Circulating soluble CD28 in patients with Behçet's disease: Relationship to clinical manifestations

K. Hamzaoui[1], A. Hamzaoui[1,2], L. Bouajina[3], H. Houman[4]

[1]*Medicine University of Tunis, Homeostasis and Cell Dysfunction Unit Research 99/UR/08-40, Tunis;* [2]*Pneumology Hospital A. Mami, Department of Respiratory Diseases, Ariana;* [3]*Farhat Hached Hospital, Department of Rheumatology Diseases, Sussa;* [4]*La Rabta Hospital, Department of Internal Medicine, Tunis, Tunisia.*

Kamel Hamzaoui, PhD; Agnès Hamzaoui, MD; Lies Bouajina, MD; Habib Houman, MD.

Please address correspondence to: Prof. Kamel Hamzaoui, PhD, Medicine University Tunis, no. 15 Rue Djebel Lakdar, 1007 Tunis, Tunisia.
E-mail: Kamel.Hamzaoui@fmt.rnu.tn

Received on August 6, 2004; accepted in revised form on March 29, 2005.

Clin Exp Rheumatol 2005; 23 (Suppl. 38): S49-S52.

© Copyright CLINICALAND EXPERIMENTAL RHEUMATOLOGY *2005.*

Key words: Behçet's disease, soluble CD28.

ABSTRACT

Objective. *This study evaluates the presence of serum soluble CD28 (sCD28) in Behçet's disease (BD) and its relationship with clinical manifestations.*

Methods. *Soluble CD28 concentration was determined by ELISA in 120 patients with BD (80 patients in active stage), 60 patients with rheumatoid arthritis (RA) and 60 healthy subjects.*

Results. *Concentrations of sCD28 were significantly higher in patients with BD and RA than in healthy subjects. Patients with active BD expressed the highest level of sCD28 in serum. Soluble CD28 exhibited a drastic increase in active BD patients, compared to BD in remission. Soluble CD28 concentrations were higher in patients with active BD patients having vasculitis. Significant positive correlation was observed in a longitudinal study of 15 BD patients, between sCD28 and C-reactive protein.*

Conclusion. *Our study suggests that fluctuations of sCD28 in BD reflects disease activity and should be assessed in evaluating disease activity.*

Introduction

Behçet's disease (BD) is an autoimmune disease (1) characterized by recurrent oro-genital ulcers, erythema nodosum-like skin lesions, uveitis, and arthritis. It affects many organ systems and sometimes causes serious complications, such as visual loss, cerebrovascular accidents or sudden death. Vasculitis is being its major pathological feature (2, 3). Uveitis, neurological involvement and pulmonary manifestations cause devastating complications (4). BD is characterized by spontaneous remissions and relapses similar to those of various autoimmune diseases. Neutrophil hyperactivity with increased superoxide production, phagocytosis, release of enzymes and implication of HSPs in BD suggest an activated innate immunity (5). Investigation of the aetiology of BD has focused predominantly on herpes simplex virus immunopathology, streptococcal infection and autoimmunity to oral or cross-reactive microbial antigens (6, 7).

In a number of situations ranging from chronic inflammatory conditions, infectious (8, 9) to autoimmune diseases (10), a dysregulation in $CD8^+$ T cells by lacking CD28 molecule (costimulatory molecule) was reported. Recently, lack of CD28 expression on BD-T cells was described (11). $CD8^+CD28^-$ T cells are characterized by morphological and functional features of activated/memory T cells (12). CD28 is constitutively expressed by T cells (13) and interacts with the molecules, CD80 (14) and CD86 (15). CD28 is recognized to play a primordial role for the priming of naive Tcells (16).

Based on the critical role played by CD28 in the immune response of certain viral and/or autoimmune diseases, the aim of this study was to evaluate whether soluble CD28 could be detected in patients with BD, and its relevance to severe BD manifestations.

Material and methods

Patients

This study was performed on 120 patients with BD [sex ratio(M/F): 97/23, mean age 32.4 years ± 6.2 (range 27.2-52)], 60 patients with RA [40 in active stage) according to the American College of Rheumatology (ACR) criteria (17), sex ratio: 18/22, mean age 52.6 years ± 10.8 (range 48-65)], and 60 healthy controls [(sex ratio: 42/18, mean age 35.8 years ± 9.5 (range 32-58)]. Active RAwas defined as the presence of at least two of the following three criteria: Thompson joint score > 10, erythrocyte sedimentation rate (ESR) ≥ 28 mm/1st h, and early morning stiffness ≥ 1 hour.

Active BD patients (n = 80) (Table I),

Table I. Characteristics of Behçet's disease patients (n = 80).

	Number of patients (%)
Age (mean ± SD, years)	32.4 ± 6.2
Sex (Male)	72 (90.0)
Duration of the disease (mean ± SD, years)	12 ± 9
HLA-B51	56 (70.0)
Frequencies of clinical manifestations	
Oral ulcer	80 (100)
Genital ulcer	80 (100)
Skin lesions	76 (95.0)
Erythema nodosum	78 (97.5)
Folliculitis	57 (71.3)
Uveitis	49 (61.2)
Skin pathergy response	68 (85.0)
Arthritis	42 (52.5)
Vasculo-Behçet	17 (21.3)
Neuro-Behcet (*)	22 (27.5)
Pulmonary manifestations (**)	15 (18.7)

*Persistent and progressive central nervous system manifestations.
**Chronic cough, pulmonary aneurysms.

were showing at least 3 of the 4 major symptoms defined by the International Study Group (18), including recurrent aphthous stomatitis, uveitis, genital ulcers, and skin lesions (erythema nodosum, folliculitis or subcutaneous thrombophlebitis). Peripheral blood was obtained from active BD before treatment. Remission BD group was composed of asymptomatic patients (mean duration of remission 2 months). Erythrocyte sedimentation rate (ESR normal range: 1-5 mm/h), and blood C-reactive protein level (CRP normal range < 5.0 mg/l) were obtained in all active patients. Fifteen BD patients were studied in both stages: active and remission.

Detection of soluble CD28 by ELISA

An immunoreactive form of CD28 was evaluated in human serum by ELISA. The anti-CD28 mAb (clone CD28.2) (BD Biosciences, Palo Alto, CA, USA) was coated (2 ng/100 µl/well) in 96 well plates (Nunc, Oslo, Norway) in 0.1M phosphate buffer pH 4.0 (16 h at 4°C) before incubation for 2 h at room temperature with PBS/BSA 1%. After washing, plates were incubated for 16 h at 4°C with undiluted serum (200µl/well). After washings, bound CD28 was detected with a biotin labelled anti-CD28 polyclonal antibody (R&D Systems, Abingdon, UK), followed by incubation with streptavidin-biotinylated HRP (used at 1/5000; Amersham Biosciences, Uppsala, Sweden) revealed with the substrate o-phenylene diamine (Sigma, St Louis, MO, USA). The specificity was determined using recombinant soluble CD28 and, as negative controls, soluble CD86 (19), soluble CTLA-4-Fc (Ancell, Bayport, MN, USA) and human cytokines (all from R&D Systems). Results (mean ± sd), are expressed in ng/ml.

Statistical analysis

We used conventional chi-square and Fisher's exact test to analyse qualitative differences, Student's test for comparison of means in large samples of similar variance, and the non-parametric Mann-Whitney U test for small samples. Values of quantitative variables are expressed as mean ± standard error of the mean (SEM). A value of $p < 0.05$ was taken to indicate statistical significance. This statistical analysis was performed by means of the SPSS program using the information stored in the data-base program.

Results

Serum soluble CD28 concentrations

Soluble CD28 was detected in the serum of all patients and controls. The levels of sCD28 were respectively of 18.04 ± 9.84 ng/ml in healthy controls, 98.55 ± 52.5 ng/ml in patients with active RA, and 248.9 ± 82.45 ng/ml in active BD patients (Fig. 1). The mean sCD28 concentration in active BD was significantly higher than sCD28 in active RA patients ($p < 0.001$). In the same way sCD28 in remission BD (57.58 ± 40.32 ng/ml) was significantly increased when compared to inactive RA (14.2 ± 10.62 ng/ml $p < 0.001$). There was no significant difference observed between healthy controls and inactive RA.

BD patients suffering from nervous or pulmonary vasculitis (n = 17) express higher levels of sCD28 (306.6 ± 92.37 ng/ml), than those free from these clinical manifestations (233.61 ± 73.20 ng/ml, $p < 0.001$).

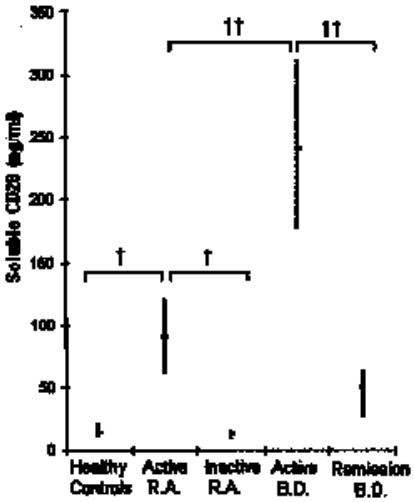

Fig. 1. Levels of soluble CD28 (sCD28) in Behçet's disease (BD) patients. Immunoreactive form of CD28 was evaluated in serum by ELISA, in healthy controls, active and inactive rheumatoid arthritis (RA), and in BD patients in active and remission stages. Values were expressed as median and the 25th-75th percentile. Comparison of median between different groups was performed using Mann-Whitney U test. (†): $p < 0.005$; (††): $p < 0.001$.

Fifteen BD patients were studied successively during active and remission stage. Active patients (Fig. 2) showed a significant decrease of sCD28 level during remission. The blood samples showed a marked elevation in ESR (16.4 ± 8.1 mm/h) and CRP (8.7 ± 2.9 mg/l) in all (n= 15) active BD ($p < 0.001$ for both). A significant correlation was observed between CRP and sCD28 (Fig. 3).

Discussion

The function of soluble forms of membrane molecules is unknown, but they may have regulatory function in inflammatory responses by binding counter receptors to interacting cell types, thereby mediating the influx of leucocytes into tissue (20). CD28 is a co-stimulatory molecule that plays a primordial role for the priming of naive T cells. Soluble CD28 may be involved in the regulatory network of T cells activation and proliferation as an altered T cell proliferation is observed in presence of soluble CD28.

In the present study, we report the de-

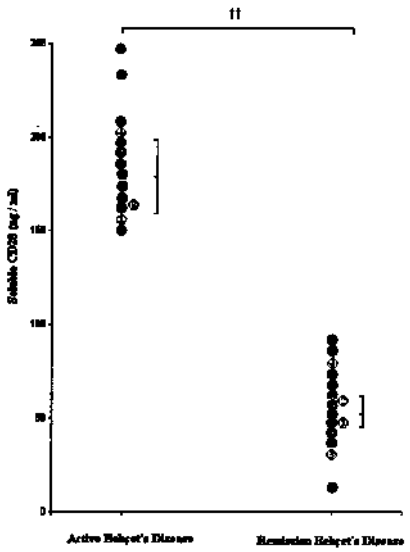

Fig. 2. Individual data points of soluble CD28 (sCD28) level in patients with Behçet's disease (BD) according to their clinical stage. Soluble CD28 was evaluated in serum by ELISA. Values were expressed as median and the 25th-75th percentile. Each number represents the same patient in a successive active and remission phase. Comparison of median between different groups was performed using Mann-Whitney U test. (††): p< 0.001.

tection of soluble CD28 in the serum of patients with BD and RA. The concentrations of soluble CD28 were significantly increased in these patients during active stages compared with healthy subjects. In patients with RA and BD, levels of soluble CD28 correlated with disease activity. Inactive RA expressed sCD28 level similar to healthy controls. During remission BD patients lowered sCD28 expression, however it persisted significantly increased compared to healthy controls and inactive RA. BD exacerbations were associated with a rise in sCD28 expression.

The higher expression of CD28 was observed in more severe BD patients, whose suffering from nervous or pulmonary vascular lesions. The association of these severe clinical manifestations with vasculitis led to a chronic course of the disease with periodic exacerbations and progressive deterioration (21). Auto-immune diseases patients (systemic sclerosis, Sjögren's syndrome and systemic lupus erythematosus) exhibit high levels of sCD28, and the increase could reflect disease severity (22).

Soluble CD28 can be produced either by membrane shedding as suggested by RTPCR analysis detecting only the full length CD28 transcript in peripheral blood cells from patients exhibiting soluble CD28 (22) or result from alternative mRNA splicing (23). The increased expression of sCD28 in active BD reflects an inflammatory state, leading probably to the shedding of the CD28 membrane form, which in the other hand explain the lack of CD28 marker on CD8 T lymphocytes (11). Purification and sequencing of circulating soluble CD28 in active BD are in progress.

Patients with active BD were characterized by T cells hyperactivity (24). Bacterial stimulation and host hypersensitivity might be involved in the symptoms and pathogenesis of BD (25). As sCD28 production is highly correlated in BD with serum CRP, it could argue in favour of microbial involvement in BD pathogenesis.

We suggest that sCD28 in BD could be involved, in T cell inflammation and vasculitis lesions. Reports of elevated serum concentrations of von Willebrand factor, plasminogen activator inhibitor-1, thrombomodulin (26) and high endothelin-1 (ET-1) expression (27), suggest the presence of vascular endothelial dysfunction in patients with Behçet's disease (26). We observed the higher levels of sCD28 in patients suffering from the more severe vascular lesions. The next step of our work will investigate the possible correlations between sCD28 and ET-1 in serum and in biological fluids in BD.

Measurements of sCD28 levels might be of potential value as surrogate markers for clinical progression or remission in BD. Active BD compared to active RA in this study find a significant difference in serum sCD28 level. Levels of sCD28 may reflect the shedding of the molecule at the end of its lifespan, as it is likely that leucocytes shed their adhesion ligands by metalloproteinase digestion (28). The frequency of CD28 negative T cells is increased in BD. Consequently, high levels in active BD patients may be due to increased shedding of cell-bound CD28-molecule.

To conclude, we showed that soluble

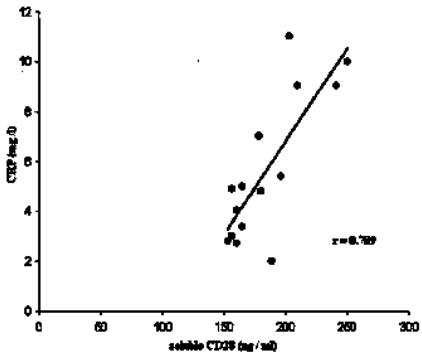

Fig. 3. Correlation between soluble CD28 (sCD28) and C-reactive protein (CRP) levels in 15 BD patients. The solid line represents the regression line and r value shown is the Spearman rank correlation. Significant positive correlation was observed (r = 0. 789, p = 0.0001).

CD28 concentrations are increased in patients with BD. The value of soluble CD28 concentrations measurement in evaluating disease activity should be assessed with reservation, until complete confirmation. Measurement of these sCD28 may increase our understanding of the relationship between *in vivo* cell activation and inflammation involved in BD.

References

1. LEE EB, JEONG YK J, YUN JL Y, PARK MH, YEONG WS Y: TNF and TNF receptor polymorphisms in korean Behcet's disease patients. *Human Immunology* 2003; 64: 614-20.
2. SARUHAN-DIRESKENELI G, YENTÜR SP, AKMAN-DEMIR G, ISIK N, SERDAROGLU P: Cytokine and chemokines in Neuro-behçet's disease compared to multiple sclerosis and other neurological diseases. *J Neuroimmunol* 2003; 145: 127-34.
3. DINC A, TAKAFUTA T, JIANG D, MELIKOGLU M, SARUHAN-DIRESKENELI G, SHAPIRO SS: Anti-endothelial cell in Behçet's disease. *Clinical Exp Rheumatol* 2003; 21: S27-S30.
4. SERDAROGLU P, ERKAN F, GÜL A, TASSALI E: Pulmonary manifestations of Behçet's disease. *Thorax* 1998; 2001; 56: 572-8.
5. DIRESKENELI H, SARUHAN-DIRESKENELI G: The role of heat shock proteins in Behçet's disease. *Clin Exp Rheumatol* 2003; 21: S44-S48.
6. EGLIN RP, LEHNER T, SUBAK-SHARPE JH: Detection of RNAcomplementary to herpes-simplex virus in mononuclear cells from patients with Behçet's syndrome and recurrent oral ulcers. *Lancet* 1982; 18: 1356-61.
7. LEHNER T: The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. I*nt Rev Immunol* 1997; 14: 21-32.
8. HISLOP AD, GUDGEON NH, CALLAN FCM *et al.*: EBV-specific CD8+ T cell memory:

relationships between epitope specificity, cell phenotype, and immediate effector function. *J Immunol* 2001; 167: 2019-29.

9. MITTRÜCKER HW, KURSAR M, KÖHLER A, HURWITZ R, KAUFMANN SHE: Role of CD-28 for the generation and expansion of antigen-specific CD8+ T lymphocytes during infection with Listeria monocytogenes. *J Immunol* 2001; 167: 5620-7.
10. SCHRIMER M, GOLDBERGER CH, WÜZNER R *et al.*: Circulating cytotoxic CD8+CD28- T cells in ankylosing spondylitis. *Arthritis Res* 2002; 4: 71-6.
11. HOUMAN H, HAMZAOUI A, BEN GHORBAL I, SMITI KHANFIR M, FEKI M, HAMZAOUI K: Tc1/Tc2 ratio in the inflammatory process in patients with Behçet's disease. *Mediators of Inflammation* 2004; 13: 247-53.
12. AROSA FA: CD8+CD28- T cells: Certainties and uncertainties of a prevalent human T-cell subset. *Immunol Cell Biol* 2002; 80: 1-13
13. GIMMI CD, FREEMAN GJ, GRIBBEN JG, GRAY G, NADLER LM: Human T cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc Natl Acad Sci USA* 1993; 90: 6586-90.
14. LINSLEY PS, CLARK EA, LEDBETTER J: T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7BB-1. *Proc Natl Acad Sci USA* 1990; 87: 5031-5.
15. AZUMA M, ITO D, YAGITA H, OKUMURA K *et al.*: B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 1993; 366: 76-9.
16. COYLE AJ, GUTIERREZ-RAMOS JC: The expanding B7 family: increasing complexity in costimulatory signals regulating T cell function. *Nat Immunol* 2001; 2: 203-9.
17. ARNETT FC, EDWORTH SM, BLOCH DA *et al.*: The American Rheumatism Association, 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
18. INTERNATIONAL STUDY GROUP FOR BEHÇET'S DISEASE: Criteria for diagnosis of Behçet's disease. *Lancet* 1990; 335: 1078-80.
19. JEANNIN P, MAGISTRELLI G, AUBRY J *et al.*: Soluble CD86 is a costimulatory molecule for human T lymphocytes. *Immunity* 2000; 13: 303-12.
20. GEARING AJ, NEWMANN W: Circulating adhesion molecules in disease. *Immunol Today* 1993; 14: 506-12.
21. VERJANS MGM, MARTIN VAN HAGEN P, VAN DER KOOI A, OSTERHAUS DME, SEERP BAARSMAG: Vg9Vd2 T cells recovered from eyes of patients with Behçet's disease recognize non-peptide peryl-pyrophosphate antigens. *J Neuroimmunol* 2002; 130: 46-54.
22. HEBBAR M, JEANNIN P, MAGISTRELLI G *et al.*: soluble CD28 in patients with systemic lupus erythematosus, primary Sjögren's syndrome and systemic scleroris. *Clin Exp Immunol* 2004; 136: 388-92.
23. MAGISTRELLI G, JEANNIN P, ELSON G *et al.*: Identification of three alternatively spliced variants of human CD28 mRNA. *Biochem Biophys Res Commun* 1999; 259: 34-7.
24. MEGEJL, DILSENN, SANGUEDOLCE V *et al.*: Overproduction of monocyte derived tumor necrosis factor alpha, interleukin (IL) 6, IL-8 and increased neutrophil superoxide generation in Behçet's disease: a comparative study with familial Mediterranean fever and healthy subjects. *J Rheumatol* 1993; 20: 1544-9.
25. KURAUCHI T, YOKOTA K, MATSUO T *et al.*: Neutrophil and lymphocyte response to oral Streptococcus in Adamantiades-Behçet's disease. *FEMS* 2005; 43: 125-31.
26. CHAMBERS JC, HASKARD DO, KOONER JS: Vascular endothelial function and oxidative stress mechanisms in patients with Behçet's Syndrome. *JACC* 2001; 37: 517-20.
27. HAMZAOUI A, HAMZAOUI K, CHABBOU A, AYED KH: Endothelin-1 expression in serum and bronchoalveolar lavage from patient with active Behçet's disease. *British J Rheumatol* 1999; 35: 357-8.
28. PRECEE G, MURPHY G, AGER A: Metalloproteinase mediated regulation of L-selectin levels on leukocytes. *J Biol Chem* 1996; 271: 11634-40.