Enhanced Expression of the Receptor for Granulocyte Macrophage Colony Stimulating Factor on Dermal Fibroblasts from Scleroderma Patients

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ABSTRACT. **Objective.** To elucidate events that initiate the involvement and stimulation of fibroblasts in systemic sclerosis (SSc).

Methods. We examined 15 patients with SSc diffuse form, 15 with CREST syndrome, and 5 healthy subjects. Cultured fibroblasts obtained from skin biopsies in SSc involved and non-involved areas and normal skin fibroblasts were cultured with different doses of granulocyte macrophage colony stimulating factor (GM-CSF) to study the effects of this factor on the expression of GM-CSF receptor (GM-CSFR) on fibroblast proliferation and cellular adhesion structures.

Results. Cultured fibroblasts obtained from biopsies of normal and SSc skin areas express GM-CSFR and such expression is increased in SSc fibroblasts. GM-CSF stimulation *in vitro* did not increase SSc fibroblast growth, in spite of a strongly increased expression of the GM-CSFR. The adhesion structures are always more abundant in SSc fibroblasts as compared to healthy cells and GM-CSF seems able to increase cell adhesion plaques.

Conclusion. We suggest that shift of fibroblasts toward a more adhesive differentiated pattern, due to or accompanied by an increased expression of GM-CSFR, may be an important event in the pathogenesis of SSc. (J Rheumatol 2002;29:94–101)

Key Indexing Terms:
FIBROBLASTS   GM-CSF RECEPTOR   SCLERODERMA

Dermal fibrosis is one of the main aspects of systemic sclerosis (SSc), a disease characterized by a marked fibroblastic hyperactivity with enhanced synthesis of various collagen types (Types I, III, IV and VII) which do not present any structural abnormality. Various clinical forms are recognized, the most frequent being the CREST syndrome (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly, telangiectasias) and the diffuse form.

The etiology of the disease is still unknown, but in the last few years a pathogenic role of activated cells (T lymphocytes and monocytes/macrophages) through the action of several cytokines[1] has been demonstrated, some-times in relation to neuroendocrine pathways[2]. In particular, some biological mediators of inflammation and vascular alteration in rheumatic diseases such as interleukin 1 (IL-1) (α and ß), IL-2, IL-6 and transforming growth factor-ß (TGF-ß)[3-6], exert a stimulating action on fibroblast collagen synthesis. Also fibronectin, increased in SSc deep dermis[7], is influenced by TGF-ß[8]. We have previously described[9] increased levels of granulocyte macrophage colony stimulating factor (GM-CSF) in the sera of patients with SSc without any difference between the CREST and diffuse forms. It is known, on the other hand, that quiescent fibroblasts can produce GM-CSF when cultured *in vitro*[10]. This cytokine induces growth and activation of monocytes and granulocytes. Stimulating effects on endothelium and connective tissue cells have also been demonstrated[11,12]. Recently, it has also been reported that transgenic mice with enhanced gene expression for GM-CSF have high serum levels of this cytokine and develop fibrotic nodules inside skeletal muscles and diaphragm: these nodules contain macrophages and fibroblasts[13].

The increased serum levels of GM-CSF in the course of SSc led us to investigate a possible role for this cytokine in the pathogenesis of the disease. This approach was supported by our previous demonstration that the receptor for GM-CSF was expressed on the surface of human fibroblasts of different origin[14]. It is interesting to observe that this receptor is made of 2 chains: the alpha chain is typical

of the GM-CSF receptor while the beta chain is shown by the IL-3 and IL-5 receptors, too. We also showed by cyto-fluorimetry and immunofluorescence that the expression of the alpha chain of this receptor on gingival fibroblasts was enhanced after stimulation with increasing doses of GM-CSF up to 100 ng/ml and that GM-CSF, at the same doses, was able to stimulate the proliferation of these cells. In contrast, low doses of GM-CSF were able to induce the production of cell adhesion structures, while an opposite effect was obtained after stimulation with high doses of the cytokine.

These data led us to investigate the behavior of dermal fibroblasts from patients with SSc, affected either by the diffuse form or by the CREST syndrome. Therefore, we compared the expression of the receptor for GM-CSF (GM-CSFR) on SSc and normal dermis fibroblasts and the differential pattern of GM-CSFR expression; we also studied the effects of GM-CSF on the cell growth of such fibroblasts. Finally, GM-CSFR expression on fibroblasts obtained from SSc involved and from apparently healthy areas not adjacent to the lesions was also investigated.

MATERIALS AND METHODS

Patients. Thirty patients (28 women, 2 men) with SSc (15 with diffuse form and 15 with CREST syndrome) were admitted to our study after obtaining their informed consent and Ethical Committee approval. All diffuse form patients denoted the involvement of the dorsal arm and/or thorax. To reduce the variability among our patients, we admitted as SSc syndrome only those patients who presented positivity for anti-nuclear antibodies by the ELISA method. In particular, all our patients with diffuse form presented serum anti-ScL70 topoisomerase I positivity and patients with CREST syndrome showed serum anti-centromere (CENP-B) positivity (both evaluated by ELISA). The mean age of the patients was 54 years (range 31–70) and the mean disease duration was about 8 years (range 4–27). All patients and normal donors were Caucasian, born in Southern Italy (where the prevalence of scleroderma is about 4–12 new cases/million/year). None of them had been exposed to any environmental or professional risk factor or had a family history of SSc. The mean value of serum GM-CSF levels as determined by a solid phase immunoenzymatic method (Genzyme, Cambridge, MA, USA) was 55 ± 7 pg/ml, strongly increased as compared to the reference value (20.1 ± 12.3). No difference was observed between serum GM-CSF levels of patients with the diffuse versus the CREST forms[9].

None of the patients had been treated with steroids or immunosuppressive drugs in the last 6 months, only patients with diffuse forms presented visceral involvement, and nobody was suffering from any other disease. In each patient, 2 fine needle punch biopsies were carried out: one from an affected skin area and the other one from an apparently healthy zone, not adjacent to involved skin areas. The criteria to differentiate involved from unaffected skin 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 (involved area) and on the dorsal forearm skin (uninvolved area); in the diffuse form patients, on the dorsal forearm skin (involved area) and on the ventral upper arm skin (uninvolved area). Surgical fragments from the same sites were obtained from 5 volunteers (3 females and 2 males, 37 ± 4 years old) who underwent aesthetic or emergency surgery and were used as normal controls.

Each fragment was divided into 2 pieces: one for paraffin-embedding and anatomo-pathological diagnosis of the disease by histological observation and the other, mechanically dissociated under the stereo microscope for *in vitro* culture.

Cell culture. SSc fibroblasts from skin biopsies were obtained as follows: surgical fragments were mechanically dissociated under a light microscope and then submitted to trypsinization for 30 minutes at 37°C. After repeated phosphate buffered saline (PBS) washing, microfragments were plated and cultured in Dulbecco's minimal essential medium (DMEM) (BIOWhittaker, Verviers, Belgium), containing 10% fetal calf serum (GIBCO, Grand Island, NY, USA), 200 mM L-glutamine, penicillin (100 mg/ml) and streptomycin (100 mg/ml). The plates were incubated at 37°C, in the presence of 5% CO_2; the medium was removed every 3 days. Surgical fragments from healthy subjects were submitted to the same treatment and cultured in the same conditions. Cell line cultures of human gingival fibroblasts, named PG-1 and kindly provided by Dr. Pontarelli (Dept. Biochemistry, Faculty of Medicine, University of Naples Federico II), were used as normal, non-dermal fibroblast controls[14].

For immunofluorescence studies, sterile glass coverslips were put into cell dishes before plating the fibroblasts, to allow cell attachment.

For cell number measurement, 2.5×10^5 cells were initially plated in each culture dish and grown in the same conditions, except for the addition of different doses of GM-CSF to the culture medium. After 24, 48 and 72 hours and after 9 days, cells were detached with EGTA (10 mM), pH 7,4, for 10 min at 37°C and 50 min at 4°C, and counted in a Neubauer hemocytometer (Neubauer Manufacturing Co, Minneapolis, MN, USA).

GM-CSF. Human recombinant GM-CSF (150 mg/ml) was obtained from Schering Plough (County Cork, Ireland). Both SSc and normal skin fibroblast cultures were stimulated for 9 days with different concentrations of GM-CSF (10 and 80 ng/ml); fresh factor at the same concentrations was added every 2 days to the culture medium. Monoclonal antibody against GM-CSF (clone BVD2-23B6) from Pharmingen (San Diego, CA, USA) was used to block GM-CSF effects on cultured cells. GM-CSF was incubated with the antibody for 40 minutes at 37°C and then added to the culture medium[11].

Immunoblotting. PG-1 cell line and primary cultures of fibroblasts from healthy donors and from SSc patients, grown to near confluency in 100 mm dishes, were incubated for 10 min at 4°C with 0.5 ml of lysis buffer (PBS, 1% Triton X-100) containing protease inhibitors. Cell lysates were collected by scraping and centrifuged at 12,000 g for 5 min at 4°C. Protein concentration in cell lysates was determined by a calorimetric assay (BioRad, Richmond, CO, USA) and 100 μg of total protein was incubated 5 minutes at 90°C in Laemmli sample buffer. Cell lysates were electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gels. Gels were electroblotted and the membranes were blocked with 5% fat-free dry milk, l% ovalbumin, 5% fetal calf serum (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 at the concentration of 10 μg/ml in washing solution. After 4 washes, filters were incubated for 30 minutes 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).

Immunocytochemistry. Glass coverslips with attached fibroblasts were fixed with 3% formaldehyde in PBS, gently washed with PBS and then incubated with monoclonal antibodies against GM-CSFR (IgM against α chain of GM-CSF receptor, clone M5D12 from Pharmingen), vinculin, Paxillin, Talin (Sigma, St.Louis, MO, USA) or with polyclonal antibodies against α-actinin (Sigma). After further washing with PBS, cells were stained with fluorescein labeled rabbit anti-mouse IgM for antibodies against GM-CSFR or with rhodamine conjugated IgG for other monoclonal antibodies and with fluorescein-conjugated goat anti-rabbit IgG for α-actinin visualization. Both steps required 1 hour incubation at room temperature, in a humid chamber.

For actin visualization, cells on glass coverslips were fixed as above, permeabilized with Triton X-100 buffer (20 mM HEPES, 300 mM sucrose,

50 mM NaCl, 3 mM $MgCl_2$, 05% Triton X-100, pH 7.4) and then stained with fluorescein-labeled phalloidin. To determine phosphotyrosine activity in adhesion plaques, permeabilized cells were stained with rhodamine-conjugated anti-phosphotyrosine monoclonal antibodies (Sigma). Coverslips were then mounted with Moviol and observed with an Axiophot microscope (Leitz, 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.

Statistical analysis. For statistical evaluation of our data on cell proliferation and GM-CSF receptor expression (evaluated as optical density, OD), we used the Student t test for unpaired data (t test on the mean), or t independent, in which the means and standard deviations of 2 independent samples are compared and each standard deviation is presumed to be from the same population; probability value of $p \leq 0.05$ was accepted as statistically significant.

RESULTS

Immunocytochemistry of GM-CSFR membrane expression. Fibroblasts from healthy dermis and PG-1 cells showed a slight immunopositivity for GM-CSFR (α-chain). In contrast, GM-CSFR was strongly expressed on the membrane of SSc fibroblasts (Figure 1).

Western blot of cell lysates. In order to identify the GM-CSF receptor α-chain (GM-CSFR α), whole cell lysates from gingival fibroblasts PG1 and cell lysates of dermal fibroblasts from both SSc forms and from normal donors were subjected to Western blot with monoclonal anti-GM-CSF receptor α-chain antibodies. As expected, the receptor migrated in a band with an apparent molecular weight of 84 kDa. This band was present, with different intensity, in all dermal fibroblast lysates (Figure 2). A quantitative analysis of the GM-CSFR bands, obtained in a series of experiments with cells from all patients, is reported in Figure 3. The basal OD values in normal fibroblasts and in PG-1 cells were 36 ± 9 and 30 ± 11, respectively.

The expression of GM-CSFR was remarkably higher in all the patients with SSc examined. Indeed, CREST fibroblasts from both involved and non-involved areas showed a more dramatic and statistically significant increase as compared to fibroblasts from diffuse form (116 ± 12 OD vs 70 ± 11 OD, $p < 0.05$). In addition, CREST fibroblasts from affected areas showed a significantly increased expression of GM-CSFR when compared to fibroblasts from non-affected areas (141 ± 6 OD vs 91 ± 12 OD, $p < 0.05$), whereas in the diffuse type of the disease both affected and apparently not affected areas showed the same increase in GM-CSFR expression (70 ± 11 OD vs 74 ± 12 OD, $p > 0.05$).

Cell proliferation following GM-CSF stimulation. Fibroblasts from cutaneous biopsies of SSc patients and of unaffected donors showed the proliferation rates after GM-CSF stimulation reported in Figure 4. The values reported represent the mean value of all the patients of the same group. The statistical significance of the data relative to each group of patients at the 9th day of culture was evaluated by the Student t test for unpaired data (Table 1).

SSc fibroblasts from both CREST and diffuse forms exhibited much slower growth rates when compared to normal dermis fibroblasts.

The growth of fibroblasts obtained from SSc biopsies of involved and apparently healthy areas showed different characteristics according to the different forms of SSc. In CREST patients, cell proliferation was higher in involved areas (doubling time: 3 days) as compared to non-involved ones, while fibroblast growth from the diffuse form was always very low in comparison to controls.

Therefore, only in the CREST form the growth rate seemed to be different according to the sclerodermic involvement of the skin area of origin, with an increase in cell proliferation of fibroblasts from involved areas.

In contrast to fibroblasts from other tissues[14], stimulation with GM-CSF at the used doses did not affect cell proliferation *in vitro*, in normal and SSc fibroblasts (Figure 5).

Cell adhesion. All fibroblasts obtained from cultures of normal dermis exhibited the common adhesion plaque structures, with strong immunopositivity for vinculin, α-actinin, Paxillin and an early, strong phosphotyrosine-related activity. We had already described such structures and their relationship with GM-CSF treatment in previous work[14]. On the other hand, expression of all adhesion plaque components was increased in SSc fibroblasts (Figure 6) and apparently was not affected by GM-CSF treatment.

Table 1. Statistical analysis of proliferation rates of fibroblasts from healthy skin and from SSc involved and non-involved skin area.

	Control	CREST Non-involved	CREST Involved	Diffuse Non-involved	Diffuse Involved
Control	—	1.97*	NS	1.99*	2.1*
CREST					
Non-involved	1.97*	—	1.7*	NS	NS
Involved	NS	1.7*	—	NS	2.2*
Diffuse					
Non-involved	1.99*	NS	NS	—	NS
Involved	2.1*	NS	2.2*	NS	—

* Student's t test was used to evaluate the statistical significance of the proliferation rates of every patient at 9th day of culture ($p < 0.05$).
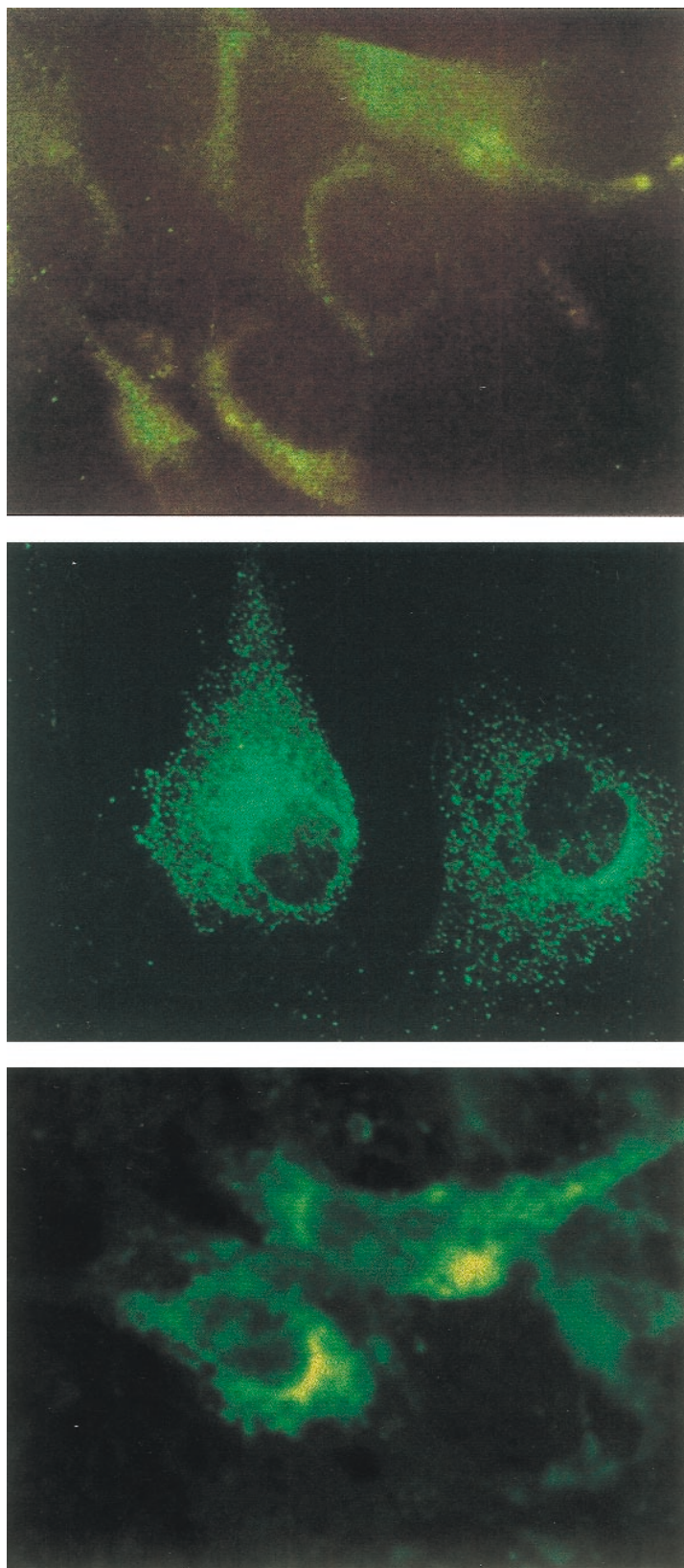
Figure 1. Immunopositivity for α-chain of GM-CSF receptor on the membrane of fibroblasts from healthy control (a); CREST syndrome (b) and SSc diffuse forms (c). Magnification: ×1030.

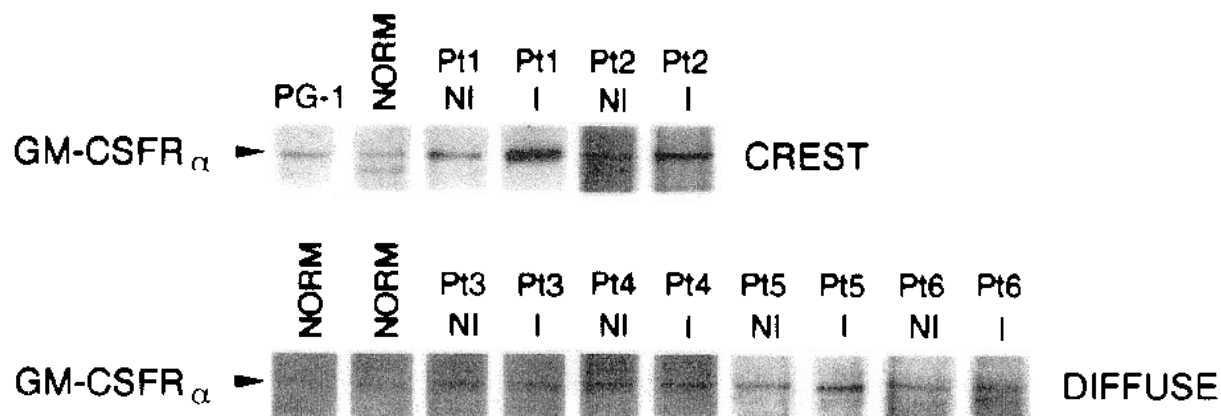*Figure 2.* Expression of GM-CSFRα as shown by Western blot with monoclonal anti-GM-CSFRα antibody in whole cell lysates from fibroblasts of 6 patients with SSc (CREST and diffuse forms), PG1 gingival fibroblasts and normal fibroblasts (NORM). NI: non-involved dermal areas; I: involved dermal areas.
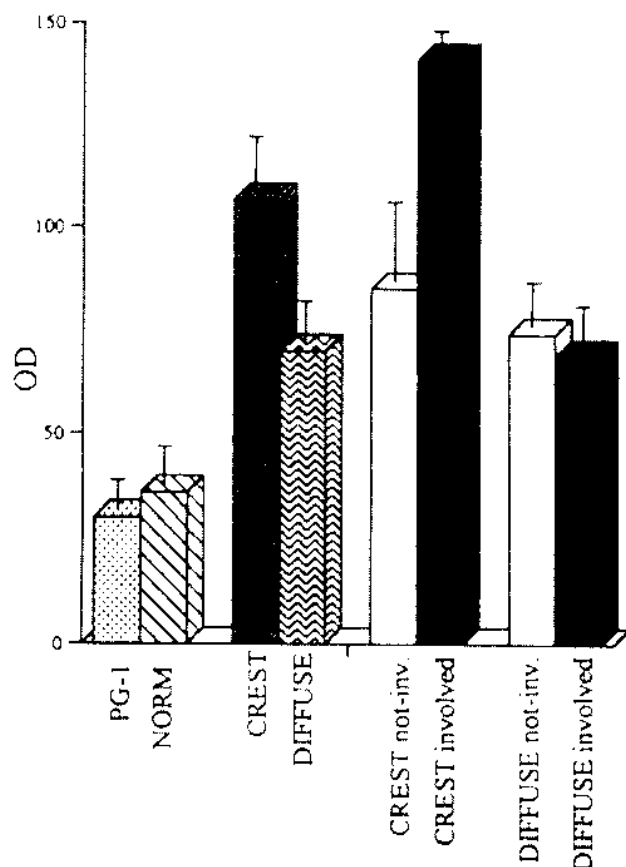

Figure 3. Mean optical densities (OD) of GM-CSFRα bands after Western blot of whole cell lysates with monoclonal anti-GM-CSFRα antibody. Columns 1 and 2: PG-1 cells and normal fibroblasts; Columns 3 and 4: SSc fibroblasts (all specimens); Columns 5–8: SSc fibroblasts obtained from involved and apparently non-involved skin areas.

DISCUSSION

GM-CSFR expression was observed in normal dermis fibroblasts by immunocytochemistry and immunoblotting and was dramatically increased in all SSc fibroblasts. In particular, CREST cells from involved areas exhibited a more than 4-fold increased expression as compared to control cells. Also diffuse forms showed an impressive increase in receptor expression, which however was less marked than in the CREST form. In the diffuse form, as expected, we did not find any major difference between involved and non-involved areas, in agreement with the systemic character of the disease. In the CREST syndrome, we also found a general skin involvement, as even fibroblasts from apparently non-involved areas showed a 2-fold increase in GM-CSFR expression in comparison with normal fibroblasts.

The results of the proliferative study also showed different behavior of SSc fibroblasts in comparison with normal ones from unaffected donors. In fact, SSc fibroblast basal growth was much lower than the proliferation of cells from normal subjects (in agreement with other reports of a high proliferative activity in the cultures of SSc fibroblasts only in serum-free conditions[15,16]); in general the doubling time of SSc fibroblasts was lower than that of normal fibroblasts. It is known that in some human and murine hemopoietic cell lines, the proliferation reaches maximal levels at the concentration of GM-CSF that results in the occupancy of virtually all GM-CSFR expressed on the cell membrane. In this condition both alpha and beta-chain intracellular domains of GM-CSFR play a role in determining the activation of the JAK/STAT proliferation pathway[17,18]. In the presence of an over-expression of GM-CSFR, GM-CSF might occupy only a small portion of the receptors, and therefore a proliferative response could be stimulated less efficiently. Although less proliferating than normal ones, SSc fibroblasts showed a proliferation rate that correlated with the level of GM-CSFR expression. In addition, fibro-
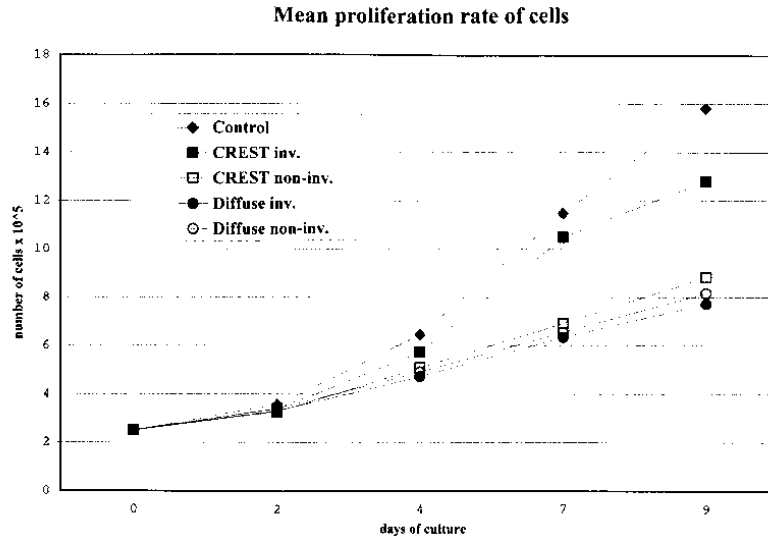
Mean proliferation rate of cells

Figure 4. Proliferation curves of fibroblasts from the dermis of healthy controls and SSc patients. Cells (2×10^5) from skin biopsies were plated and cultured as described. Cells were harvested and counted at the indicated times.
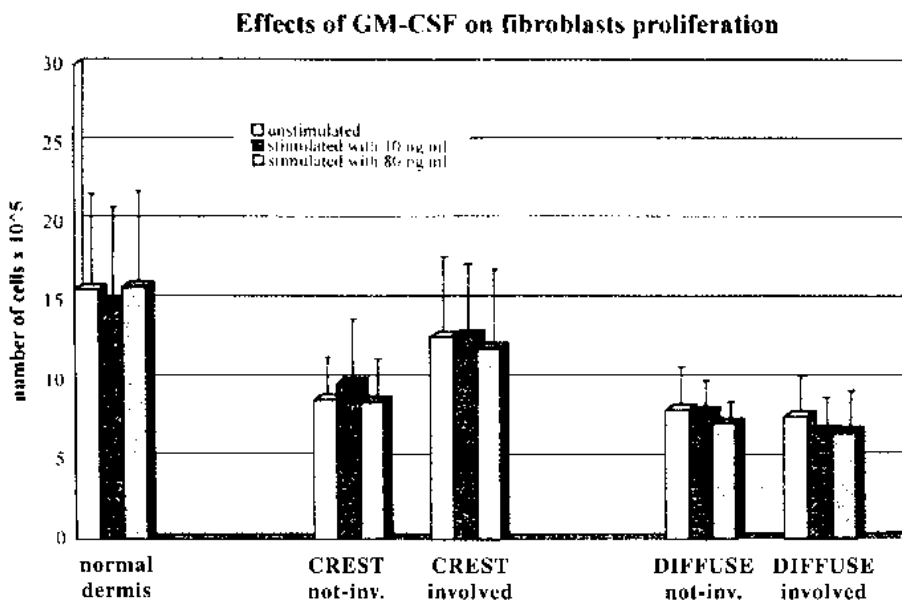
Effects of GM-CSF on fibroblasts proliferation

Figure 5. Cell proliferation after stimulation with GM-CSF. Cells (2×10^5) were plated and cultured in the presence or in the absence of GM-CSF as described. Cells were harvested at Day 9 and counted.

blasts from CREST involved areas showed a statistically significant increase in proliferation rate and in GM-CSFR expression as compared to fibroblasts from non-involved areas. In contrast, no statistically significant difference was found for the proliferation rate and GM-CSFR expression in fibroblasts from involved and non-involved areas of the SSc diffuse form, that showed, however, lower values compared to the CREST form ($p < 0.05$).

The results on GM-CSFR expression and cell proliferation will prompt us to investigate the biosynthetic activity of

SSc fibroblasts. Even though there is a general agreement on the dramatic increase in collagen[19,20], fibronectin[7] and tenascin[15] synthesis by SSc fibroblasts accompanied by an altered expression of integrins and adhesion molecules both in serum and on the fibroblast surface[16,21-23], data on these characteristics under GM-CSF stimulation are still lacking.

Both SSc types exhibit a strongly adhesive phenotype and this finding could explain their poor growth rates[11]. In this regard, the relatively high proliferation rate of CREST cells from involved areas remains to be explained. We
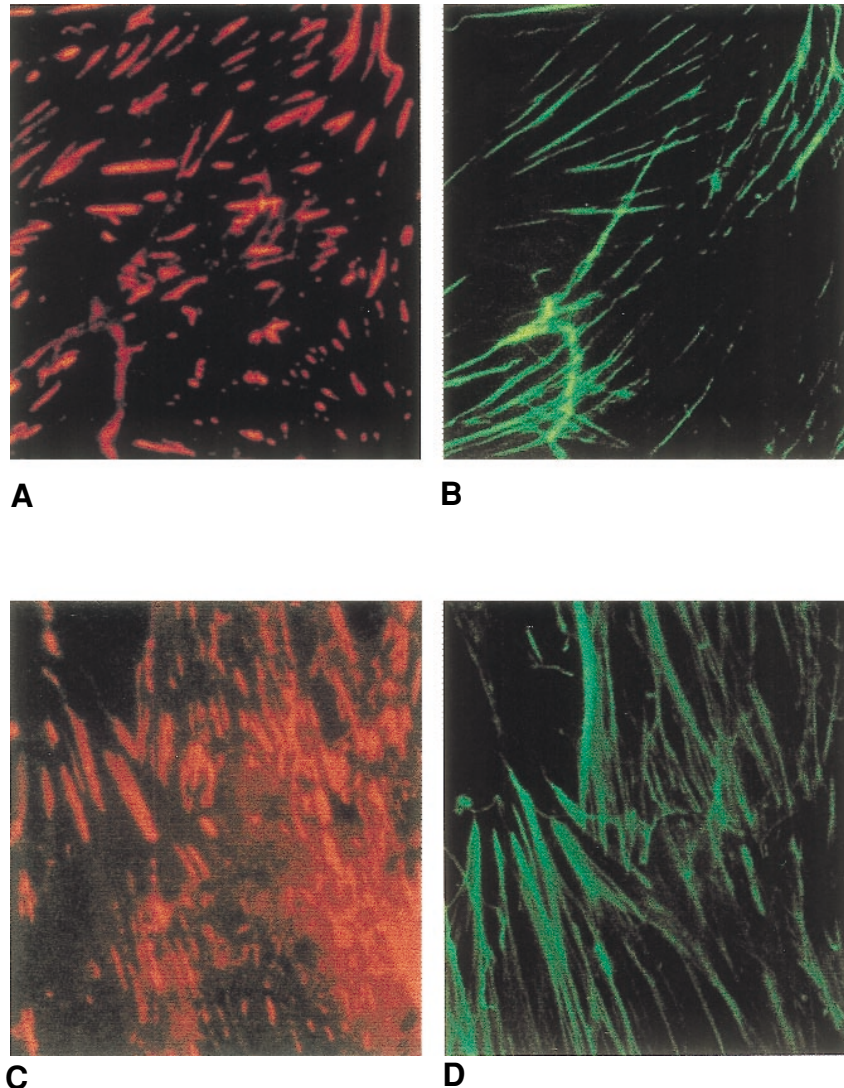
Figure 6. Cellular adhesion structures in normal and SSc fibroblasts *in vitro*. Cellular adhesion structures containing vinculin (a,c) and actin fibers (b,d) are widely distributed on the cell surface which adheres to glass coverslips in the culture dishes. In particular vinculin is more abundant and well structured in SSc fibroblasts (lower panel) as compared to normal dermis (upper panel). Magnification: ×1060.

suggest that these represent transition areas where fibroblasts undergo a shift toward a more differentiated state, due to or accompanied by an increased expression of GM-CSFR.

It is interesting that in contrast to normal fibroblasts, in some epithelial cells the expression of an adhesive phenotype stimulates cell proliferation. It is therefore clear that the relationship between cell adhesion and proliferation is a complex one, depending on the cell type and the effect of several factors: our data strongly suggest that GM-CSF is indeed one of the relevant cytokines.

We suggest that the over-expression of GM-CSFR on SSc cutaneous fibroblasts might play an important pathogenetic role, presumably by influencing extracellular matrix deposition more than cell proliferation. The effects of GM-CSF on the differentiation of SSc dermal fibroblasts, mediated by the over-expression of the GM-CSF receptor, are probably amplified by the interaction with the ECM. It has been demonstrated that extracellular matrix can bind GM-CSF and present it in a biologically active form[24]. It is also possible that fibroblasts play the double role of cells that express the receptor for GM-CSF and produce the ligand, like many other cells involved in autocrine mechanisms.

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