Increased plasma soluble CD40 ligand concentrations in systemic sclerosis and association with pulmonary arterial hypertension and digital ulcers

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Methods: Quantitative sandwich ELISA was used to measure plasma sCD40L in systemic sclerosis (n = 50) and matched healthy controls (n = 20). Patients with systemic sclerosis had limited cutaneous disease (29), digital ulcers (14), pulmonary arterial hypertension (PAH) (10), pulmonary fibrosis on CT (23), positive anti-Scl70 (14), and anti-centromere antibodies (10). Calcium channel blockers were discontinued 72 hours before measurements.

Results: Median (range) sCD40L concentration (pg/ml) was higher in systemic sclerosis than in controls (495 (10 to 7720) vs 79 (50 to 118); p = 0.003), in limited cutaneous disease vs diffuse disease (620 (20 to 7720) vs 250 (10 to 2690); p = 0.005), in patients with digital ulcers vs those without (1430 (36 to 7720) vs 370 (10 to 2320); p = 0.002), and in those with PAH vs those without (995 (15 to 3850) vs 400 (10 to 7720); p = 0.048). sCD40L correlated with pulmonary arterial pressure estimated by Doppler echocardiography (r = 0.41; p = 0.005).

Conclusions: The soluble form of CD40L is increased in plasma in systemic sclerosis and may be associated with vascular complications of the disease.

Assay of soluble CD40L

According to previous recommendations, we used plasma samples. Blood samples (10 ml) were collected in tubes containing ethylenediamine-tetraacetic acid. They were centrifuged at 3000 xg for 10 minutes within 30 minutes of collection. After separation, plasma were stored at −80°C until use. Plasma sCD40L were measured by ELISA (R&D systems Abingdon, UK). Briefly, diluted (1:5) plasma samples were applied to 96-well plates precoated with a polyclonal antibody specific for CD40 ligand and incubated for two hours. After washing, a horseradish peroxidase linked polyclonal antibody specific for CD 40 ligand was added to the wells and incubated. Subsequently, plates were washed

Abbreviations: DCO, lung diffusing capacity for carbon monoxide; PAH, pulmonary artery hypertension; PAPs, systolic pulmonary artery pressure
and antibody binding determined by colorimetry using 3,3',5,5'-tetramethylbenzidine substrate. Absorbance was read using a Dynatech MR 5000 microplate reader (Dynex Technology) at 450 nm within 30 minutes. According to the manufacturer, intra-assay precision was 5.1% for 430 pg/ml (n = 20) and 5.4% for 2638 pg/ml (n = 20); interassay precision was 6.4% for 437 pg/ml (n = 40) and 6.2% for 2612 pg/ml (n = 40).

### Statistical analysis

Data were analysed with the following non-parametric tests: Mann–Whitney and Wilcoxon tests for comparison of groups and Spearman’s rank correlation test for assessment of the relation between quantitative variables. Probability (p) values less than 0.05 were considered significant. All quantitative data are expressed as median (range).

### RESULTS

We included 50 successive patients with systemic sclerosis. There were 44 women and six men, mean (SD) age, 57 (11) years, range 34 to 80. Mean disease duration was 7 (7) years; in 18 patients disease duration was less than three years. The clinical and laboratory data for these patients are presented in table 1.

The median (range) concentration of sCD40L was higher in the patients with systemic sclerosis than in the healthy controls, at 495 (10 to 7720) pg/ml (p = 0.003; Mann–Whitney U test; fig 1). Median sCD40L was increased in patients with limited cutaneous disease compared with those with diffuse disease, at 620 (20 to 7720) pg/ml (p = 0.048; Mann–Whitney). CD40L correlated with PAPs and the finding needs to be confirmed in patients with severe PAH.

Concentrations of sCD40L were significantly higher in SSC patients with PAH. These patients had early and mild PAH, as the upper limit of normal, 37 of the 50 patients (74%) had increased concentrations. Patients with pulmonary arterial hypertension and digital ulcers had the highest concentrations of sCD40L than those without, at 1430 (36 to 7720) pg/ml (p = 0.002; Mann–Whitney; fig 1). In the subgroup of patients with limited disease (n = 29), median sCD40L was higher in patients with PAH (n = 5) than in those without PAH (n = 24), at 2140 (1390 to 3850) pg/ml (p = 0.049; Mann–Whitney). CD40L correlated with PAPs estimated by Doppler echocardiography (r = 0.41; p = 0.005, Spearman’s test). Patients with systemic sclerosis and digital ulcers had higher concentrations of sCD40L than those without, at 1390 (36 to 7720) pg/ml (p = 0.048; Mann–Whitney). Age, disease duration, concurrent cutaneous skin score, pulmonary fibrosis, carbon monoxide diffusion, autoantibody status, and treatment (antiaggregant, corticosteroids, angiotensin converting enzyme inhibitors) were not associated with sCD40L concentrations.

### DISCUSSION

We report a significant increase in the soluble form of CD40L in the plasma of patients with systemic sclerosis. If the 90th centile of the control concentrations (107.8 pg/ml) was taken as the upper limit of normal, 37 of the 50 patients (74%) had increased concentrations. Patients with pulmonary arterial hypertension and digital ulcers had the highest levels of sCD40L.

It has recently been suggested that an abnormal cell mediated response between fibroblasts and T cells in the skin lesions of systemic sclerosis may implicate the CD40-CD40L system. In our study, we report higher values of sCD40L in patients with limited cutaneous disease than in those with diffuse disease, and sCD40L concentrations did not correlate with the skin score. Our results do not support the view that sCD40L reflects fibroblast activation. It has recently been reported that membrane tumour necrosis factor may be involved in the inhibition of fibroblast synthesis by Th2 lymphocytes; the involvement of CD40L in this phenomenon should be investigated.

We report here that the presence of digital ulcers was associated with sCD40L concentrations in the whole study population and also in the subgroup of patients with limited disease, which was stratified to limit the influence of cutaneous subtype and disease duration on sCD40L levels. Concentrations of sCD40L were significantly higher in patients with PAH. These patients had early and mild PAH, and the finding needs to be confirmed in patients with severe PAH.

Compared with previous studies, our mean (SD) serum concentration of sCD40L in systemic sclerosis (918 (1384) pg/ml) was lower than in systemic lupus erythematosus (2610
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(2150) pg/ml) or mixed connective tissue disease (median 999 pg/ml) but higher than in rheumatoid arthritis (730 (1.11) pg/ml). The sCD40L values in our patients with systemic sclerosis were also higher in the healthy controls in those previous studies (mean (SD), 25 (40) pg/ml in serum, 170 (190) pg/ml in plasma, and median 179 pg/ml in the final study). The values in our control group (median, 79 pg/ml; mean (SD), 81 (19)) are in accord with those data. However, differences in the various methods used do not allow strict comparisons between those results and ours; in particular, we used plasma samples with high speed centrifugation to limit all sources of contamination by platelets.

In systemic lupus erythematosus, sCD40L is thought to reflect T cell activation or a chronic inflammatory condition; however, recent data also suggest a link with antiphospholipid antibodies and platelet activation in this condition. We could not identify this link in our patients with systemic sclerosis.

CD40L is believed to be deeply involved in the immune response. In systemic sclerosis, T–B collaboration is essential for the autoimmune response; however, we could not determine an association between autoantibody status and sCD40L concentration. Upon ligation with CD40, CD40L is endocytosed and its mRNA expression is downregulated. Although this mechanism is thought to be the principal way in which the CD40–CD40L interaction is regulated, the production of sCD40L could also act as an antagonist in the immune response and may therefore represent a secondary event in systemic sclerosis.

Although CD40–CD40L has been studied intensively in T–B cell activation, CD40L also binds to other CD40 positive cells, such as endothelial cells, leading to the synthesis of adhesion molecule, chemokines, and matrix metalloproteinases. Ligation of CD40L on various vascular cells contributes to the pathogenesis of atherosclerotic, thrombotic, and inflammatory processes. Vascularopathy resulting from endothelial cell activation and activated lymphocyte recruitment is a crucial feature of systemic sclerosis, and coagulation abnormalities are well recognised features; investigation of CD40L and sCD40L in these areas is warranted.

Conclusions
Our study showed increased plasma soluble CD40 ligand concentrations in systemic sclerosis and an association between the increased values and pulmonary arterial hypertension and digital ulcers. Our results, along with those previously reported, suggest that CD40L signalling may play an important role in this disease.

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