4^+$ T cells was evaluated by flow cytometry in a FACSCalibur flow cytometer (Beckton & Dickinson, Mountain View, CA, USA) after incubating an aliquot of the cell fractions ($5x10^4$ cells) with 2 μL of an anti-CD4-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at 4ºC. Purity of $CD4^+$ T was generally higher than 90%.

– Real-Time quantitative-polymerase chain reaction (RT-PCR)

Reverse transcription reactions with RNA from $CD4^+$ T cells were performed as previously described (12). ITGAL, PRF1, and CD70 were quantified by using Taqman Gene expression assays from Applied Biosystems (Cheshire, UK). Reactions for determining the expression of the gene of interest and β-actin (reference gene) were carried out as duplex PCRs on an ABI PRISM 7000 Sequence Detection System. Each assay included a standard curve for both genes. The standard curve was constructed with serial dilutions of reverse transcription products corresponding to different concentrations of total RNA from a reference cell line (Jurkat). Unknown sample expression levels were then determined from the standard curves and reported in equivalent quantity of total RNA from the reference cell line. Expression ratios between the gene of interest and β-actin were calculated.

Statistical analysis

Either the Mann-Whitney U-test or the independent samples *t*-test for equality of means (along with the Levene's test for equality of variances) were used to compare values. Spearman's rank correlation was used to examine the relationship between two continuous variables. *p*-values less than 0.05 were considered significant. All analyses were performed with the Prism GraphPad 5.0 software.

Results and discussion

Overexpression of ITGAL, PRF1, and CD70 in $CD4^+$ lupus T cells

Gender was not relevant for the expression of ITGAL and PRF1 in the healthy controls and SLE patients. Only a tendency towards showing a higher expression of CD70 in SLE women was observed (1.65±1.67 *vs.* 0.68±1.29, p=0.051). Oelke *et al*. and Kozlowska *et al*. (6, 13) included basically only women in their studies (13 out of 14, and 39 out of 41, respectively). Since in our work the mean levels of CD70 for male SLE and controls were similar, we can not rule out the possibility that the overexpression of CD70 may be found only in female patients.

Although no correlation was observed between age and the expression of the three genes in the SLE population, Spearman's rank negative correlations coefficients were obtained with respect to ITGAL (r=-0.583, p=0.007) in the control group. An impaired ITGAL production may lead to the depressed lymphocyte response of immunosenescence**.** Other T-cell markers have been reported to decrease with age (14). Nevertheless, it should be pointed out that Zhang *et al*. (15) found that ITGAL mRNA levels were higher in older subjects. Their study included a group of middle-aged people (23–50 yrs) and comparisons were established with two other groups: newborns and individuals over 65 years of age. Thus, it is possible that the increase on ITGAL levels observed by these authors may be due to their comparisons being performed considering elderly age groups. Furthermore, they evaluated the whole fraction of T cells, whereas we focused on $CD4^+$ T cells. Consequently, it is

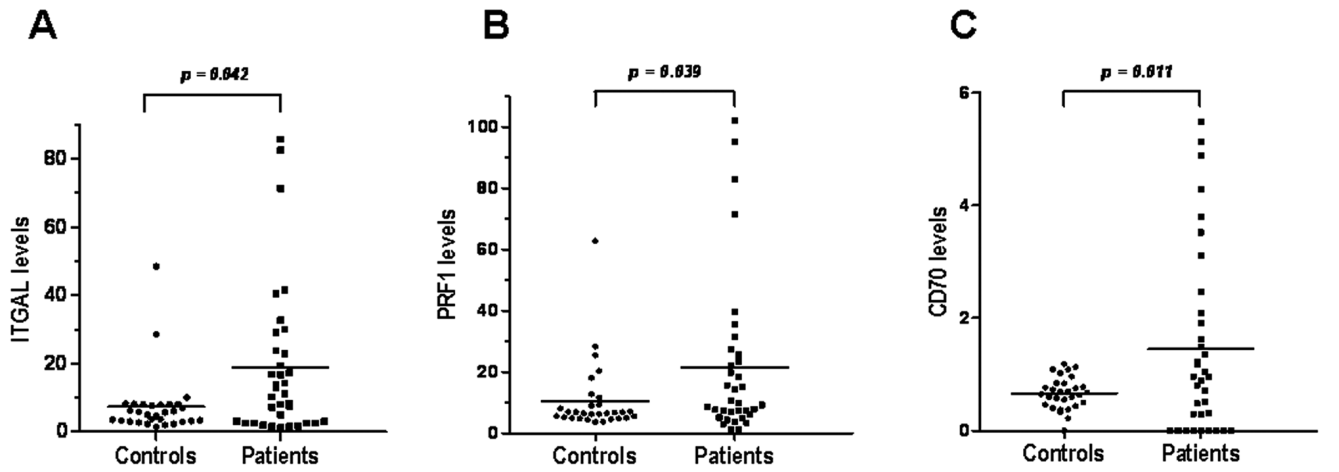

Fig. 1. Comparisons of transcript levels of ITGAL (**A**), PRF1 (**B**), and CD70 (**C**) in CD4+ T cells from healthy controls and SLE patients. Means are indicated as horizontal lines for each group.

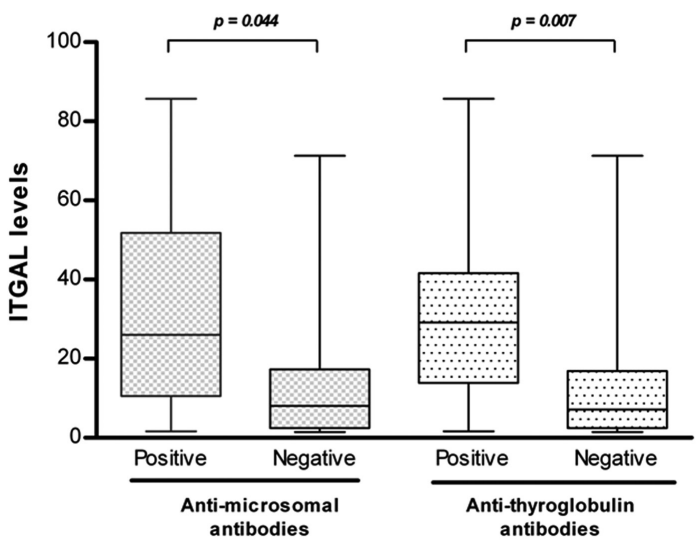

Fig. 2. Higher ITGAL transcript levels were detected in SLE patients with anti-microsomal and anti-thyroglobulin antibodies compared to those who tested negative for these antibodies.

possible that other subsets of T lymphocytes might have accounted for the effect they observed.

As seen in Figure 1, SLE patients had significantly elevated transcript levels of ITGAL (18.61±22.17 *vs*. 7.33±9.17, p=0.042), PRF1 (21.67±26.34 *vs*. 10.67±11.65, p=0.039), and CD70 (1.45±1.63 *vs*. 0.67±0.28, p=0.011). ITGAL could help to overstabilise the normally low affinity interaction between the T-cell receptor (TCR) and the Major Histocompatibility Complex MHC class II molecules (thus decreasing the antigen threshold dose on the presenting cell for T-cell activation) and PRF1 would exert its cytolitic activity. It all would promote the overt apoptosis observed in these patients (16).

Laboratory parameters, clinical manifestations, disease's activity, and treatment: associations with transcript levels of ITGAL, PRF1, and CD70

Patients were grouped according to the presence or absence of the antibodies shown in Table IA (see Supplementary File I for individual SLE patient information). Since the overexpression of CD70 seems to be linked to an overstimulation of IgG synthesis mediated by lupus T cells *in vitro* (6), we thought that an association between the presence of SLE antibodies and the mean levels of CD70 could be established. We did not find such correlation for any of the antibodies but lupus anticoagulant (LA). The three patients with lupus anticoagulant (LA) showed higher transcript levels of CD70 than the thirty patients without LA (3.42±0.91 *vs*. 1.22±1.61, p=0.037). Obviously, the sample number in this case is too small and conclusions are difficult to draw. As for ITGAL and PRF1, we found that transcript levels were not statistically different between those patients who tested positive for any of the characteristic antibodies found in SLE patients and those who were negative.

Interestingly, patients who presented anti-microsomal antibodies had higher transcript levels of ITGAL than those who did not test positive for these antibodies (33.41±30.14 *vs*. 13.58±16.43, p=0.044). Furthermore, patients with anti-thyroglobulin antibodies also had higher levels of ITGAL than patients who were negative for these types of antibodies (34.01±27.66 *vs*. 11.90±16.17, p=0.007) (see Fig. 2). Most of the patients (n=9) tested positive for both antibodies and 5 out of those 12 with any of these antibodies suffered from clinical hypothyroidism. LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligands, ICAMs 1–3. Follicular thyroid cells of autoimmune thyroiditis patients express ICAM-1 (17). Thus, an overexpression of ITGAL in CD4+ T cells may contribute to enhance their interaction with ICAM-1 which, in turn, could lead to gland destruction. Besides thyroid function impairment, it would trigger the formation of the above mentioned anti-thyroid autoantibodies.

As for the other serological and haematological parameters, we must point out that similar transcript levels of the three genes were obtained when comparisons were established between those patients who had low lymphocyte counts, platelet counts, or complement levels, and those who had high levels (see Supplementary File II).

Although almost all of our patients suffered from arthritis (and, hence, derived statistical meanings must be considered with caution), it is worth mentioning that we found higher levels of mRNA ITGAL in patients with arthritis than in those without such clinical manifestation. Thus, the mean levels of ITGAL when considering the patients with arthritis were 20.77±22.69, whereas those of the patients without this clinical feature were 1.88±0.82 (p=0.007). Using murine models it has been demonstrated that LFA-1 is absolutely required for the development of arthritis (18). Consequently, it seems logical to think that the increase of ITGAL observed on $CD4^+$ T cells of subjects affected with SLE may help to induce arthritis by supporting the usual leukocyte infiltration of synovial fluid and tissues so characteristic of inflammatory arthritis.

Patients with a SLEDAI <6 and patients with a SLEDAI ≥6 had similar levels of the three genes. Moreover, no correlations were found between the SLEDAI scores and the levels of any of them. Several reports have indicated that the expression of ITGAL, PRF1, and CD70 is usually correlated with disease activity (3, 5, 6). This discrepancy may be due to the fact that we included more patients in our study. Actually, our results are in accordance with those of Kozlowska *et al*. (13, 19), who also recruited a considerable number of SLE patients (n=41) and did not find any correlation either between SLEDAI and CD70 or PRF1 expression. In our opinion, it was the clinical status that marked the tendency to show higher transcript levels of the genes, especially of ITGAL and PRF1. Accordingly, the four asymptomatic patients (and therefore, truly inactive patients) included in our study showed lower ITGAL (2.24±0.82) and PRF1 levels (4.36±2.58) than those patients at flare (20.73±22.73 for ITGAL, p=0.013; 23.90±27.21 for PRF1, p=0.013).

Finally, no differences were observed between the 25 treated patients and the 10 patients whose blood sample was withdrawn at disease onset (non-treated group). Previous reports have not found any association between drugs and the transcript levels of ITGAL, PRF1, and CD70 (3, 5, 6, 13). Therefore, the changes in expression of the three genes are unlikely to be caused by medication.

Our investigation demonstrates that the expression of ITGAL, PRF1, and CD70 is upregulated in $CD4^+$ T cells from SLE patients. Nevertheless, it is not linked to the typical clinical and serological parameters associated with the disease. The role that ITGAL may play in autoimmune thyroiditis deserves further investigation.

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