Effect of Granulocyte Macrophage-Colony Stimulating Factor on Extracellular Matrix Deposition by Dermal Fibroblasts from Patients with Scleroderma

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ABSTRACT. ***Objective***. To investigate the *in vitro* effect of granulocyte macrophage-colony stimulating factor (GM-CSF) on the deposition of extracellular matrix (ECM) in fibroblasts obtained from the skin of patients with systemic sclerosis (Ssc), compared to healthy controls.

Methods. Dermal fibroblasts obtained from 14 patients with SSc (7 with the diffuse form and 7 with CREST syndrome) and from 7 controls were studied. Both SSc and normal skin fibroblast cultures were stimulated for 4 and 8 days with 100 ng/ml GM-CFS. GM-CSF receptor (GM-CSFR) expression was determined by Western blot of cell lysates. Immunofluorescence was used to determine GM-CSFR expression and to investigate the deposition of ECM (type I collagen, fibronectin, and tenascin). Quantitative analysis of ECM was performed by ELISA. Expression of type I collagen and metalloproteinase 1 (MMP-1) mRNA was determined by real-time quantitative PCR.

Results. Deposition of ECM by normal fibroblasts appeared not to be influenced by stimulation with GM-CSF; in contrast, after stimulation with GM-CSF SSc fibroblasts showed increased deposition of fibronectin and tenascin, while type I collagen production was decreased; these results were found with both immunofluorescence and ELISA. Quantitative PCR revealed that GM-CSF inhibited the expression of mRNA type I collagen in SSc fibroblasts but not in normal fibroblasts, whereas levels of the main collagenolytic enzyme, MMP-1, were not affected.

Conclusion. These results suggest that in SSc fibroblasts GM-CSF exerts a blocking effect on the deposition of type I collagen, through an inhibitory action on mRNA, while the production of other components of ECM such as fibronectin and tenascin is increased by stimulation with this cytokine. (J Rheumatol 2005;32:656–64)

Key Indexing Terms:
GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR FIBROBLASTS
GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR RECEPTOR
SYSTEMIC SCLEROSIS EXTRACELLULAR MATRIX

Scleroderma (systemic sclerosis, SSc) is a multisystem disorder of connective tissue characterized by extensive thickening and fibrosis of the skin and by a proliferative reaction in blood vessel walls, often accompanied by a failure of internal organs, such as lungs, heart, or kidneys[1-4]. Various clinical forms are recognized, the most frequent being the CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias) and the diffuse form. The pathological alterations of the disease are due to excessive deposition of extracellular matrix (ECM) proteins, predominantly type I collagen, in the affected tissues. Excessive ECM production is the consequence of abnormal interactions between endothelial cells, fibroblasts, and mononuclear cells.

Immunological abnormalities are present very early in the development of SSc. Mononuclear cells, particularly macrophages and T lymphocytes, play a prominent role in fibroblast activation and ECM synthesis. Due to activation signals from lymphocytes and monocytes, the properties of endothelial cells and platelet fibroblasts are altered either directly, by cell contact, or indirectly, via specific cytokines. In SSc, the pathogenic role of cytokines such as interleukin 1

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(IL-1), IL-2, IL-6, transforming growth factor-ß (TGF-ß), and IL-4 has been emphasized in many studies[5-8]. In particular, stimulation of fibroblast activity and upregulation of the synthesis of some components of ECM, such as tenascin and type I collagen, by cytokines have been reported[9,10]. We have reported the presence of enhanced concentrations of granulocyte macrophage-colony stimulating factor (GM-CSF) in sera of patients with SSc[11]. GM-CSF is a cytokine that induces growth and activation of monocytes and granulocytes. Stimulating effects on endothelium and connective tissue cells have also been described[12-16]. Moreover, it has recently been reported that transgenic mice with enhanced expression of the GM-CSF gene have high serum concentrations of this cytokine and develop fibrotic nodules inside skeletal muscles and diaphragm: these nodules contain macrophages and fibroblasts[17].

We previously demonstrated the presence of a specific receptor for GM-CSF (GM-CSFR) on the surface of normal human skin fibroblasts and its increased expression, in basal condition, during SSc[18]. This receptor consists of 2 chains: the alpha chain is typical of GM-CSFR, while the beta chain is also shown by the IL-3 and IL-5 receptors[19].

On the basis of these results that suggest a role of GM-CSF in the pathogenesis of SSc, we investigated the *in vitro* effects of GM-CSF on GM-CSFR expression in fibroblasts obtained from the skin of patients with SSc. Production of ECM components by normal and scleroderma skin fibroblasts was also studied, with respect, in particular, to fibronectin, tenascin, and type I collagen. The gene expression of the main important collagenolytic enzyme, metalloproteinase 1 (MMP-1), was also studied.

MATERIALS AND METHODS

Patients and controls. Fourteen patients (12 women, 2 men) with SSc (7 with the diffuse form, 7 with CREST syndrome) were admitted for study, after giving informed consent and with Ethical Committee approval. No patient had genetically determined connective tissue pathologies, and none reported a similar pathology in his or her family. All patients with the diffuse form had involvement of dorsal arm and/or thorax. To reduce variability among patients, we admitted for study only SSc patients positive for antinuclear antibodies (ANA) showing a speckled pattern as evaluated by indirect immunofluorescence and successively by ELISA. Since ANA positivity may be considered not sufficient for subtype characterization, other clinical measures were investigated; in particular, all patients with diffuse disease presented serum anti-Scl-70 topoisomerase I positivity, and patients with CREST syndrome showed serum anticentromere (CENP-B) positivity. The mean age of patients was 54 years (range 31–70) and the mean disease duration was about 8 years (range 4–27). It is commonly held that scleroderma related processes are not influenced substantially after 4 years' disease duration[20]. Our observations confirm this opinion. Patients and healthy controls were Caucasians, born in Southern Italy, where the prevalence of SSc is about 4–12 new cases/million/year. None had been exposed to any environmental or professional risk factor or had a family history of SSc.

No patient had been treated with steroids or immunosuppressive drugs in the last 6 months. Only patients with diffuse disease presented visceral involvement, and none had other diseases. In each patient, a fine needle-punch biopsy was carried out from an affected skin area. The criteria to differentiate involved skin areas from unaffected areas were skin elasticity and the presence of accessory organs of the skin. In the CREST patients, biopsies were performed on the dorsal finger skin, and in the diffuse-form patients on the dorsal forearm skin. Surgical fragments from the same sites were obtained from 7 volunteers (5 women, 2 men, ages 37 ± 4 yrs) undergoing aesthetic or emergency surgery who were used as controls. Specimens from healthy controls were microdissected in very small fragments comparable to needle-punch biopsies. The length of time and number of passages required to obtain adequate cell numbers were therefore similar for control specimens and SSc fibroblasts.

Each fragment was divided into 2: one was embedded in paraffin and used by the pathologist for histological diagnosis, the other was mechanically dissociated under a stereo microscope and used for *in vitro* culture.

Cell culture. SSc fibroblasts from skin biopsies were obtained as follows: surgical fragments were mechanically dissociated and then submitted to tripsynization for 30 min at 37˚C. After repeated washing in phosphate buffered saline (PBS), microfragments were plated and cultured in Dulbecco's minimal essential medium (DMEM; BioWhittaker, Verviers, Belgium), containing 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA), 200 mM L-glutamine, penicillin (100 mg/ml), and streptomycin (100 mg/ml). Plates were incubated at 37˚C in 5% CO_2; the medium was replaced every 3 days.

Surgical fragments from healthy subjects were submitted to the same treatment and cultured in the same conditions. For immunofluorescence studies, sterile glass coverslips were put into cell dishes before plating the fibroblasts, to allow cell attachment.

GM-CSF. Human recombinant GM-CSF (150 mg/ml) was obtained from Schering Plough (Cork, Ireland). SSc and normal skin fibroblast cultures were stimulated for 4 and 8 days with 10, 50, 100, and 200 ng/ml of GM-CSF; fresh factor at the same concentration was added every 2 days to the culture medium.

GM-CSF showed no statistically significant effect on ECM production by normal and SSc fibroblasts at any concentration, with the exception of collagen type I deposition by SSc fibroblasts (see Results). Since the differences in collagen type I deposition became statistically significant at 100 ng/ml, we used this concentration for the rest of our study.

Immunoblotting of cell lysates. Primary cultures of fibroblasts from healthy donors and SSc patients, grown to near confluence in 100 mm dishes, were incubated 10 min at 4˚C with 0.5 ml lysis buffer (PBS, 1% Triton X-100), containing proteases inhibitors. Cell lysates were collected by scraping and centrifuged at 12,000 g for 5 min at 4˚C. Protein concentrations in cell lysates were determined by a colorimetric assay (BioRad, Richmond, CO, USA) and 100 µg of total protein was incubated 5 min at 90˚C in Laemmli sample buffer. Cell lysates were electrophoresed on 10% SDS-polyacrylamide gels. Gels were electroblotted and the membranes were blocked with 5% fat-free dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine. After 3 washes with washing solution (0.1% fat-free dry milk, 0.1% ovalbumin, 1% FCS in PBS), the membranes were incubated overnight at 4˚C with monoclonal anti-GM-CSFR antibody, concentration 10 µg/ml, in washing solution. After 4 washes, filters were incubated for 30 min at room temperature with horseradish peroxidase conjugated goat anti-mouse antibody (BioRad), diluted 1:3000 in PBS. After 3 washes as above and 3 additional washes in TTBS (50 mM Tris, pH 7.5, 0.5 M NaCl, 0.2% Tween-20), the membranes were stained with an ECL system (Amersham, Little Chalfont, England). As well, membranes were incubated overnight at 4˚C with anti-actin antibody (as internal standard). A quantitative analysis was performed by scanner densitometry (Snapscan 1212, Agfa).

Immunocytochemistry. Glass coverslips with attached fibroblasts were fixed with 3% formaldehyde in PBS, gently washed with PBS, and then incubated with mouse monoclonal antibodies against GM-CSFRα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), collagen I and tenascin, and rabbit polyclonal antibody against fibronectin (Sigma, St. Louis, MO, USA). After further washings with PBS, cells were stained with fluorescein or rhodamine labeled goat anti-mouse antibodies or with goat anti-rabbit

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fluoresceinated antibodies. Both steps required 1 h incubation at 37˚C in a humid chamber. Nuclear staining with bisbenzimide was performed for 10 min at room temperature.

Coverslips were then mounted with Vectashield (Vector, Newcastle, UK) and observed with a DM LB microscope (Leica, Solms, Germany) equipped for epifluorescence and microphotography. Every sample was evaluated by 3 independent observers, using a 4 point arbitrary scale ranging from 0 to 3.

In situ ELISA. Quantitative analysis of some ECM components (collagen I, fibronectin, tenascin) produced by SSc and normal fibroblasts was perfomed by *in situ* ELISA. In particular, quantitative assessment of extracellular and intracytoplasmic collagen I was performed by ELISA, since Western blot is not suitable for collagen quantification. The ELISA procedure has largely been accepted for quantitative assessment of collagen expression, in lieu of the hydroxyproline assay[21]. Western blotting was employed to quantify fibronectin and tenascin, in addition to the ELISA, but no difference was observed between the 2 methods (data not shown).

Fibroblast cells were plated in 96 well plates at a density of 5000 cells/well and analyzed on the fourth and eighth days of culture. Cells were fixed by acetone/methanol (v/v) for 10 min at room temperature, then incubated in 0.5% PBS/BSA, 0.2% Tween 20 for 2 h at 4˚C to minimize aspecific binding sites, and washed in PBS.

Primary antibodies were then added (anticollagen I from goat, antifibronectin, anti-tenascin from rabbit; Chemicon, Temecula, CA, USA) and cells were incubated 30 min at room temperature with horseradish peroxidase conjugated anti-rabbit IgG and anti-goat IgG (Santa Cruz Biotechnology).

After further washes in PBS, the specific substrate was added (1 mg/ml OPD/0.1 mol/l citrate buffer, pH 5.0, 0.006% H_2O_2) and cells were incubated 30 min at 37˚C in the dark. The reaction was then stopped using 1 N H_2SO_4, and the absorbance was read at 450 nm in a spectrophotometer.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from cells cultured at 8 days using Trizol reagent according to the manufacturer's instructions (Invitrogen, Paisley, UK). Contaminating DNA was digested with DNase using a DNase Kit (Invitrogen) and 2 µg of total RNA was reverse transcribed with 100 U SuperScript II Rnase H-Reverse Transcriptase (Invitrogen) in a volume of 40 µl, using 100 mM random hexamer primers (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Primer sequences for RT-PCR were as follows: collagen type I forward primer, 5'-GAG GAA GGC CAA GTC GAG G-3'; collagen type I reverse primer, 5'-CCG AGT GAA GAT CCC CTT TTT A-3'; and generated an 86 base-pair fragment.

The amplification was established using a DNA Thermal Cycler (Perkin Elmer Cetus) for 35 cycles as follows: denaturation, 95˚C for 45 s; annealing, 60˚C for 30 s; extension, 72˚C for 30 s.

At the beginning of the reaction, a cycle at 95˚C for 5 min was carried out to activate Taq polymerase.

Real-time quantitative PCR. A quantitative assay for type I (alpha 1) collagen mRNA expression and matrix MMP-1 mRNA expression was established using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). All measurements were normalized to an endogenous control using the 101 bp fragment at 3' of the beta-glucuronidase region. PCR oligo primers were: h-COL1A1 forward primer 5'-GAG GAA GGC CAA GTC GAG G-3', and h-COL1A1 reverse primer 5'-ACG TCT CGG TCA TGG TAC CTG-3' generating an 86 bp fragment; MMP-1 forward primer 5'-AGAGCAGATGTGGACCATGC-3' and MMP-1 reverse primer 5'-ACCGGACTTCATCTCTGTCG -3'; h-GUSB forward primer 5'-GAA AAT ATG TGG TTG GAG AGC TCA TT-3' and h-GUSB reverse primer 5'-CCG AGT GAA GAT CCC CTT TTT A-3'.

Real-time PCR was performed using the SYBR Green PCR Master Mix 2X (Applied Biosystems) and 50 ng of cDNA in a total volume of 15 µl. Each sample was run in triplicate, for both type I (alpha 1) collagen and beta-glucuronidase. The PCR cycling profile consisted of AmpErase UNG incubation for 2 min at 50˚C and AmpliTaq Gold activation for 10 min at 95˚C, as predenaturation steps, and 50 two-step cycles at 95˚C for 15 s and at 60˚C for 60 s. For type I (alpha 1) collagen mRNA and MMP-1 mRNA relative quantification, the comparative Ct method was used according to the manufacturer's instructions (Applied Biosystems).

Statistical analysis. For statistical evaluation, we used the Student t test for unpaired data (t test on the mean), or the independent t test, in which the means and standard deviations of 2 independent samples are compared. Probability values of $p \leq 0.05$ were accepted as statistically significant.

RESULTS

Immunoblotting of cell lysates. To identify the GM-CSF receptor–α chain (GM-CSFRα), whole-cell lysates from controls and cell lysates of dermal fibroblasts from both SSc forms were subjected to Western blot with monoclonal anti-GM-CSFRα chain antibodies. As expected, the receptor migrated in a band with an apparent molecular weight of 84 kDa.

In agreement with our previous study[18], GM-CSFR expression was higher in cell lysates from SSc fibroblasts in basal conditions compared to normal fibroblasts. Expression of the receptor appeared significantly increased ($p < 0.05$) in lysates from normal cells after GM-CSF stimulation, while no significant effect of the cytokine was observed in lysates from SSc fibroblasts (Figure 1) from either the diffuse form SSc or the CREST syndrome samples.

Immunocytochemistry. GM-CSFR was expressed on the membrane of normal and SSc cells as shown by immunofluorescence (Figure 2). The deposition of ECM component at Day 8 in treated and untreated cells is summarized in Table 1.

Fibronectin and tenascin deposition was increased in all SSc cultures, compared to fibroblasts from controls, and was apparently slightly increased after treatment with GM-CSF. However, the difference between treated and untreated SSc cells was not significant (Table 1).

Type I collagen production showed quite different behavior. Indeed, deposition was increased in SSc fibroblasts compared to normal ones, as expected, while GM-CSF treatment appeared to reduce the production of this component; only a few cells exhibited type I collagen immunopositivity. Therefore, untreated SSc cells appeared to be more active than treated ones, at least for deposition of this type of collagen (Table 1, Figure 3).

In situ ELISA

Collagen synthesis. Results obtained with this assay confirmed the immunofluorescence observations. In basal conditions, scleroderma fibroblasts showed enhanced type I collagen deposition activity compared to normal fibroblasts, with a significant increase after 8 days.

In contrast, scleroderma fibroblasts (from both CREST and diffuse samples) stimulated with GM-CSF concentrations greater than 100 ng/ml showed a statistically significant decrease of type I collagen deposition after 8 days compared to SSc untreated cells.

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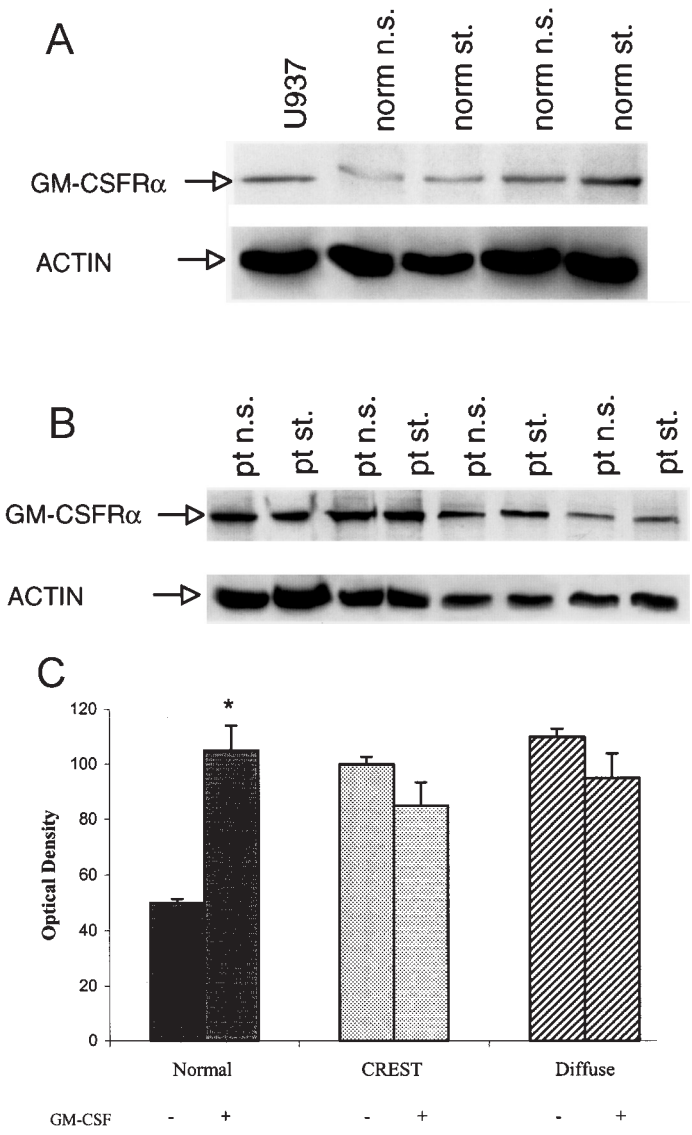

Figure 1. Effect of GM-CSF stimulation on the expression of GM-CSFR. Primary cell cultures derived from tissues of 7 healthy individuals and 14 SSc patients (7 CREST syndrome, 7 diffuse form) were stimulated with (st.) or without (n.s.) 100 ng/ml GM-CSF for 8 days, and then lysed; 100 µg total protein was analyzed by 10% SDS-PAGE and Western blot with an anti-GM-CSFRα antibody. The filter was then hybridized with an anti-actin antibody, as a control for protein loading. Two representative patterns of normal individuals (norm, A) and 4 patterns of SSc patients (pt, B) are shown. A lysate from U937 cells was employed as a positive control for GM-CSFRα production. C: mean optical densities of GM-CSFRα bands from all 21 samples. No difference was observed between SSc patients with diffuse and CREST form. *$p < 0.05$.

Normal fibroblasts were not influenced by GM-CSF stimulation (Figure 4A, 4B).

Fibronectin and tenascin synthesis. In basal conditions, as expected, scleroderma fibroblasts showed a significant increase in fibronectin and tenascin deposition compared to normal fibroblasts. This activity was not influenced by GM-CSF treatment in either normal or SSc fibroblasts (Figure 5A, 5B). Indeed, differences between scleroderma fibroblasts untreated and treated with GM-CSF were not statistically significant. The same data were obtained with different GM-CSF concentrations (10, 50, or 200 ng/ml; data not shown).

RT-PCR and real-time quantitative PCR. Type I collagen deposition was investigated also by RT-PCR and real-time quantitative PCR. The results show that GM-CSF inhibited the expression of type I collagen mRNA in SSc fibroblasts, while it had no statistically significant effect on healthy control fibroblasts (Figure 6), in agreement with the data obtained by immunofluorescence and *in situ* ELISA. No statistically significant differences were found in the expression of MMP-1 mRNA between fibroblasts from healthy controls and from SSc patients both in basal conditions and after stimulation (Figure 7).

DISCUSSION

Increased deposition of type I collagen by dermal fibroblasts is one of the main factors in the pathogenesis of SSc[22], and the role played by various cytokines in this process has been described in several studies[23-25]. We have reported that SSc patients present increased plasma concentrations of GM-CSF[11]. Further, we have also demonstrated an increased expression of GM-CSFR on the membrane of dermal fibroblasts from scleroderma patients[18].

The biological role of the GM-CSF/GM-CSFR system is an intriguing problem. Since *in vitro* stimulation of SSc fibroblasts with GM-CSF failed to influence cell proliferation[18], we suggested that this cytokine, through its receptor, exerts some effect on other cellular activities, in particular on the synthesis of ECM components.

Our results indicate that GM-CSF has a stimulating effect on collagen deposition *in vitro* by normal fibroblasts, but not by SSc fibroblasts, which in contrast show a decreased type I collagen deposition after treatment with GM-CSF compared to SSc unstimulated cells. Since the basal synthetic activity of unstimulated SSc fibroblasts increases in the first 8 days of culture, it is reasonable to suggest a direct inhibiting effect of GM-CSF on this activity, in contrast to the stimulating effect observed in normal fibroblasts.

Since SSc fibroblasts *in vitro* show increased expression of GM-CSFR[18], we also suggest a correlation with the increased plasma level of the cytokine. We have demonstrated that the addition of GM-CSF to the culture medium induces increased expression of its specific receptor on the cell membrane of fibroblasts, together with increased deposition of ECM components, without stimulating cell proliferation. On the other hand, our data show that GM-CSFR is not upregulated by GM-CSF in SSc fibroblasts *in vitro*, suggesting that in these cells the expression of GM-CSFR is already maximal and cannot be stimulated further by GM-CSF.

As a consequence, GM-CSF cannot exert its stimulating action on the synthetic activity of SSc fibroblasts.

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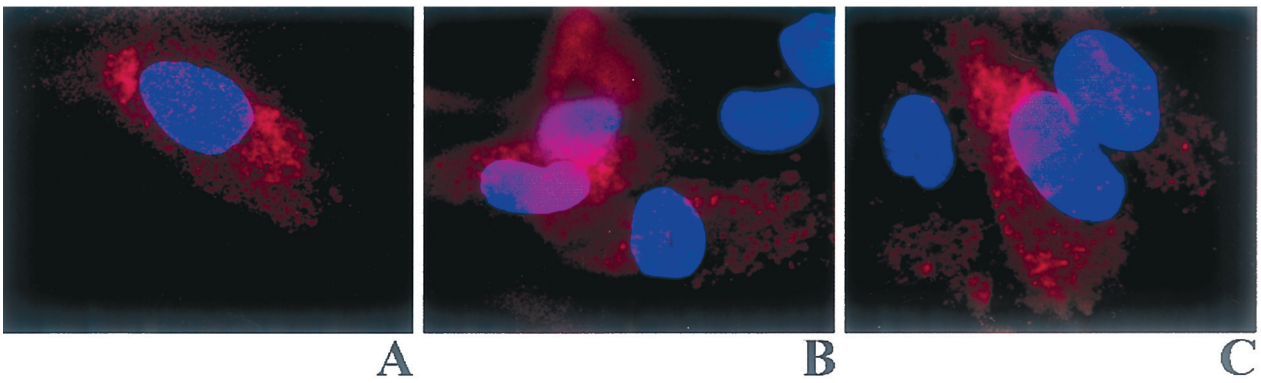

Figure 2. GM-CSFR expression in healthy control fibroblasts (A), and in SSc untreated (B) and treated (100 ng GM-CSF/ml, C) fibroblasts, shown by immunofluorescence. At Day 8 of culture, cells were treated with mouse mAb against GM-CSFRα and stained with rhodamine-labeled goat anti-mouse IgG antibodies. As expected, the receptor is uniformly distributed on the cell membrane; nuclei are stained with bisbenzimide (light blue; original magnification x1000).

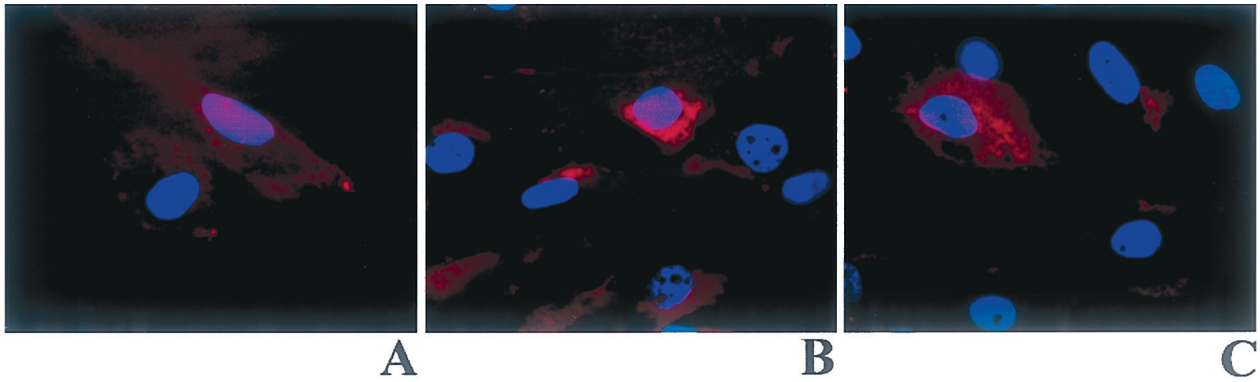

Figure 3. Type I collagen deposition, shown by immunofluorescence, is similar in control (A) and SSc untreated (B) fibroblasts, uniformly distributed in all cells. Cells cultured 8 days in the presence or absence of 100 ng GM-CSF/ml were reacted with mouse anti-collagen I mAb and stained with rhodamine-labeled goat anti-mouse antibodies. In GM-CSF-treated SSc fibroblasts (C) deposition is decreased and only a few cells exhibit immunopositivity (original magnification x400).

The observed decrease of collagen I after GM-CSF stimulation is difficult to explain. In this regard, it is important to note that 2 different cytokines, interferon-γ (IFN-γ) and interleukin 10, interfere with collagen metabolism[26,27]. IFN-γ acts by inhibiting transcriptional processes, as shown by the reduced levels of mRNA of type I, II, and III procollagen after IFN-γ stimulation[27]. IL-10, on the other hand, acts through a stimulating action on the enzymes (metalloproteinases) directly involved in collagen catabolism[26]. We have, therefore, measured the levels of type I collagen mRNA and the levels of MMP-1 mRNA, after stimulation of GM-CSF, to investigate whether this cytokine acts on collagen and MMP-1 gene expression.

Table 1. Immunocytochemical evaluation of deposition of components of the extracellular matrix by control and SSc fibroblasts in basal conditions and after GM-CSF treatment. Primary cell cultures derived from 7 controls and 14 SSc patients were treated 8 days in the presence or absence of 100 ng/ml GM-CSF. Evaluation was as described in Materials and Methods.

	Control		SSc	
	Basal Conditions	GM-CSF Treated	Basal Conditions	GM-CSF Treated
Fibronectin	++	+++	+++	+++
Tenascin	++	+++	+++	+++
Type I collagen	++	+++	++++	++

Our data show that type I collagen mRNA levels are decreased in SSc fibroblasts and are unaffected in normal cells after GM-CSF stimulation, thus showing a direct correlation between mRNA and protein levels. We therefore suggest an inhibitory role of GM-CSF on the transcriptional processes of SSc cells, through a suppressing effect on type I collagen mRNA synthesis. Vice versa, there was no significant variation, either in basal conditions or after stimulation, in SSc fibroblasts compared to normal samples.

It has been shown that SSc fibroblasts present unaltered MMP-1 gene expression, as compared to normal fibroblasts, in spite of reduced enzymatic activity in SSc cells, indicating that MMP-1 mRNA levels do not necessarily reflect the activity of the enzyme[28].

Our results are also in agreement with a previous study[29] demonstrating that MMP-1 mRNA levels in fibroblasts from patients with SSc of more than 6 years' duration showed no significant difference from the levels found in normal fibroblasts.

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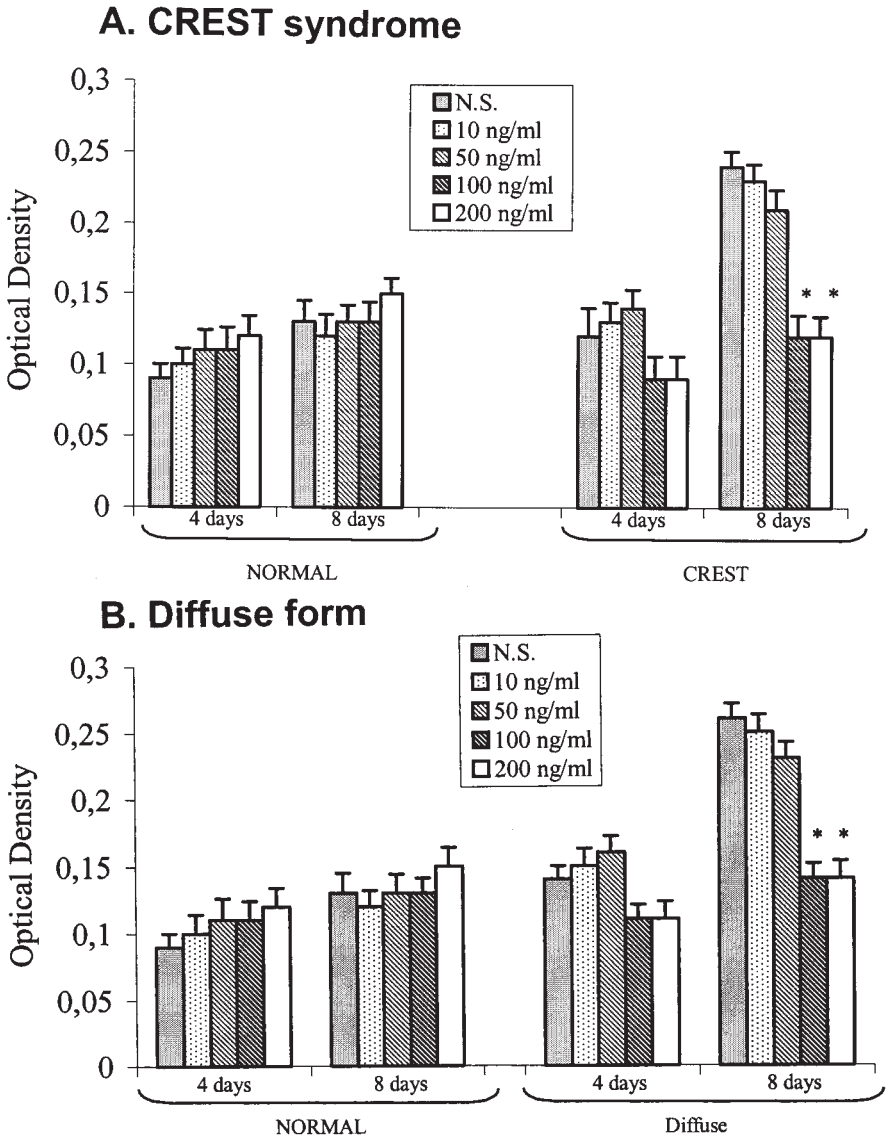

Figure 4. Type I collagen deposition in normal and SSc fibroblasts (from CREST syndrome and diffuse form). (A) Fibroblasts from 7 patients with CREST syndrome and 7 healthy individuals were stimulated with GM-CSF concentrations as indicated (NS: control, not stimulated). After 4 and 8 days, cells were assayed for collagen deposition by ELISA, using a mouse anti-collagen I mAb (*$p < 0.05$). (B) Fibroblasts from 7 patients with diffuse SSc and 7 healthy individuals were assayed by ELISA for collagen I deposition, as in panel A. Each column represents the average of the 7 SSc patients and the 7 controls; bars indicate standard deviation. Difference between untreated and treated SSc fibroblasts was statistically significant at GM-CSF concentrations of 100 and 200 ng/ml, after 8 days of stimulation (*$p < 0.05$).

It has recently been shown that an increased expression of heat shock protein 47 (HSP-47), a collagen-binding stress protein able to act as a collagen-specific molecular chaperone during the biosynthesis and secretion of procollagen[30], is present in SSc but not in normal fibroblasts, and it is associated with an overproduction of type I procollagen by SSc fibroblasts. Moreover, stimulation of these cells with IFN-γ results in a significant decrease in HSP-47 mRNA levels, in a dose-and time-dependent manner[30].

We therefore plan to test in a forthcoming study whether GM-CSF is able to reduce HSP-47 expression, and consequently type I procollagen mRNA levels, with a mechanism similar to that of IFN-γ.

On the other hand, the Smad system may be another interesting pathway to investigate, in order to clarify GM-CSF involvement in the fibrotic process of scleroderma. The Smad are intracellular signal transducers mediating fibroblast activation and fibrotic response by inducing transcription of TGF-ß, which in turn promotes collagen deposition[31-33]. In particular, murine skin fibroblasts affected by

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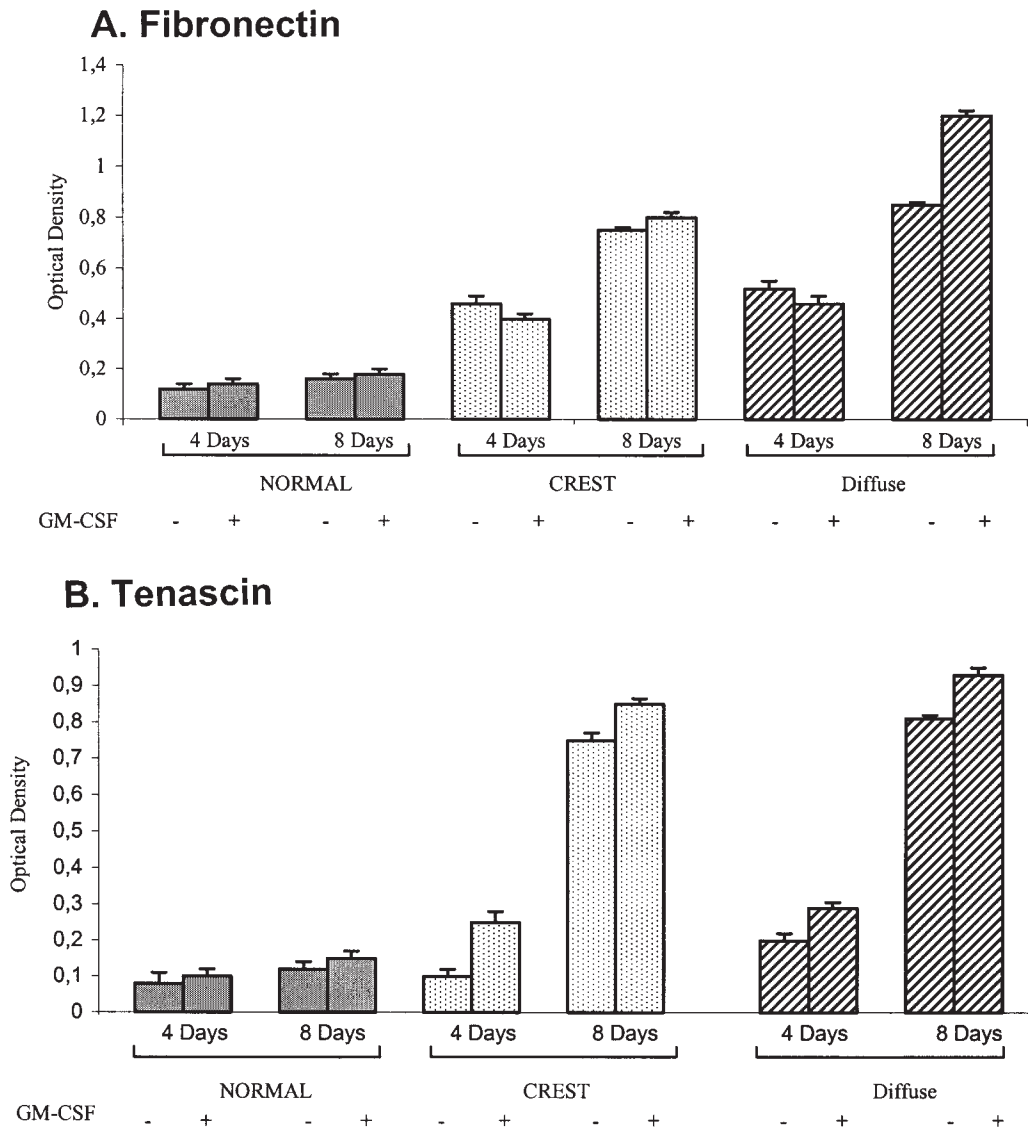

Figure 5. ECM protein deposition in control and SSc fibroblasts from CREST syndrome and diffuse form. Fibroblasts from the same controls and SSc patients shown in Figure 4 were cultured 4 and 8 days in the presence or absence of 100 ng GM-CSF/ml and analyzed by ELISA for fibronectin (A) and tenascin (B) deposition. Each column represents the average of 7 cultures from control or SSc fibroblasts; bars indicate standard deviation. No statistically significant differences were observed between treated and untreated cells.

bleomycin-induced scleroderma showed increased expression of Smad 2/3; in contrast the expression of Smad 7, the endogenous inhibitor of Smad signaling, was reduced in the same experimental conditions[34]. We suggest a possible intracellular signaling axis — GM-CSF/Smad — that deserves further investigation.

It is not clear why the effect of GM-CSF is confined only to SSc fibroblasts. As reported, different factors are implicated in the regulation of type I collagen gene expression in healthy and SSc fibroblasts[35]. GM-CSF probably acts in SSc fibroblasts by activating signal transduction pathways different from those involved in normal fibroblasts. For instance, the different concentrations of the αVß5 integrins observed in scleroderma fibroblasts could influence the response of these cells to stimulation[36].

We are aware that the *in vivo* transposition of *in vitro* results is often very difficult; we must, however, consider that the GM-CSF concentrations used in our experiments correspond to about 50 mg of GM-CSF given "*in vivo*", and that this cytokine is currently employed in therapy, while the high toxicity of IFN-α in humans has strongly hampered its therapeutic use. Moreover, a dose-dependent decrease of collagen deposition (80%) by topical GM-CSF used in human experimental wounds has recently been reported[37]. We are therefore confident that GM-CSF could be taken into serious consideration for treatment of SSc.

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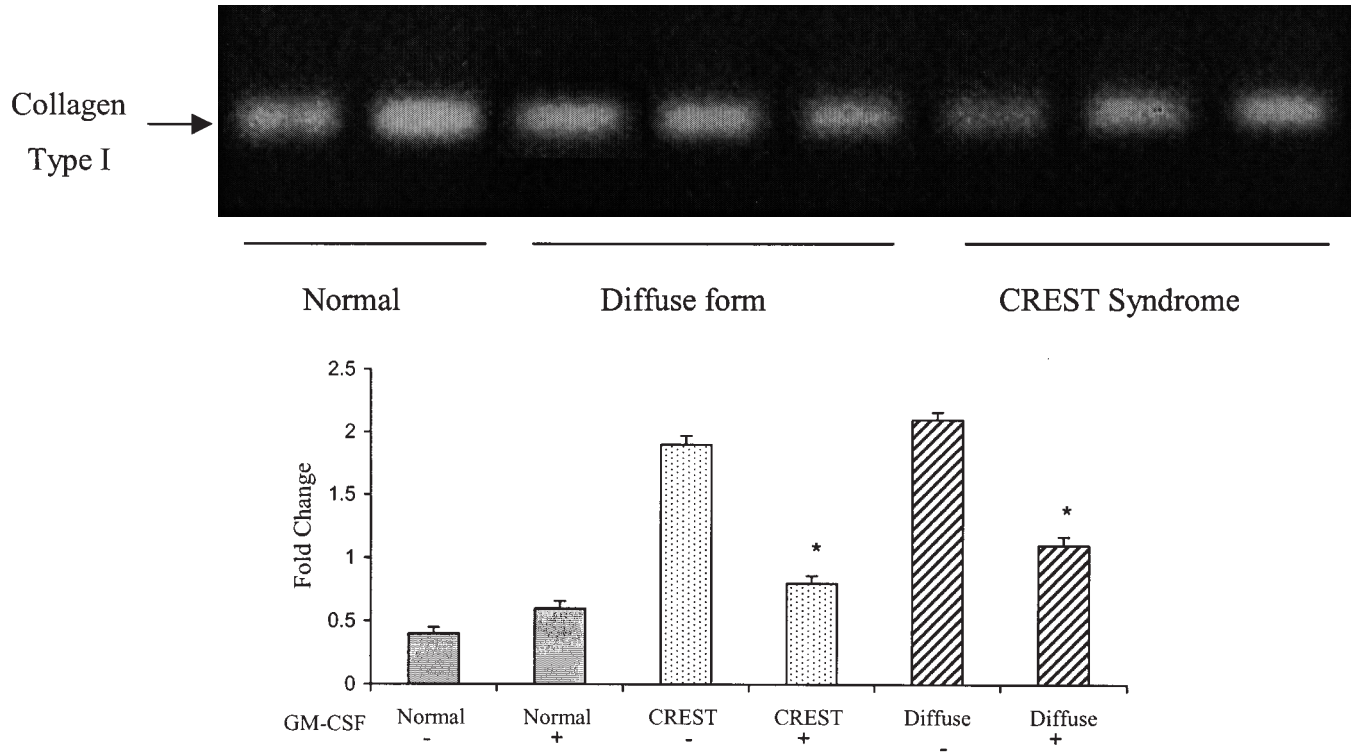

Figure 6. Expression of collagen type I mRNA in control and SSc fibroblasts as shown by RT-PCR and real-time PCR. Fibroblasts from the same controls and SSc patients shown in Figure 4 were cultured 8 days in the presence (+) or absence (–) of 100 ng GM-CSF/ml and analyzed for collagen I mRNA. The top panel shows a typical RT-PCR gel. Bottom panel, results of the real-time PCR. Each column represents the average of 7 cultures; bars indicate standard deviation. The difference between fibroblasts stimulated with (ST) and without (NS) GM-CSF was statistically significant (*$p < 0.05$).

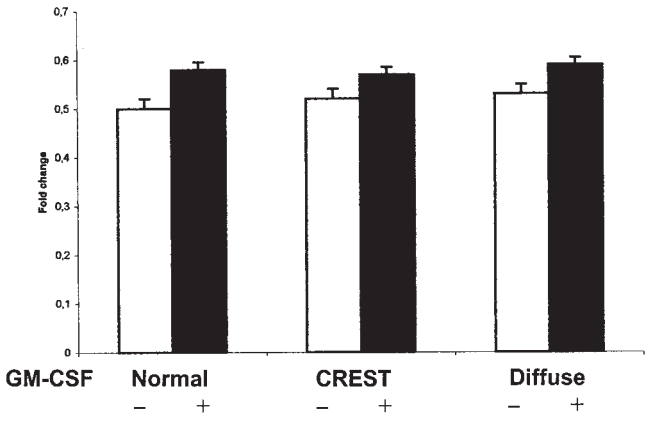

Figure 7. Expression of MMP-1 mRNA in control and SSc fibroblasts, as shown by real-time PCR. Fibroblasts from the same controls and SSc patients shown in Figure 4 were cultured 8 days in the presence (+) or absence (–) of 100 ng GM-CSF/ml and analyzed for MMP-1 mRNA by real-time PCR. Each column represents the average of 7 cultures; bars indicate standard deviation. The difference between untreated and GM-CSF treated fibroblasts was not statistically significant.

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