CIRCULATING INTERCELLULAR ADHESION MOLECULE-1 IN THE SERA OF PATIENTS WITH SYSTEMIC SCLEROSIS: ENHANCEMENT BY INFLAMMATORY CYTOKINES

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SUMMARY
We measured serum levels of circulating intercellular adhesion molecule-1 (cICAM-1) in patients with systemic sclerosis (SSc) and normal controls. The levels of cICAM-1 were determined by sandwich enzyme-linked immunosorbent assay in sera from 88 patients with SSc and in 20 healthy controls. In addition, these levels were examined in the supernatants of cultured peripheral blood mononuclear cells (PBMC) and dermal fibroblasts from 10 patients with SSc and 10 healthy control subjects. Serum levels of cICAM-1 were significantly higher in patients with SSc than in healthy controls. Serum cICAM-1 levels were significantly higher in patients with diffuse cutaneous SSc (dcSSc) than in patients with limited cutaneous SSc (lcSSc). These serum levels were correlated with the presence of contracture of phalanges, pulmonary fibrosis, joint involvement and increased erythrocyte sedimentation rate. The release of cICAM-1 was significantly increased in the supernatants of cultured PBMC from patients with SSc. Moreover, inflammatory cytokines (interferon-γ, interleukin-1 and tumour necrosis factor-α) enhanced the release of cICAM-1 in vitro in SSc cells. These findings suggest that cICAM-1 may be involved in immune reactions in this disease.

KEY WORDS: Fibroblasts, Peripheral blood mononuclear cells, Pulmonary fibrosis, Inflammatory cytokines.

SCLERODERMA, or systemic sclerosis (SSc), is a generalized connective tissue disease which involves sclerotic changes in the skin and many other organ systems [1]. Although the pathogenesis of SSc is still unknown, the basic mechanism appears to involve endothelial cell injury, overproduction of extracellular matrix and aberrant immune activation [2–4]. The presence of mononuclear cell infiltrates and increased serum levels of certain cytokines have highlighted the importance of adhesion interactions with leucocytes and the control of adhesion molecule expression in SSc [5–7].

Intercellular adhesion molecule-1 (ICAM-1; CD54), a 76–114 kDa cell surface glycoprotein and member of the immunoglobulin supergene family, plays an important role in a variety of inflammatory and immune-mediated mechanisms, including lymphocyte recruitment and targeting, antigen presentation and recognition, and lymphocyte cytotoxicity [8–10]. ICAM-1 is the counter-receptor for leucocyte function-associated antigen-1 (LFA-1, or CD11a/CD18) and Mac-1 (CD11b/CD18) [8]. ICAM-1 is expressed on various cell types, such as vascular endothelial cells, epithelial cells, fibroblasts, tissue macrophages and peripheral blood leucocytes [10]. Upon stimulation with interferon-γ (IFN-γ), interleukin-1 (IL-1) or tumour necrosis factor-α (TNF-α), there is a rapid and strong increase of expression of ICAM-1 on lymphoid cells, keratinocytes, fibroblasts and endothelial cells [10].

In addition to membrane-bound ICAM-1, a circulating form of this molecule has been described [11]. This circulating ICAM-1 (cICAM-1) is functionally active and can be detected in the sera of healthy subjects with an enzyme-linked immunosorbent assay (ELISA). High levels of cICAM-1 have been noted in a variety of inflammatory and neoplastic conditions [12–14].

In the present study, we measured serum levels of cICAM-1 in patients with SSc and investigated whether these levels were correlated with clinical or serological features of this disease. Furthermore, we investigated levels of cICAM-1 in the supernatants of cultured mononuclear cells and dermal fibroblasts, and examined the effects of the cytokines such as IL-1β, TNF-α and IFN-γ on the release of this molecule. Cultured mononuclear cells from patients with SSc released greater amounts of cICAM-1 both basally and in response to inflammatory cytokines.

PATIENTS AND METHODS

Patients
Eighty-eight patients with SSc and 20 healthy control subjects were studied. The patients with SSc were classified according to the classification system proposed by LeRoy et al. [15]: 44 patients had limited cutaneous SSc (lcSSc) and 44 had diffuse cutaneous SSc (dcSSc), as previously described [16]. All patients with dcSSc and 39 with lcSSc fulfilled the criteria proposed by the American College of Rheumatology.
Control fibroblasts were obtained by biopsy from 10 SSc patients. All the patients had elevated cICAM-1 levels. The cells were separated according to sex, age and biopsy site. Control subjects matched with each SSc patient were healthy donors. The sclerotic changes were confirmed histologically.

Fibroblast cultures

Fibroblasts were obtained by skin biopsy from affected skin of 10 patients with dcSSc with a <2 yr duration of skin thickening. All the patients had thickening of the forearms at the time of biopsy and the sclerotic changes were confirmed histologically. All the patients had elevated cICAM-1 levels. Control fibroblasts were obtained by biopsy from 10 healthy donors matched with each SSc patient according to sex, age and biopsy site. Control subjects had normal cICAM-1 levels. The cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Fibroblasts between passages 2 and 4 were used for experiments.

For cytokine treatment, the fibroblasts were grown to confluence in 24-well plates. The culture medium was replaced with serum-free MEM and fibroblasts were cultured either in the absence or in the presence of the cytokines. The recombinant human cytokines, IL-1β (Boehringer-Mannheim, Indianapolis, IN, USA), IFN-γ and TNF-α (R&D Systems Inc., Minneapolis, MN, USA), were used at 50 ng/ml, 100 U/ml and 10 ng/ml, respectively. These concentrations were considered to have maximum effects according to the dose-response curves. After incubation for 48 h and 7 days, the fibroblast supernatants were collected, centrifuged for 5 min at 6000 g, and concentrated 10-fold on Centricon devices (Amicon, Beverley, MA, USA) at 4°C.

Clinical assessment

Clinical and laboratory data reported in the present study were obtained at the time the blood samples were drawn. Patients were evaluated for the presence of gastrointestinal, pulmonary, cardiac, renal or muscle involvement as described previously [16,19]: (a) oesophageal hypomotility was defined as distal oesophageal hypomotility on barium radiography; (b) pulmonary interstitial fibrosis was defined as bibasilar interstitial fibrosis on chest radiograph (high kV film) and high-resolution CT scan, and pulmonary function tests were also performed; (c) cardiac involvement was defined as any of the following: symptomatic pericarditis, clinical evidence of left ventricular congestive heart failure or arrhythmias requiring treatment; (d) renal involvement was defined as malignant hypertension and/or rapidly progressive renal failure; (e) skeletal muscle involvement was defined as proximal muscle weakness and elevated serum creatine kinase level, plus abnormal electromyographic findings consistent with myopathy and/or histopathological changes of inflammatory myopathy; (f) joint involvement was defined as inflammatory polyarthralgia or arthritis.

ANA analyses

Antinuclear antibodies (ANA) were detected by immunofluorescence, using HEp-2 cells as the substrate, and double immunodiffusion [20].

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Separation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from the same patients and control subjects listed above, as described previously [18]. Briefly, heparinized venous blood was obtained from patients and healthy volunteers, as described above. Mononuclear cells were separated by layering over Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) in 50 ml plastic tubes. Tubes were centrifuged for 10 min at 330 g, and plasma and mononuclear cells were collected using a Pasteur pipette. PBMC were subsequently washed three times with RPMI 1640, counted, and adjusted to a concentration of 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS. PBMC were cultured either in the absence or in the presence of the cytokines described above. After incubation for 48 h, the supernatants were collected, centrifuged and concentrated as described above.

Circulating ICAM-1 assay

cICAM-1 levels were measured with a specific ELISA kit (T cell Diagnostics, Cambridge, MA, USA), as described previously [5]. Briefly, serum samples diluted 1:100 (25 μl) were applied to the polystyrene microwells pre-coated with murine monoclonal antibody against human cICAM-1. A horse-radish peroxidase-conjugated anti-mouse monoclonal antibody with functional neutralizing properties that binds to the cICAM-1 captured by the primary antibody was then added. Following incubation for 2 h at room temperature and extensive washing, the reaction product was developed in tetramethylbenzidine for 30 min, and then the enzyme reaction was terminated with 2 N sulphuric acid. Absorbances for samples and cICAM-1 were determined with a Titertek Multiskan (Flow Laboratories, McLean, VA, USA) at 490 nm. cICAM-1 levels in serum samples were determined by comparing the mean absorbance of duplicate samples with the standard curve for each assay. The sensitivity of this assay was 0.3 ng/ml. Serum cICAM-1 levels > 2 s.d. above the mean level in the normal controls were regarded as elevated.

Statistical analysis

Statistical analysis was carried out with Student’s t-test or the Mann–Whitney test for the comparison of means. Fisher’s exact probability test for the analysis of frequency, and Newman–Keuls’s test for multiple comparisons. Correlations with clinical data were assessed by Spearman’s rank correlation coefficient. P values of < 0.05 were considered significant.
RESULTS

Serum levels of cICAM-1

Serum levels of cICAM-1 in samples derived from patients with SSc and normal individuals are illustrated in Fig. 1. Compared with levels in the healthy control subjects (mean ± s.d. 192.0 ± 49.0 ng/ml), serum levels of cICAM-1 were significantly elevated in the patients with SSc (316.7 ± 170.3 ng/ml; P < 0.0001). As seen in Fig. 1, patients with dcSSc had significantly higher serum cICAM-1 levels (370.7 ± 171.6 ng/ml) than those with lcSSc (262.7 ± 152.6 ng/ml; P < 0.001). The cut-off value (mean ± 2 s.d.) was set at 290.0 ng/ml, based on the data for the 20 healthy control sera. Elevated serum levels of cICAM-1 were found in 28/44 (64%) patients with dcSSc and 12/44 (27%) patients with lcSSc.

Correlation of serum cICAM-1 levels with clinical and serological features of patients with SSc

The clinical and serological features in the patients with elevated and with normal cICAM-1 levels are shown in Table I. The presence of pulmonary fibrosis was found in a significantly greater proportion of patients with elevated cICAM-1 levels than in those with normal levels (63% vs 33%; P < 0.02). The presence of decreased %VC and %DLco was also significantly correlated with elevated cICAM-1 levels (38% vs 16%, P < 0.05; 81% vs 49%, P < 0.05, respectively). The patients with elevated cICAM-1 levels had significantly lower %VC values than those with normal levels (mean ± s.d. 51.6 ± 24.0% vs 67.9 ± 20.2%; P < 0.05). Similarly, the patients with elevated cICAM-1 levels displayed significantly lower %DLco values than those with normal levels (mean ± s.d. 75.6 ± 17.5% vs 93.5 ± 11.0%; P < 0.05). Moreover, increased serum cICAM-1 levels were correlated with decreased %VC and %DLco even in dcSSc patients with pulmonary fibrosis (P < 0.05).

The presence of joint involvement was significantly greater in patients with elevated cICAM-1 levels than in those with normal levels (51% vs 27%; P < 0.05). The presence of increased erythrocyte sedimentation rate (ESR) levels was also significantly correlated with elevated cICAM-1 levels (79% vs 50%, P < 0.01), and the levels of ESR were significantly correlated with serum cICAM-1 levels (r = 0.392, P < 0.01). However, there was no significant correlation between elevated cICAM-1 levels and the presence of elevated CRP levels.

![Fig. 1. Serum levels of cICAM-1. The dashed line represents the cut-off value (mean ± 2 s.d.), which was set at 290.0 ng/ml, based on the data for the 20 healthy control sera. Elevated serum levels of cICAM-1 were found in 28/44 (64%) patients with dcSSc and 12/44 (27%) patients with lcSSc. DSSc, diffuse cutaneous systemic sclerosis; LSSc, limited cutaneous systemic sclerosis.](image-url)

**TABLE I**

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<tr>
<th>Correlation of serum cICAM-1 levels with clinical and serological features of patients with SSc</th>
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<tr>
<td>Patients with elevated cICAM-1 level (n = 40)</td>
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<td>Mean age (yr)</td>
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<td>Duration (yr)</td>
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<td>Clinical features</td>
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<td>Pitting scars/ulcers</td>
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<td>Short sublingual frenulum</td>
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<td>Contracture of palphanges</td>
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<td>Organ involvement</td>
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<td>Lung</td>
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<td>Decreased %VC</td>
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<td>Anti-U1 RNP</td>
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<td>Laboratory findings</td>
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<td>Elevated ESR</td>
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<td>Increased CRP</td>
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Unless noted otherwise, values are percentages. VC, vital capacity; DLco, diffusive capacity for carbon monoxide; ANA, antinuclear antibodies; Anti-topo I, anti-topoisomerase 1 antibody; ACA, anticentromere antibody; ESR, erythrocyte sedimentation rate. *P < 0.05; **P < 0.02; ***P < 0.01.
Levels of cICAM-1 released into culture supernatants

The supernatant media of PBMC from patients with SSc and normal healthy controls contained detectable amounts of cICAM-1 (Table II). The levels of cICAM-1 were significantly higher in the culture supernatants from SSc cells than in those from normal cells (mean ± s.d. 2.5 ± 0.5 ng/ml vs 1.0 ± 1.2 ng/ml; *P < 0.05). However, the supernatants of SSc and normal fibroblasts did not have detectable amounts of cICAM-1.

Next, we examined the effects of IL-1β, TNF-α and IFN-γ on the cICAM-1 levels released by PBMC and fibroblasts. All of these cytokines markedly increased the levels of cICAM-1 released by PBMC in a dose-dependent manner (data not shown). The levels of cICAM-1 were significantly increased, by 70, 106 and 132%, in supernatants of PBMC from SSc treated with IL-1β, TNF-α and IFN-γ, respectively. Culture of normal PBMC in the presence of these cytokines was also found to give rise to similar upregulation of cICAM-1 release, which was raised by 167, 100 and 152% when treated by IL-1β, TNF-α and IFN-γ, respectively. Furthermore, the levels of cICAM-1 were significantly higher in the supernatants of PBMC from patients with SSc than in those from normal cells in the presence of IL-1β, TNF-α and IFN-γ, respectively.

Although the supernatants of SSc and normal fibroblasts did not have detectable amounts of cICAM-1, the levels of cICAM-1 release were detectable in normal and SSc fibroblasts in the presence of these cytokines. However, there were no significant differences between these levels.

Furthermore, cICAM-1 levels released by PBMC were correlated with serum levels in patients with SSc (*\( r = 0.85, \ P < 0.01 \)).

DISCUSSION

cICAM-1 contains most of the structure and function of the extracellular portion of cell-bound ICAM-1. Thus, cICAM-1 is thought to be generated through proteolytic cleavage of cell-bound ICAM-1 close to the cell membrane [11]. It appears that the main sources of cICAM-1 in vivo are mononuclear cells and endothelial cells, because it was demonstrated that cICAM-1 is released by activated mono-


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<th>TABLE II</th>
<th>Levels of cICAM-1 released into culture supernatants</th>
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<td><strong>cICAM-1 released by PBMC</strong></td>
<td><strong>cICAM-1 released by fibroblasts</strong></td>
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<tr>
<td>Treatment</td>
<td>Normal PBMC (( n = 10 ))</td>
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<tr>
<td>Alone</td>
<td>1.03 ± 1.24*</td>
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<tr>
<td>IL-1β</td>
<td>2.75 ± 0.19**</td>
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<tr>
<td>TNF-α</td>
<td>2.07 ± 0.23**</td>
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<tr>
<td>IFN-γ</td>
<td>2.60 ± 0.26*</td>
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Values are mean ± s.d. (ng/ml).
cICAM-1, circulating intercellular adhesion molecule 1; SSc, systemic sclerosis; IL-1β, interleukin-1β (50 ng/ml); TNF-α, tumour necrosis factor-α (10 ng/ml); IFN-γ, interferon-γ (100 U/ml).
* *P < 0.05, ** *P < 0.01.

nuclear cells and activated endothelial cells in vitro [11, 21, 22]. cICAM-1 has been reported to function as a competitive inhibitor of membrane-bound ICAM-1; cICAM-1 inhibits LFA-1/ICAM-1-mediated cell adhesion in vitro [11, 21, 23], and abrogates the non-MHC-restricted cytotoxicity mediated by natural killer cells and lymphokine-activated killer cells [24], suggesting that cICAM-1 may have important effects on the immune system.

In this study, we demonstrated that serum levels of cICAM-1 were significantly higher in patients with SSc than in healthy control subjects. This suggests ICAM-1 upregulation in patients with SSc, since the amount of cICAM-1 is correlated with the level of surface expression of ICAM-1 in vitro [21]. Indeed, increased expression of cell-bound ICAM-1 has been documented immunohistochemically at the sites of mononuclear cell infiltrates and fibroblasts in the involved skin of patients with SSc [25–27]. Several studies have shown that the shedding of cICAM-1, as well as upregulation of cell-bound ICAM-1, is induced by activation signals and cytokines, such as TNF-α, IL-1 and IFN-γ [10, 11]. Thus, elevated serum levels of cICAM-1 may be a consequence of chronic systemic exposure to these cytokines in patients with SSc.

Patients with dcSSc had significantly higher serum cICAM-1 levels than those with lcSSc. Moreover, serum cICAM-1 was significantly correlated with the presence and the severity of pulmonary fibrosis, the presence of joint involvement, and ESR. This indicates that serum cICAM-1 may reflect the severity of this disease. The previous study also indicated that serial measurement of this molecule may have potential value as a marker for clinical progression or remission in this disease [28].

We detected significantly higher levels of cICAM-1 in the culture supernatants from lcSSc PBMC than in those from normal cells. Furthermore, the levels of cICAM-1 were significantly higher in the supernatants of PBMC from patients with SSc than in those from normal cells in the presence of IL-1β, TNF-α and IFN-γ, respectively. However, the relative enhancement was less for SSc PBMC. These data suggest that SSc PBMC were already activated and less responsive to exogenous inflammatory cytokines.
It is also notable that although there were no significant differences in cICAM-1 levels between the supernatants of SSc fibroblasts and those of normal cells in the presence or absence of these cytokines, we detected significantly higher cICAM-1 in the supernatants of SSc PBMC than in those of normal cells. It could be postulated that elevated serum levels of cICAM-1 in patients with SSc may reflect the elevated release of this molecule from PBMC of the patients. The previous study indicated that cICAM-1 levels were higher in the culture supernatants of SSc fibroblasts than in media of normal cells [29]. This is different from our result. The difference may be due to the patient population or assay conditions. Further studies are needed to clarify the cICAM-1 levels in the culture supernatants of SSc fibroblasts and normal cells.

The data reported in this study suggest that the presence of inflammatory mediators directly affects the elevated cICAM-1 levels from SSc PBMC, which potentially influence leucocyte interactions in vivo and the pathogenesis of SSc. Analysis suggests that serum cICAM-1 levels are linked to the severity of this disease. Further studies are needed to document the conditions of release and the potential immunoregulatory role of cICAM-1 in SSc. Prospective serial studies are also required to define the potential use of the assay in clinical staging and management.

ACKNOWLEDGEMENT

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