Clin Exp Immunol 1997; **108**:42–46

Circulating levels of soluble CD30 are increased in patients with systemic sclerosis (SSc) and correlate with serological and clinical features of the disease

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(Accepted for publication 27 December 1996)

SUMMARY

Activated Th2 lymphocytes express the surface molecule CD30 and release a soluble form of the same molecule which can be detected both *in vivo* and *in vitro*. In the present study, high levels of soluble CD30 were found in the peripheral blood of patients with SSc, and a significant correlation with skin score and erythrocyte sedimentation rate (ESR) was detected. Furthermore, we observed a higher spontaneous release of soluble CD30 in the supernatants of unstimulated cultures of peripheral blood mononuclear cells from our patients compared with healthy controls. Taken together, these data suggest a possible involvement of Th2 cells in the immunopathogenesis of SSc, and the dosage of CD30 soluble in the peripheral blood may be helpful in following the outcome of the disease.

Keywords systemic sclerosis CD30 T cell

INTRODUCTION

SSc is a connective tissue disease in which autoimmune mechanisms, such as autoantibody production and lymphocytic infiltration, seem to be related to disease pathogenesis. In fact, several reports suggest that an ongoing T cell activation can mediate many of the serological and clinical findings of SSc, and it is well known that most infiltrating lymphocytes in the target organs show a CD4 phenotype predominance [1]. There is evidence that $CD4^+$ helper T cells (Th) include at least two different subsets, generally referred to as Th1 and Th2, which produce a different pattern of cytokines following activation and exhibit different functions [2,3]. Th1 cells mainly secrete IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor (TNF), induce DTH reactions and immunoglobulin IgG2a isotype switching. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, provide help for B cell activation, antibody production and IgE isotype swtching [4].

In recent years, the role of these two cellular subsets in various experimental autoimmune diseases has been studied, and increasing evidence suggests their importance in the development of human autoimmune diseases [5]. T cell clones derived from patients affected by multiple sclerosis have a Th1 cytokine profile, and increased levels of TNF in plasma or cerebrospinal fluid are predictive of relapses [6]. A homogeneous Th1 profile has been observed in $CD4^+$ T cell clones obtained from retroorbital infiltrates of patients with Graves' ophthalmopathy during autoimmune thyroid disease [7]. Peripheral blood lymphocytes from untreated systemic lupus erythematosus (SLE) patients produce high amounts of IL-10, and transfer of these cells into severe combined immunodeficient (SCID) mice induced the production of IgG anti-DNA antibodies, which was specifically abrogated following administration of anti-IL-10 antibodies [8].

Recent studies suggest that the CD30 molecule, a member of the TNF/nerve growth factor receptor superfamily [9], is generally expressed on activated human T $CD4^+$ cell clones producing Th2-type cytokines [10]. Its surface expression is also inducible on a small proportion of T $CD8^+$ cells [11] and on B lympocytes activated by lectin or virus stimulation [12]. Under normal conditions T cells expressing CD30 are barely detectable *in vivo* [13]. The CD30 extracellular portion is proteolytically cleaved to produce a soluble form (sCD30), released by activated CD30-expressing cells both *in vivo* and *in vitro* [14]. High levels of sCD30 can be detected in peripheral blood and synovial fluid from patients affected by rheumatoid arthritis (RA) [15] and in sera of patients with SLE. Moreover, these levels correlate with the disease activity [16].

Polyclonal B cell activation and hypergammaglobulinaemia [17], autoantibody production [18] and increased levels of IL-4 [19] and IL-6 in serum [20] and supernatants of peripheral blood mononuclear cell (PBMC) cultures [21] have been shown in SSc patients, suggesting Th2 cell involvement in the pathogenesis of the disease. These data prompted us to evaluate sCD30 levels in sera of SSc patients and to correlate these findings with clinical (skin score, diffusing capacity of the lung for carbon monoxide (DLCO), Raynaud's phenomenon) and laboratory (ESR) features of the disease. Furthermore, we evaluated in the same patients the percentage of circulating T lymphocytes expressing the CD30

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Table 1. Clinical and laboratory details of SSc patients (diffuse disease nos 1–4; limited disease nos 5–12)

Patient	Age (years)	Skin score	ESR (mm/h)	Centromere antibody	Topoisomerase antibody	Organ involvement	sCD30 (U/ml)
1	70	35	120	Neg.	Pos.	E, P, I	200·0
2	62	16	95	Neg.	Pos.	E, P, I	100·0
3	50	24	55	Neg.	Neg.	E, P	8·6
4	65	20	30	Pos.	Neg.	E, P	15·9
5	58	10	80	Pos.	Neg.	E, P	63·0
6	45	10	20	Pos.	Neg.	E, P	7·8
7	54	22	30	Pos.	Neg.	E, P, I	16·2
8	61	12	25	Pos.	Neg.	E, P	14·4
9	59	12	20	Pos.	Neg.	P	10·3
10	73	4	35	Pos.	Neg.	E, P	26·6
11	49	20	22	Neg.	Pos.	E, P	12·5
12	31	22	18	Neg.	Pos.	E, P, I	8·5

E, Oesophageal; P, pulmonary; I, intestinal; ESR, erythrocyte sedimentation rate.

molecule and sCD30 levels in supernatants of both unstimulated and stimulated cultures of their PBMC, in order to investigate whether a Th2 response is involved in SSc.

PATIENTS AND METHODS

Patients and controls

After informed consent was obtained, 12 women, aged 31–73 years (mean age 54·4 years), with SSc diagnosed according to the preliminary criteria of the American Rheumatism Association were studied [22]. Four patients were classified as having diffuse and eight as having limited disease according to LeRoy *et al.* [23]. Skin assessment was performed utilizing a modified Rodnan (m-Rodnan) total skin thickness score technique, in which 17 anatomical areas are scored, using a 0–3 scale [24]. All patients had a detailed clinical assessment for involvement of the internal organs. All but one exhibited oesophageal dismotility, evaluated by manometry. Pulmonary fibrosis (diagnosed by conventional chest x-ray, vital capacity < 80%, and DLCO < 70% predicted) and Raynaud's phenomenon were invariably present. Four patients showed symptoms referable to intestinal involvement (steatorrhoea with increased faecal fat excretion and weight loss). Eight patients had never been treated with cytotoxic drugs or biologic response-modifying agents during the 12 months preceding the study; four patients who were receiving steroids (prednisone 10 mg/day) discontinued the treatment 30 days before immunological assays. Laboratory data of the SSc patients are summarized in Table 1. Twelve healthy volunteers, mean age 58·2 years, were enrolled among the department personnel as controls.

Blood samples

In order to avoid chronobiological variations of sCD30 release, blood samples were collected from SSc patients and controls in a synchronized fashion during the previous spring. Sera were recovered and aliquoted into vials, stored frozen at −80°C and defrosted immediately before assay.

Cell cultures

Heparinized venous blood was obtained from all patients and controls and PBMC were isolated by centrifugation on Ficoll–Hypaque gradient, counted, and their viability, as assessed by trypan blue dye exclusion, was routinely > 95%. PBMC, at a density of 2×10 cells/ml (in RPMI 1640, with 10% heat-inactivated fetal calf serum (FCS) supplemented with 1% L-glutamine, 1100 U/ml penicillin, 200 μg/ml streptomycin and 100 μg/ml gentamycin) were incubated in the presence of medium alone or medium plus phytohaemagglutinin (PHA; Biochrom KG, Berlin, Germany) at a final concentration of 10 μg/ml, in 96-well culture plates and cultured at 37°C in a 5% CO_2 atmosphere. Supernatants of unstimulated and stimulated cells were harvested after 24 h and 48 h of culture.

Assay for sCD30 in supernatants and blood samples

Supernatants and sera were harvested and sCD30 was measured by ELISA test kits (Dako CD30-Ki antigen ELISA; Dako A/S, Glostrup, Denmark). These assays were based on the dual immunometric sandwich principle and were performed according to the manufacturer's instructions.

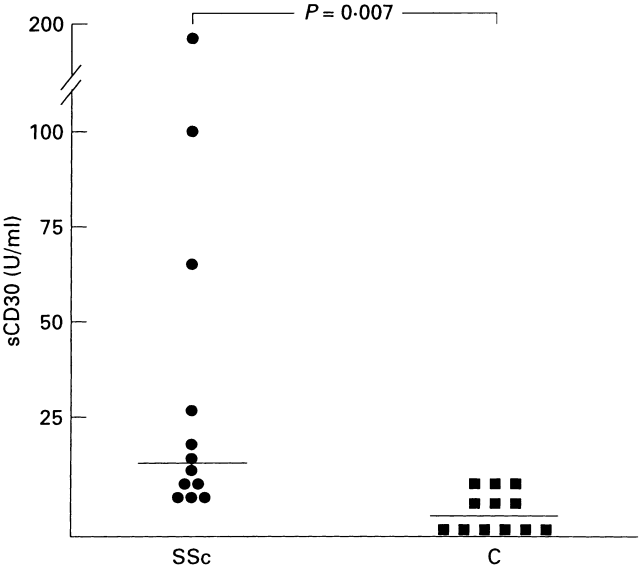

Fig. 1. Serum levels of sCD30 in SSc patients (●) and controls (■). Bars represent medians. The difference is statistically significant ($P < 0{\cdot}007$, Wilcoxon's tailed test).

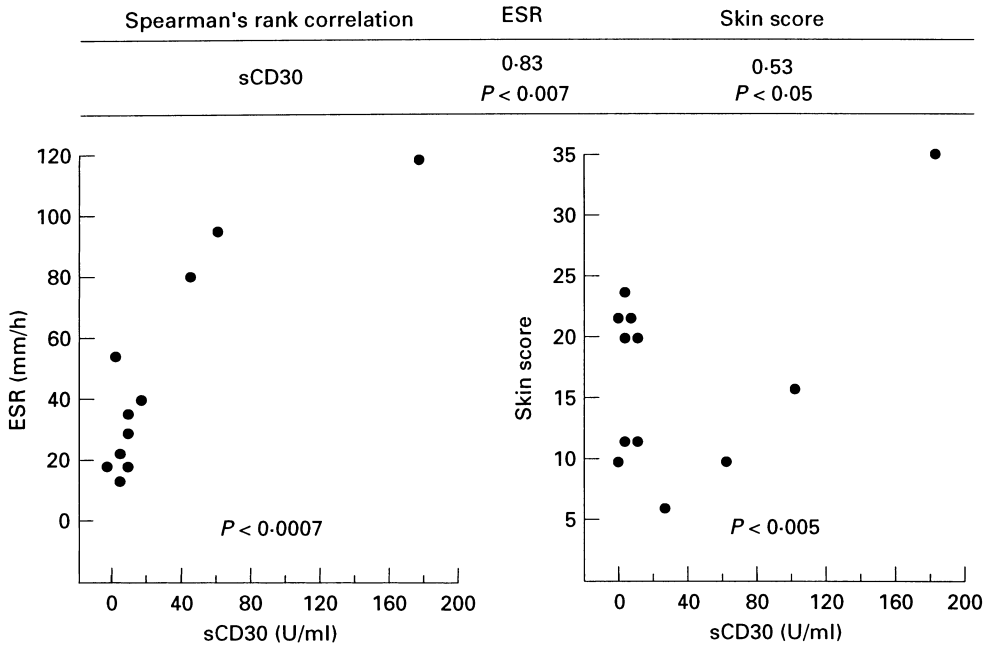

Fig. 2. Significant associations identified by Spearman's rank correlation of the relationship of sCD30 with erythrocyte sedimentation rate (ESR) ($P < 0{\cdot}007$) and with the skin score ($P < 0{\cdot}05$). The two illustrations show the correlations identified by simple linear regression between sCD30 levels and the same serological and clinical features (ESR $P < 0{\cdot}0007$; skin score $P < 0{\cdot}005$).

Surface phenotype of PBMC

Coexpression of CD30 on circulating lymphocytes was investigated by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) after incubation with an FITC-conjugated anti-CD30 MoAb (Ki-1 antigen, CD30/FITC; Dako) in combination with PE-conjugated MoAbs against CD3, CD4 and CD8 molecules (Leu-4, Leu-3 and Leu-2, respectively; Becton Dickinson).

Statistical analysis

The non-parametric Wilcoxon's two-tailed test (normal approximation), the one-way analysis of variance (ANOVA), the Spearman's correlation coefficient and simple linear regression associated to the least squares method and χ^2 were used as appropriate for statistical analysis of data.

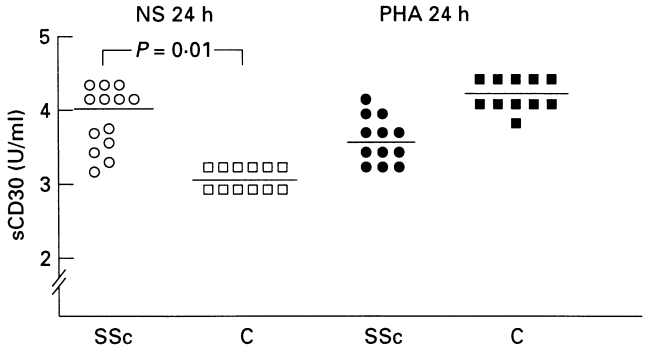

Fig. 3. sCD30 levels in supernatants of both unstimulated and phytohaemagglutinin (PHA)-stimulated cultures of SSc patients and controls (unstimulated cultures of patients (○) and controls (□); PHA-stimulated cultures of patients (●) and controls (■)) after 24 h. A significant increase of sCD30 levels in unstimulated cultures of SSc patients can be detected ($P < 0{\cdot}01$, Wilcoxon's tailed test). No differences were observed between patients and controls after 24 h of PHA stimulation.

RESULTS

Detection of sCD30 in sera and clinical correlations

Increased circulating sCD30 levels were observed in our patients, and this finding correlates with clinical and laboratory features of the disease. In fact, our results, expressed as median and range, showed a significant increase of sCD30 in sera of SSc patients (16·2 U/ml, range 7·8–200·0 U/ml) compared with controls (4·5 U/ml, range 3·1–6·8 U/ml, $P < 0{\cdot}007$; Fig. 1).

As shown in Fig. 2, a statistically significant correlation between sCD30 levels and both skin score and ESR could be detected, in our patients, by Spearman's rank method and simple linear regression. In contrast, no correlations were observed between sCD30 and the other two clinical features (DLCO and Raynaud's phenomenon) studied.

Detection of sCD30 in supernatants

Raised sCD30 levels were detected both in 24 h (Fig. 3) and 48 h (Fig. 4) unstimulated cultures of PBMC of SSc patients compared with controls (C) (24 h: 4·0 U/ml, range 3·0–4·3 U/ml in SSc *versus* 3·0 U/ml, range 2·9–3·0 U/ml in C, $P < 0{\cdot}01$; 48 h: 4·2 U/ml, range 3·2–11·2 U/ml in SSc *versus* 3·3 U/ml, range 3·0–4·0 U/ml in C, $P < 0{\cdot}05$). Furthermore, we observed that sCD30 levels in supernatants of both 24 h and 48 h PHA-stimulated cultures of SSc patients did not differ significantly from the levels of their unstimulated cultures. In contrast, in controls both 24 h and 48 h PHA-stimulated cultures showed a significant increase of sCD30 compared with unstimulated cultures (24 h: 3·0 U/ml, range 2·9–3·0 U/ml in unstimulated cultures *versus* 4·1 U/ml, range 4·0–4·2 U/ml in PHA-stimulated cultures, $P < 0{\cdot}007$; 48 h: 3·3 U/ml,

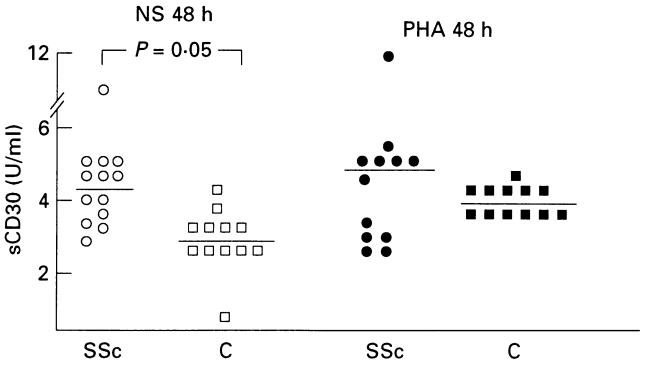

Fig. 4. sCD30 levels in supernatants of both unstimulated and phytohaemagglutinin (PHA)-stimulated cultures of SSc patients and controls (unstimulated cultures of patients (○) and controls (□); PHA-stimulated cultures of patients (●) and controls (■)) after 48 h. A significant increase of sCD30 levels in unstimulated cultures of SSc patients can be detected ($P < 0{\cdot}05$, Wilcoxon's tailed test). No differences were observed between patients and controls after 48 h of PHA stimulation.

range 0·0–4·0 U/ml in unstimulated cultures *versus* 3·9 U/ml, range 3·6–4·2 U/ml in PHA-stimulated cultures, $P < 0{\cdot}01$), suggesting a normal kinetic of sCD30 release.

Cell surface phenotype

Irrespective of the sCD30 levels, a low percentage of circulating cells expressing CD30 on their surface was observed both in patients and controls ($CD30^+/CD3^+$: 1·5%, range 0·3–1·9% in patients compared with 0·9%, range 0·4–1·7% in C; $CD30^+/CD4^+$: 0·6%, range 0·1–1·3% in patients compared with 0·6%, range 0·1–1·2% in C; $CD30^+/CD8^+$: 0·3%, range 0·1–0·5% in patients compared with 0·2%, range 0·1–0·3% in C). All these results were not statistically significant.

DISCUSSION

Recent studies showed that an imbalance of Th1/Th2 response may have an impact on the pathogenesis of some pathologic conditions, such as allergy [25], infectious diseases [26] and autoimmunity [27]. CD30 surface expression on T cells and the release of sCD30 are generally considered a feature of Th2 activation and proliferation [10]. Thus, the detection of this soluble form may be used as a tool to investigate, *in vivo* and *in vitro*, the possible occurrence of Th2 cell activation in these diseases. Our study is the first to demonstrate that sCD30 levels are increased in sera of SSc patients and correlate with clinical and serological features, suggesting a possible involvement of Th2-type immune response in SSc. Th2 cells provide efficient help for autoantibody B cell production and polyclonal B cell activation [3]. Furthermore, these cells seem to be critically implicated in the pathogenesis of autoimmune manifestations observed in the context of allogeneic reactions. In fact, alloreactive T cells in graft-*versus*-host disease, a natural model of SSc, produce both IL-4 and IL-10, but not IL-2 or IFN-γ [28]. High levels of circulating IL-4 were found in SSc patients [19] and in the supernatants of their *in vitro* stimulated PBMC [21]. In contrast, IFN-γ was not found in the sera of these patients [19], and its mean level, observed in the supernatant of SSc-stimulated PBMC, was significantly lower than in healthy controls [29]. These two cytokines are known to be involved in the modulation of connective tissue synthesis and exhibit the ability to activate endothelial cells. IL-4 stimulates proliferation and extracellular matrix production by human fibroblasts *in vitro* [30] and increases endothelial cell adhesion for T cells [31]; IFN-γ inhibits excessive collagen production [32] and induces MHC antigen expression on endothelial cells [33]. Taken together, these data and the higher circulating levels of several cytokines, such as IL-2, IL-4 and IL-6, observed in this disease clearly indicate that chronic T cell activation is present in SSc patients. The increased levels of sCD30 in our patients probably mirror the increased number and functional activity of Th2 cells *in vivo*. The slight, but not statistically significant, increase of circulating $CD30^+/CD3^+$ T cells does not contradict this possibility, because the cells releasing sCD30 into the circulation might well be confined to the specific target organs, where the inflammatory process is taking place. However, we observed spontaneous production of sCD30 *in vitro* by PBMC of patients with SSc, suggesting that these cells have already been primed *in vivo*. No further increase in sCD30 levels was observed after 24 h and 48 h PHA stimulation. On the contrary, healthy controls showed normal kinetics in the release of sCD30 after mitogen stimulation [10]. A possible explanation of these findings is that, in SSc the slightly increased $CD30^+$ subset, already maximally activated *in vivo* and unresponsive to further *in vitro* mitogen stimulation, may be the source of the sCD30 detected in the unstimulated cultures.

Laboratory tests are considered to be of little value in monitoring the activity, extent or prognosis of SSc. On the contrary, skin score methods have shown that extensive skin involvement (i.e. a high skin score) is related to decreased survival and a greater risk of developing extensive involvement of internal organs [34]. Thus, the observed correlation of circulating sCD30 levels with skin score may suggest this serum measurement to be helpful in clinical evaluation of SSc patients, as a simple, reliable and sensitive test to assess the outcome of the disease, similar to what has been reported in other autoimmune diseases, such as SLE [16] and RA [15]. With this in mind, we are currently undertaking long-term investigation of the same patients to establish whether sCD30 measurement may prove valuable in monitoring the outcome of SSc. Furthermore, as far as the correlation between sCD30 and ESR is concerned, it may be hypothesized that this soluble molecule is a marker of systemic inflammation, although ESR might be modulated not only by acute-phase reactants but also by a rise in serum plasma proteins, such as immunoglobulins. It must be pointed out that our SSc patients showed hypergammaglobulinaemia (data not shown). Furthermore, in sera of patients affected by inflammatory bowel diseases, such as Crohn's disease (CD) and ulcerative colitis (UC), increased values of sCD30 were observed only in UC patients, independently of clinical status (manuscript in preparation). Thus, further studies are needed to clarify the relationship between sCD30 and inflammation.

The specific role of CD30 and its related ligands in the pathophysiology of Th2 cells has not yet been clarified, although this complex could be relevant to their functional activities. Probably, an improved knowledge of these activities, and their related effects, will eventually open new therapeutic opportunities in autoimmune diseases, through manipulation of the cytokine network.

ACKNOWLEDGMENTS

This work was supported by MURST 40%, 1994. The authors wish to thank Mr M. Termine for his excellent technical assistance.

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