Systemic Sclerosis Fibroblasts Show Specific Alterations of Interferon-γ and Tumor Necrosis Factor-α-induced Modulation of Interleukin 6 and Chemokine Ligand 2

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ABSTRACT. ***Objective.*** We evaluated the effect of interferon-γ (IFN-γ) and/or tumor necrosis factor-α (TNF-α) on the secretion of prototype proinflammatory cytokine interleukin 6 (IL-6), compared to T-helper 1 [Th1; chemokine (C-X-C motif) ligand 10 (CXCL10)] or Th2 [chemokine (C-C motif) ligand 2 (CCL2)] chemokines, in primary cultured fibroblasts from patients with systemic sclerosis (SSc) at an early stage of the disease.

Methods. Fibroblast cultures from 5 SSc patients (disease duration < 2 yrs) and 5 healthy controls were evaluated for the production of IL-6, CXCL10, and CCL2 at the basal level and after stimulation with IFN-γ and/or TNF-α.

Results. SSc fibroblasts basally produced higher levels of IL-6 than controls, while no difference was observed about CCL2 and CXCL10. TNF-α was able to dose-dependently induce IL-6 and CCL2 secretion in SSc, but not in control fibroblasts. By stimulation with increasing doses of IFN-γ, SSc fibroblasts were induced to secrete CCL2 and CXCL10, while no effect was observed on IL-6. The combination of IFN-γ and TNF-α induced a strong secretion of IL-6 and CCL2 in SSc fibroblasts but not in controls. In contrast, the synergistic effect of IFN-γ and TNF-α on CXCL10 secretion was similar in SSc fibroblasts and in controls.

Conclusion. SSc fibroblasts participate in the self-perpetuation of inflammation by releasing IL-6, CXCL10, and CCL2 under the influence of IFN-γ and/or TNF-α. SSc fibroblasts are more active than controls in the secretion of IL-6 at baseline, and in the production of IL-6 and CCL2 under the combined IFN-γ/TNF-α stimulation. (J Rheumatol First Release March 15 2012; doi:10.3899/jrheum.111132)

Key Indexing Terms:
SCLERODERMA INTERLEUKIN 6 CCL2 CXCL10 FIBROBLASTS CYTOKINES

Systemic sclerosis (SSc) is a connective disease characterized by features of autoimmunity, vasculopathy, inflammation, and fibrosis. The disease typically starts with Raynaud's phenomenon, followed by skin thickening in the extremities due to inflammation and fibrosis. Fibrosis results from excessive collagen production by fibroblasts, which constitutes the final common pathway of complex cellular interactions including B cells[1].

Serum levels of interleukin 6 (IL-6), an important proinflammatory cytokine, have been shown to correlate with skin fibrosis. B cells, among other cells, secrete IL-6 that stimulates fibroblasts to produce collagen. In a mouse scleroderma model, B cell depletion using an anti-mouse CD20 monoclonal antibody led to a decrease in IL-6 messenger RNA (mRNA) levels and a reduction of fibrosis[2]. IL-6 serum levels and spontaneous IL-6 production by peripheral blood mononuclear cells are higher in patients with SSc compared with healthy controls. In addition, it was shown that IL-6 serum levels correlate with skin fibrosis[3]. Thus, IL-6 may be an important target for the treatment of SSc in humans.

SSc fibroblasts produce IL-6 spontaneously[4,5,6] or under a variety of stimuli, such as IL-1α[7,8], ligation of CD40 by recombinant human CD154[9], autoantibodies to fibroblasts[10], platelet-derived growth factor[11], and IL-1ß[12]. To our knowledge, no study has evaluated the effect of interferon-γ (IFN-γ) alone or in combination with tumor necrosis factor-α (TNF-α) on the secretion of IL-6 in fibroblasts from patients with SSc.

Even if proinflammatory cytokines play a central role in the pathogenesis of SSc, T-helper 1 (Th1) and Th2 chemokines have an important effect mediating the interactions among leukocytes, endothelial cells, and fibroblasts, and are likely to be central to the pathogenesis of the disease[13].

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Among Th2 chemokines, it is likely that chemokine (C-C motif) ligand 2 (CCL2) and its ligand, chemokine (C-C motif) receptor 2 (CCR2), play a central role in the pathogenesis of SSc[14]. However, other CC, CXC, and CX3C chemokines may also cooperatively contribute to the development of this disease[15,16].

CCL2 is produced by macrophages, fibroblasts, endothelial cells, and other cells and it is a predominant chemoattractant and activator of monocytes and T cells. Moreover, this chemokine induces Th2 cell polarization[17] and stimulates collagen production by fibroblasts through specific receptors and endogenous upregulation of expression of transforming growth factor-ß (TGF-ß). Promoter polymorphism in the CCL2 gene is associated with susceptibility and CCL2 expression of skin fibroblasts in SSc[18].

CCL2 levels were elevated in patients with SSc compared to healthy controls[19]. CCL2 was strongly expressed in the epidermis, inflammatory mononuclear cells, and endothelial cells in the sclerotic skin of patients with SSc, but was not expressed in normal skin, by immunohistochemical analysis[19]. Consistent with this report, a critical role of CCL2 was revealed in transendothelial leukocyte migration using an *in vitro* assay system[20]. That study suggested that SSc fibroblasts promote leukocyte migration across endothelial cell monolayers through a CCL2-dependent mechanism.

Cultured SSc fibroblasts displayed increased expression of CCL2 mRNA and protein, compared to control fibroblasts[21,22,23]. Further, treatment with platelet-derived growth factor significantly stimulated CCL2 expression in dermal SSc fibroblasts compared with that from normal controls[21,24].

Recently, it was shown that serum chemokine (C-X-C motif) ligand 10 (CXCL10), a Th1 chemoattractant, was detected more frequently in patients with SSc than in normal controls[25]. In accordance, we previously reported that serum CXCL10 levels are significantly elevated in the early stage of disease, with a progressive regression to reach almost the control level 5 years after disease onset[15,16].

To our knowledge, no study to date has evaluated the effect of IFN-γ, TNF-α, or the combination of IFN-γ and TNF-α stimulation on the prototype proinflammatory cytokine IL-6, in comparison with Th1 (CXCL10) or Th2 (CCL2) chemokine secretion in primary cultured fibroblasts from patients at an early stage of SSc. We evaluated the effect of IFN-γ and/or TNF-α stimulation on IL-6, CXCL10, and CCL2 secretion in fibroblast cells obtained from patients with SSc.

MATERIALS AND METHODS

Patients. Five patients with SSc (3 women, 2 men, median age 59 yrs, range 30–75 yrs), classified according to the 1980 American College of Rheumatology criteria[26], and 5 sex- and age-matched healthy controls were enrolled in our study. All patients with SSc had a disease duration < 2 years and presented with antitopoisomerase I antibodies. Three subjects had the diffuse cutaneous disease subset and 2 the limited subset.

Fibroblast cell cultures. Skin tissue samples were obtained from 5 patients with SSc. The biopsies were performed at the arm, so that 2/5 samples derived from nonsclerotic cutis. SSc tissue explants were minced and placed directly in plastic culture dishes to permit fibroblast proliferation as described[10,11]. Cells were propagated in medium 199 with 20% fetal bovine serum (FBS; Gibco Invitrogen, Paisley, UK), penicillin 100 IU/ml, and gentamycin 20 μg/ml in a humidified 5% CO_2 incubator at 37°C, and maintained with medium 199 containing 10% FBS and antibiotics.

Control fibroblasts were obtained from unaffected dermal tissues of 5 healthy subjects, who were undergoing skin biopsy for diagnostic purposes, in whom any immune-mediated disorder was excluded.

All subjects gave their informed consent for our study, which was approved by the local Ethical Committee.

IL-6, CXCL10, and CCL2 secretion assays. The tests on fibroblast cultures were performed at the fifth passage. For IL-6, CCL2, and CXCL10 secretion assays, cells were seeded in 96-well plates at a concentration of 30,000 cells/ml in a final volume of 100 μl per well, in growth medium, which was removed after 24 h. Then cells were washed in phosphate buffered saline, and incubated 24 h in phenol red and serum-free medium with IFN-γ (0, 500, 1000, and 5000 IU/ml; R&D Systems, Minneapolis, MN, USA) or TNF-α (0, 1, 5, and 10 ng/ml; R&D Systems), alone or in combination. After 24 h, the supernatant was collected and kept frozen at –20°C until assayed.

Supernatants were assayed by ELISA for IL-6, CCL2, and CXCL10 concentrations. The experiments were repeated 3 times with the 10 different cell preparations.

ELISA for IL-6, CCL2, and CXCL10. IL-6 levels in culture supernatants were assayed by a quantitative sandwich immunoassay using a commercial kit (R&D Systems) with a sensitivity of 0.7 pg/ml. The intraassay and interassay coefficients of variation (CV) were 2.5% and 3.0%, respectively.

CCL2 levels in culture supernatants were assayed by a quantitative sandwich immunoassay using a commercial kit (R&D Systems). The mean minimum detectable dose was 4.5 pg/ml. The intraassay and interassay CV were 4.4% and 5.7%.

Culture supernatant levels of CXCL10 were assayed by a quantitative sandwich immunoassay using a commercial kit (R&D Systems). The mean minimum detectable dose was 1.35 pg/ml. The intraassay and interassay CV were 3.2% and 6.7%.

Data analysis. Values are given as mean ± SD for normally distributed variables (in the text) or mean ± SEM (in the figures), and otherwise as median (interquartile range). Mean group values were compared using 1-way ANOVA for normally distributed variables, otherwise by the Mann-Whitney U or Kruskal-Wallis test. Proportions were compared by chi-square test. Posthoc comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test. We used StatView software, version 5.0 (SAS Institute, Cary, NC, USA) for statistical analysis.

RESULTS

IL-6 secretion in SSc and control fibroblasts. IL-6 was detectable at a higher level in the supernatants collected from primary SSc fibroblast cultures than in control fibroblasts (617 ± 173 vs 213 ± 123 pg/ml, respectively; ANOVA, $p < 0.001$; Figure 1A).

IFN-γ alone (0, 500, 1000, 5000 IU/ml) had no effect on the IL-6 release in either SSc fibroblasts or controls.

TNF-α alone dose-dependently induced the release of IL-6 in SSc fibroblasts (IL-6: 609 ± 184, 723 ± 243, 1079 ± 297, and 1436 ± 326 pg/ml, respectively, with TNF-α 0, 1, 5, 10 ng/ml; Figure 2), while no effect was observed in controls.

The combination of IFN-γ and TNF-α had a significant

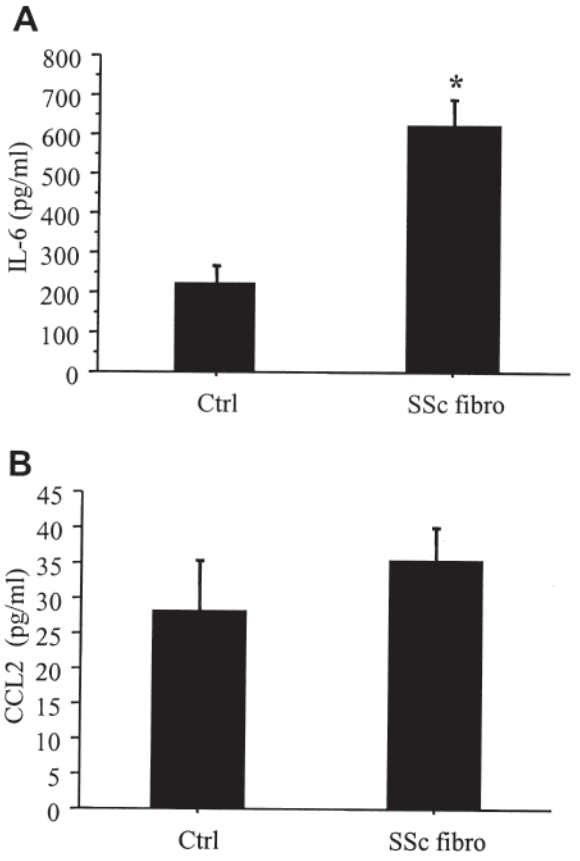

Figure 1. Interleukin 6 (IL-6) levels (A) in systemic sclerosis (SSc) fibroblast cultures (SSc fibro) at baseline were higher compared to control fibroblasts (*p < 0.001, ANOVA). Chemokine ligand 2 (CCL2) levels (B) in SSc fibroblast cultures at baseline were not significantly different from those in control fibroblasts (ANOVA). Bars show mean ± SEM.

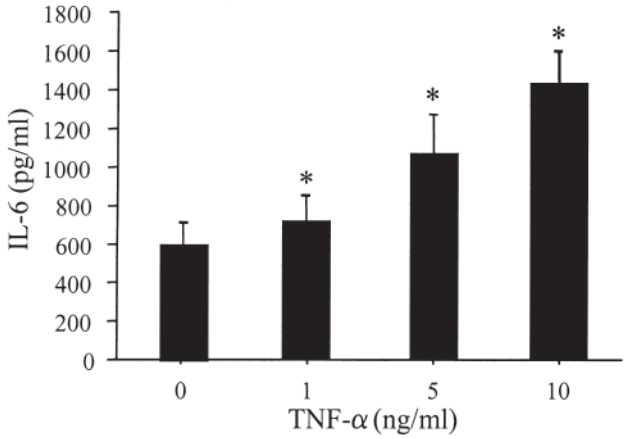

Figure 2. Tumor necrosis factor (TNF)-α alone (0, 1, 5, 10 ng/ml) dose-dependently induced interleukin 6 (IL-6) release by primary systemic sclerosis fibroblasts (p < 0.0001, ANOVA). Bars show mean ± SEM. *p < 0.05 or less vs TNF-α concentration 0 ng/ml (Bonferroni-Dunn test).

synergistic effect on the IL-6 secretion in SSc fibroblasts (ANOVA, p < 0.0001; Figure 3), while no effect was observed in controls.

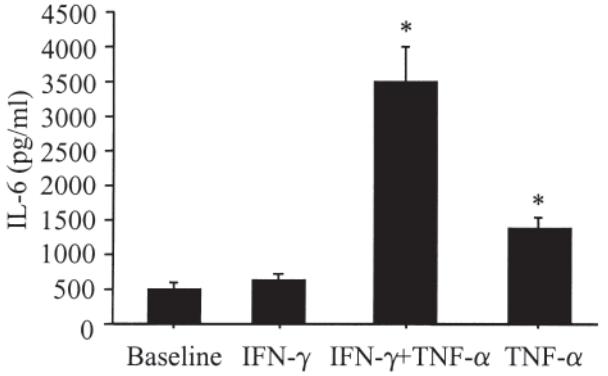

Figure 3. Interleukin 6 (IL-6) levels in systemic sclerosis fibroblast cultures at baseline and after stimulation with a combination of interferon (IFN)-γ (1000 IU/ml) and tumor necrosis factor (TNF)-α (10 ng/ml), or IFN-γ (1000 IU/ml) alone, or TNF-α (10 ng/ml) alone (p < 0.0001, ANOVA). Bars show mean ± SEM. *p < 0.05 or less vs baseline (Bonferroni-Dunn test).

CCL2 secretion in SSc and control fibroblasts. CCL2 was detectable (35 ± 11 pg/ml) in the supernatants collected from primary SSc fibroblast cultures, similarly to amounts collected in the control cultures (28 ± 15 pg/ml; Figure 1B).

IFN-γ dose-dependently induced the release of CCL2 in SSc fibroblasts (CCL2: 34 ± 15, 121 ± 68, 243 ± 132, and 378 ± 167 pg/ml, respectively, with IFN-γ 0, 500, 1000, 5000 IU/ml; ANOVA, p < 0.001), at a level similar to that in controls (CCL2: 29 ± 14, 109 ± 72, 217 ± 121, and 364 ± 126 pg/ml, respectively, with IFN-γ 0, 500, 1000, 5000 IU/ml; ANOVA, p < 0.001).

TNF-α alone dose-dependently induced the release of CCL2 in SSc fibroblasts (CCL2: 31 ± 14, 243 ± 95, 702 ± 206, and 1150 ± 311 pg/ml, respectively, with TNF-α 0, 1, 5, 10 ng/ml; Figure 4); while TNF-α alone had no effect on the CCL2 release in control fibroblasts.

The combination of IFN-γ and TNF-α had a significant synergistic effect on CCL2 secretion in SSc fibroblasts (ANOVA, p < 0.0001; Figure 5A), and in controls (Figure 5B). However, the combination of IFN-γ and TNF-α had a significantly stronger synergistic effect on CCL2 secretion (ANOVA, p < 0.0001) in SSc fibroblasts than in controls.

CXCL10 secretion in SSc and control fibroblasts. CXCL10 was absent in SSc fibroblasts under basal conditions. A significant secretion of CXCL10 was induced in the cellular preparations by stimulation with increasing doses of IFN-γ (CXCL10: 0, 128 ± 61, 297 ± 91, and 478 ± 121 pg/ml, respectively, with IFN-γ 0, 500, 1000, 5000 IU/ml; ANOVA, p < 0.001). The results obtained studying fibroblasts from the healthy subjects were not statistically different from those obtained using SSc fibroblasts.

Stimulation of fibroblasts with TNF-α alone (TNF-α 0, 1, 5, 10 ng/ml) was not able to induce secretion of the chemokine in either SSc or control fibroblasts.

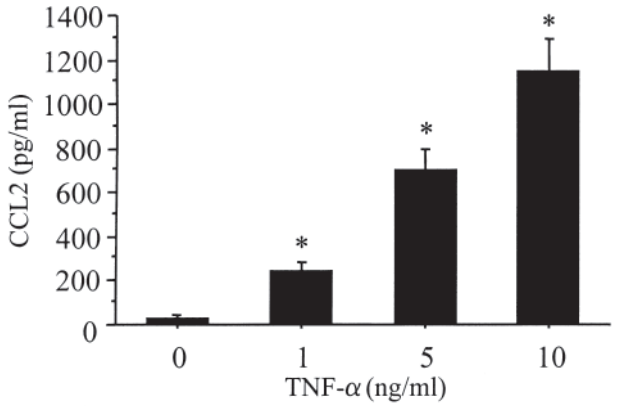

Figure 4. Tumor necrosis factor (TNF)-α alone (0, 1, 5, 10 ng/ml) dose-dependently induced chemokine ligand 2 (CCL2) release by primary systemic sclerosis fibroblasts ($p < 0.0001$, ANOVA). Bars show mean ± SEM. *$p < 0.05$ or less vs TNF-α concentration 0 ng/ml (Bonferroni-Dunn test).

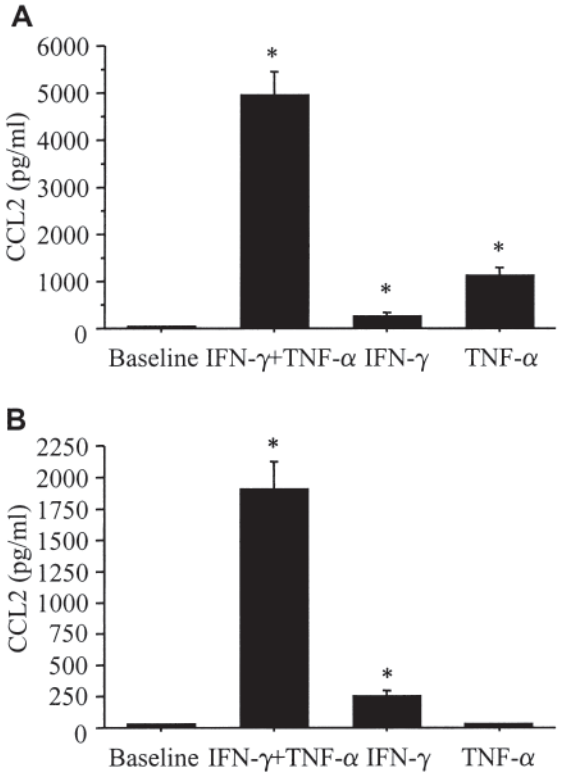

Figure 5. Chemokine ligand 2 (CCL2) levels in systemic sclerosis fibroblast cultures (A) and in control fibroblasts (B), at baseline and after stimulation with a combination of interferon (IFN)-γ (1000 IU/ml) and tumor necrosis factor (TNF)-α (10 ng/ml), or IFN-γ (1000 IU/ml) alone, or TNF-α (10 ng/ml) alone ($p < 0.0001$, ANOVA). Bars show mean ± SEM. *$p < 0.05$ or less vs baseline (Bonferroni-Dunn test).

Combinations of IFN-γ and TNF-α synergistically increased the secretion of CXCL10 compared to IFN-γ alone in SSc fibroblasts (CXCL10: 307 ± 101 vs 1349 ± 197 pg/ml, respectively, with IFN-γ, or IFN-γ combined with TNF-α; ANOVA, $p < 0.001$). The results obtained studying fibroblasts from the healthy subjects were not statistically different.

DISCUSSION

Some peculiarities of SSc fibroblasts upon stimulation with IFN-γ or TNF-α or the combination of IFN-γ and TNF-α were found in our study. Indeed, primary cultured fibroblasts from patients with SSc produced higher levels of IL-6 in basal condition than controls, while no difference was observed for CCL2 and CXCL10. TNF-α was able to dose-dependently induce secretion of IL-6 and CCL2 in SSc but not in control fibroblasts. SSc fibroblasts were induced by stimulation with increasing doses of IFN-γ to secrete CCL2 and CXCL10, while no effect was observed on IL-6. Further, the combination of IFN-γ and TNF-α induced a stronger secretion of IL-6 and CCL2 in SSc fibroblasts than in controls. In contrast, the synergistic effect of IFN-γ combined with TNF-α on the secretion of CXCL10 was similar in SSc fibroblasts and in controls.

Several clinical and laboratory studies suggest a crucial role of the immune system in the pathogenesis of SSc, which may involve both T and B lymphocytes and some "pathogenetic" autoantibodies[27,28]. A different combination of such alterations of the immune system may explain the appearance of different clinico-serological phenotypes of SSc. Moreover, the natural history of the disease, often characterized by unpredictable progression and outcomes, suggests a multistep pathogenetic process. It may be hypothesized that modulation of the immune system, due to various pathogenetic factors and possibly to therapeutic interventions, is responsible for markedly variable disease expression. Together, these considerations may explain the contrasting data in the literature, particularly concerning the possible role of a proinflammatory Th1/Th2 balance[28,29,30]: a variable polarization of T lymphocytes with different cytokine/chemokine repertoires might be present during the progression of SSc, varying from prevalent vascular to fibrotic alterations[28,29,30]. Therefore, in order to obtain more reproducible results, we used only fibroblasts obtained from patients with SSc at an early disease stage, not treated with any drug.

SSc fibroblasts produce IL-6 spontaneously, at a higher level than control fibroblasts, as suggested by some studies[4,5,6]. Our finding of a dose-dependent production of IL-6 under TNF-α stimulation is in agreement with results in another study[12].

However, to date, no study has evaluated the effect of IFN-γ in combination with TNF-α on the secretion of IL-6 in fibroblasts from patients with SSc. Our findings show that the combination of IFN-γ and TNF-α had a potent synergistic effect in SSc fibroblasts, whereas no effect was observed in controls.

SSc fibroblasts themselves might be a source of the circulating IL-6 chemokine in patients with SSc. However, we

cannot exclude that immune cells or endothelial cells may participate in the production of high levels of the circulating IL-6 in patients with SSc.

Our results, and the studies noted above, show the importance of IL-6 in the progression of SSc, suggesting that modulation of IL-6 may be a viable pathway for the development of a novel therapeutic intervention in SSc. This could be addressed in a prospective study with tocilizumab, a recombinant humanized monoclonal antibody against IL-6 receptor, in patients with SSc[31].

CCL2 may play an important role in the induction of fibrosis through its direct effect on fibroblasts, together with its indirect effect mediated by a number of cytokines released from leukocytes recruited into the lesional tissues. Experiments in animal models also support the role of CCL2 in tissue fibrosis. In the murine sclerodermatous graft versus host disease (Scl GVHD) model, increased expression of CCL2 precedes the development of skin and lung fibrosis[32]. In a rat model of bleomycin-induced pulmonary fibrosis, mRNA and protein levels of CCL2 are elevated[33]. Administration of anti-CCL2 neutralizing antibody reduced skin sclerosis in bleomycin-treated mice[34]. Anti-CCL2 gene therapy attenuated pulmonary fibrosis in bleomycin-treated mice[35]. Mice deficient for CCR2 are protected from fluorescein isothiocyanate-induced and bleomycin-induced lung fibrosis[36,37].

We recently described high serum levels of CXCL10 (Th1) and CCL2 (Th2) chemokines in newly diagnosed SSc[15,16]. High values of CXCL10 were associated with a more severe clinical phenotype (lung and kidney involvement). CXCL10 declined during the followup, while CCL2 remained unmodified, suggesting that the disease progresses from the early Th1 inflammatory condition to the advanced Th2-like stage.

The mechanisms that induce CCL2 secretion in the first phases of SSc are not known. Our results show that IFN-γ in early SSc is able to induce secretion of both CCL2 and CXCL10 chemokines by scleroderma fibroblasts. The IFN-γ-induced CCL2 secretion is unexpected, since Th2 chemokines are poor responders to IFN-γ, but it is in agreement with results from other studies. Yamana, *et al* showed that IFN-γ and lipopolysaccharide synergistically induced CCL2 and nitric oxide release in primary murine dermal fibroblasts[38]. Moreover, Struyf, *et al* showed in human fibroblasts that IFN-γ is a potent stimulus for CCL2 release[39].

As for CXCL10, it is known that IFN-γ-inducible CXC chemokines can be produced by several types of normal mammalian cells, such as endothelial cells[40], thyrocytes[41], and fibroblasts[42]. These cells are able to produce the CXC chemokines, not in basal conditions, but only after stimulation by cytokines such as IFN-γ and TNF-α that are secreted in a Th1 inflammatory site. This process has been suggested to be involved in the initiation and perpetuation of the inflammation in several autoimmune diseases, and on the basis of our results could be applied even to SSc.

This view is in agreement with recent results from Zhou, *et al*, who investigated the molecular mechanisms governing skin fibrosis in Scl GVHD, a model for human SSc[43]. Our findings of high levels of CXCL10 in patients with SSc are consistent with this model of murine Scl GVHD.

In the early phase of SSc, high serum levels of both CXCL10 (Th1) and CCL2 (Th2) were found; successively, serum CXCL10 declined, while CCL2 levels remained unmodified, with a consequent increase of the Th2/Th1 balance (roughly evaluated through the CCL2/CXCL10 ratio). Thus, the disease progresses from early inflammatory (Th1-like) to later noninflammatory (Th2-like) stages, when the inflammation leads to fibrosis. Our results in SSc fibroblasts suggest that fibroblasts themselves are a source of the circulating chemokine CCL2 and CXCL10 in patients with SSc. However, we cannot exclude that immune cells[44] or endothelial cells[40] may participate in the production of high levels of circulating CXCL10 in patients with SSc.

Stimulation of fibroblasts with TNF-α alone was able to induce CCL2 chemokine secretion in SSc fibroblasts, but not in controls. This result is in agreement with another study that showed TNF-induced CCL2 expression of fibroblasts isolated from SSc patients[18]. The production of CCL2 induced by TNF-α in SSc fibroblasts suggests a deregulation of chemokine production induced by cytokines in SSc; the mechanisms for this remain to be investigated.

TNF-α alone was not able to induce CXCL10 chemokine secretion in either control or SSc fibroblasts, in agreement with other studies[40,41,42].

Stimulation of fibroblasts with combinations of IFN-γ and TNF-α synergistically increased CCL2. Interestingly, the synergistic effect of IFN-γ and TNF-α on CCL2 secretion was more than double in SSc fibroblasts than in controls. In contrast, the synergistic effect of IFN-γ and TNF-α on CXCL10 secretion was similar in SSc fibroblasts and in controls, in agreement with results obtained in fibroblasts in other autoimmune diseases[42,44], and confirming our previous studies in human SSc fibroblasts[45].

The stronger synergistic effect of the combinations of IFN-γ and TNF-α on CCL2 secretion in SSc fibroblasts than in controls may be related, in part, to the production of CCL2 induced by TNF-α alone in SSc fibroblasts, reinforcing the hypothesis of a deregulation of chemokine production induced by cytokines in SSc; the mechanisms involved remain to be investigated.

Our results provide evidence for the involvement of proinflammatory cytokines (IL-6) and Th1 (CXCL10) or Th2 (CCL2) chemokines in the development of SSc. Indeed, the Th2 cytokine/chemokine response, with production of CCL2 (and also IL-4, IL-10, and TGF-ß), leads to tissue fibrosis, whereas the Th1 and proinflammatory cytokines/chemokines promote inflammation in patients with SSc[46].

Development of cytokine modulators able to restore the cytokine/chemokine balance could be considered for new therapeutic approaches in SSc[46].

It should be considered that our results were obtained from a small case series, although homogenous patients were recruited. Therefore larger studies are needed. Moreover, this was an *in vitro* study, and these results need to be confirmed *in vivo*.

SSc fibroblasts might participate in the self-perpetuation of inflammation by releasing proinflammatory cytokines (IL-6) or Th1 (CXCL10) and Th2 (CCL2) chemokines under the influence of IFN-γ and TNF-α. Some peculiarities of SSc fibroblasts were observed with respect to controls: (1) SSc fibroblasts produced basally larger amounts of IL-6; (2) TNF-α was able to induce IL-6 and CCL2 secretion only in SSc fibroblasts; and (3) the synergistic effect of IFN-γ and TNF-α on secretion of IL-6 and CCL2 was stronger in SSc fibroblasts than in controls. However, the mechanisms involved remain to be investigated.

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