Autocrine Activation by Interleukin 1α Induces the Fibrogenic Phenotype of Systemic Sclerosis Fibroblasts

YASUSHI KAWAGUCHI, SUSAN A. McCARTHY, SIMON C. WATKINS, and TIMOTHY M. WRIGHT

ABSTRACT. ***Objective.*** To explore the cellular localization of interleukin 1α (IL-1α) in cultured fibroblasts from lesional skin of patients with systemic sclerosis (SSc) and to study the role of intracellular IL-1α in the activation of fibroblasts.

Methods. Dermal fibroblasts were derived from 12 patients with SSc. Expression of IL-1α mRNA was examined using reverse transcriptase-polymerase chain reaction (RT-PCR). The cellular distribution of IL-1α was examined by subcellular fractionation, flow cytometry, and immunocytochemistry. A full-length IL-1α cDNA was subcloned into the pcDNA3 vector to create sense and antisense-encoding constructs. Normal and SSc fibroblasts were stably transfected with the sense and antisense-encoding constructs, respectively. Stably transfected fibroblast clones were analyzed for the production of procollagen and IL-6 protein by ELISA, α1(I) procollagen mRNA by Northern blot hybridization, and proliferation by [^{3}H]thymidine incorporation.

Results. SSc-affected fibroblasts constitutively expressed intracellular IL-1α, which was predominantly located in the nucleus. Inhibition of IL-1α expression in SSc-affected fibroblasts using antisense constructs resulted in decreased proliferation, IL-6 production, and procollagen synthesis. Conversely, overexpression of IL-1α in normal fibroblasts resulted in development of the SSc fibroblast phenotype.

Conclusion. IL-1α is an important autocrine fibrogenic factor in SSc, suggesting that inhibition of intracellular IL-1α may be a novel strategy for the treatment of SSc. (J Rheumatol 2004;31:1946–54)

Key Indexing Terms:
SYSTEMIC SCLEROSIS HUMAN SKIN FIBROBLASTS
INTERLEUKIN 1α FIBROSIS

Systemic sclerosis (SSc) is an idiopathic disorder of connective tissue characterized by increased production and deposition of collagen in the skin and internal organs such as the lungs, gastrointestinal tract, and heart[1]. Although SSc is associated with specific cellular and humoral autoimmune responses[2-5], the main feature of this illness that distinguishes it from other autoimmune diseases is the extensive deposition of collagen and other matrix proteins by fibroblasts, resulting in extensive tissue fibrosis[6]. In order to design new therapies for SSc, an understanding of the pathophysiologic mechanisms underlying this fibrotic process is essential.

The initiating events leading to excessive matrix deposition by SSc fibroblasts are poorly understood. It is known, however, that mononuclear cells infiltrate SSc skin and internal organs and that patients with SSc have elevated concentrations of soluble interleukin 2 (IL-2) receptor in their serum and bronchoalveolar lavage fluid indicative of ongoing T cell activation[7-9]. The inflammatory cells may activate fibroblasts through the elaboration of cytokines and growth factors, or through cell-cell interactions resulting in increased fibroblast proliferation and matrix protein production[10,11]. It is well established that fibroblasts derived from lesional skin of patients with SSc have an abnormal phenotype in culture that is characterized by increased expression of IL-6[12], transforming growth factor-ß (TGF-ß) receptor, IL-1 receptor type I[13], glycosaminoglycan[14], and collagen[15]. These findings raise the possibility that an intrinsic fibroblast abnormality or autocrine activation pathway, perhaps initiated by infiltrating inflammatory cells or tissue hypoxia, may be responsible for the fibrosis observed in SSc.

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One candidate for an autocrine factor regulating the phenotype of SSc fibroblasts is the proinflammatory cytokine IL-1α. We and others have reported that IL-1α is constitutively expressed in fibroblasts cultured from lesional SSc skin (SSc-affected fibroblasts)[16-18]. Here we describe further analysis of the subcellular localization of IL-1α in SSc-affected fibroblasts and the role of this cytokine in causing the abnormal fibrogenic phenotype of these cells.

MATERIALS AND METHODS

Cell culture. Human dermal fibroblasts were derived from skin biopsies obtained from 7 Caucasian and 5 Japanese patients with SSc who fulfilled the American College of Rheumatology preliminary classification criteria for SSc[19]. Control fibroblasts were derived from biopsies obtained from 4 Caucasian and 3 Japanese healthy donors. Primary explant cultures were established and maintained in 100 mm culture dishes in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 10 units/ml of penicillin, and 10 µg/ml streptomycin (Gibco). In this study, the cells in the 3rd through 5th passages were used.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Confluent monolayer fibroblasts were cultured in serum-free medium (QBSF-51; Sigma) for 48 h, followed by total RNA extraction using the Trizol™ method (Gibco). RT-PCR was performed using an RNA PCR kit according to the manufacturer's instructions (Perkin-Elmer, Norwalk, CT, USA) using specific primers for IL-1α (included in the kit) or ß-actin. Thirty-five cycles of PCR were performed, with denaturing at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min 30 s. A 10 µl aliquot of each PCR sample was resolved by electrophoresis in 2% agarose gels. Amplification of the same RNA with ß-actin primers confirmed that equal amounts of RNA were reverse-transcribed. The sequences of primers for ß-actin were sense primer: 5'-AAGAGAGGCATCCTCACCCT-3' and antisense primer: 5'-TACATGGCTGGGGTGTTGAA-3'.

Measurement of IL-1α in subcellular fractions. IL-1α protein levels were assayed in membrane, cytosol, and nuclear fractions by ELISA using a commercial kit (R&D Systems, Minneapolis, MN, USA). Membrane, cytosol, and nuclear fractions were prepared from human fibroblasts as described[20], with minor modifications. Briefly, confluent fibroblasts were cultured in 75 cm^2 culture flasks with serum-free medium, and were harvested by trypsinization. Cells (1×10^7) were washed with cold phosphate buffered saline (PBS) and resuspended in 7 ml ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.6, and 0.5 mM $MgCl_2$) with protease inhibitors (10 µg/ml leupepsin, 10 µg/ml aprotinin, 1 mM PMSF). Cells were allowed to swell for 10 min and then were disrupted in a Dounce homogenizer. Tonicity restoration buffer (10 mM Tris-HCl, pH 7.6, 0.5 mM $MgCl_2$, and 0.6 M NaCl) with protease inhibitors was added to the homogenized cells to adjust the concentration of NaCl to 0.15 M. Cell disruption was confirmed by trypan blue staining and was > 95%. Nuclei were collected by centrifugation at 500 g for 5 min at 4°C. The nuclear pellet was resuspended in RIPA buffer (1% NP-40, 0.1% sodium deoxycholate, 150 mM Tris-HCl, pH 7.6, 10 µg/ml leupepsin, 10 µg/ml aprotinin, and 1 mM PMSF), and was dispersed by repeated pipetting. The supernatants were removed and EDTA was added to a final concentration of 5 mM. Samples were centrifuged at 100,000 g for 1 h at 4°C. The supernatants (cytosolic fraction) after ultracentrifugation were collected and analyzed by ELISA. The membrane pellet was resuspended in RIPA buffer and was dispersed by repeated pipetting. Insoluble material was pelleted by centrifugation at 10,000 g for 15 min. The solubilized membrane proteins were analyzed by ELISA.

Flow cytometry. Fibroblasts were cultured in 75 cm^2 culture flasks with serum-free medium for 48 h and were harvested by gentle trypsinization. Cells were washed 3 times with cold PBS and incubated at 4°C for 10 min with or without 2% paraformaldehyde (PFA, Sigma) alone or 2% PFA plus 0.1% Triton X-100. Indirect immunofluorescence staining was performed using anti-human IL-1α antibody (10 µg/ml; Genzyme, Cambridge, MA, USA) combined with biotinylated anti-rabbit IgG (H+L) antibody (Gibco) and FITC-conjugated streptavidin (Boehringer Mannheim). Samples were analyzed using a FACScan (Becton-Dickinson, Mountain View, CA, USA) equipped with Lysis II software.

Immunocytochemical staining. Monolayer fibroblast cultures (5×10^4 cells/well) were grown for 48 h in serum-free medium on 4-chamber slides (Lab-tek, Nunc, Naperville, IL, USA). Fibroblasts were washed twice with cold PBS, and fixed with 2% PFA or 2% PFA plus 0.1% Triton X-100 in PBS. The primary antibodies used in these experiments were polyclonal rabbit anti-human IL-1α antibody, monoclonal mouse anti-histone antibody (Boehringer Mannheim), monoclonal mouse anti-human HLA-A, B, C antibody (Dako, Carpinteria, CA, USA), and monoclonal mouse anti-human prolyl-4-hydroxylase antibody (Dako). Cells were incubated with the primary antibody (anti-IL-1α 10 µg/ml; anti-histone, 4 µg/ml; antihuman HLA-A, B, C, 3 µg/ml; or anti-prolyl-4-hydroxylase, 3 µg/ml) or, as controls, preimmune rabbit IgG (10 µg/ml; Immunovision, Springdale, AR, USA) or isotype-matched mouse monoclonal antibodies with irrelevant specificities for 1 h at 4°C. The primary antibody was detected by incubation with biotinylated anti-rabbit IgG (H+L) antibody or biotinylated anti-mouse IgG antibody (Pierce, Rockford, IL, USA) for 30 min at room temperature, followed by incubation with avidin:biotinylated enzyme complex (ABC; Pierce) and development with 3,3' diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate (Sigma Fast™; Sigma) for 10 min. For analysis of specimens by confocal laser scanning microscopy, incubation with FITC-conjugated streptavidin (Boehringer Mannheim) was substituted for the ABC. The chamber slides were dried and mounted with Immuno-mount™ (Shandon, Pittsburgh, PA, USA) and were analyzed by light and, as indicated, by fluorescence microscopy. Subcellular distribution of IL-1α staining was analyzed using a 2001 inverted laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA, USA).

Plasmid construction and stable transfections. We obtained a full-length 2.4 kb human IL-1α cDNA from the American Type Culture Collection (Bethesda, MD, USA). The cDNA insert was excised by BamHI and subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA, USA) to create the IL-1α sense and antisense-encoding constructs. Stable transfections were performed as described[21]. Briefly, 2 µg of DNA (twice cesium chloride banded) and 8 µl of LipofectAmine (Gibco) were added to 1 ml of Opti-MEM (Gibco), and then added to fibroblasts (5×10^4) on 35 mm culture dishes. After 5 h, 1 ml of DMEM containing 20% FBS was added to the dishes. After an additional 15 h, the medium was changed to 2 ml of complete medium. Seventy-two hours after transfection, medium was replaced and cells were trypsinized and transferred to ten 100-mm dishes with 10 ml of complete medium supplemented with 450 µg/ml of G418 (Gibco). Continuous G418 selection for about 4 weeks resulted in generation of drug-resistant colonies. Individual colonies were harvested using cloning rings and expanded for further analysis.

Immunoblotting of IL-1α. Confluent fibroblasts were maintained in serum-free medium for 48 h, and cells were then trypsinized and washed with PBS. Cell lysates were prepared by sonication as described above. The cell lysates were resolved in 15% polyacrylamide gels under reducing conditions according to the method of Laemmli[22] and transferred to nitrocellulose membranes. The membranes were incubated with rabbit anti-IL-1α polyclonal antibody (Genzyme) for 1 h. Horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) was applied to the membrane and incubated for 1 h. The blot was incubated with a chemiluminescent substrate (Perkin-Elmer) and exposed to x-ray film.

Measurement of IL-1α, IL-6, and procollagen protein expression in stably transfected fibroblast clones. Fibroblasts (5×10^5 cells) were grown to con-

fluence in 75 cm^2 culture flasks and incubated with serum-free medium for 48 h. The supernatants were collected to measure IL-6 and procollagen type I C-peptide. The cells were harvested, counted in a hemacytometer, and sonicated in PBS with 1 mM PMSF and 10 µg/ml aprotinin for 30 s to prepare lysates for measurement of intracellular IL-1α. The amounts of IL-1α, IL-6, and procollagen type I C-peptide were assayed by ELISA using commercial kits (R&D Systems; Genzyme; and Takara Shuzo, Otsu, Japan, respectively). The results were normalized to cell number.

Fibroblast proliferation assay. Fibroblasts (5000 cells/well) were cultured in 96-well flat bottom tissue culture plates (Corning, Corning, NY, USA). Cells were cultured in complete medium for 48 h, and then incubated with 1 µCi/well of [^{3}H]-thymidine during the final 24 h of culture. Cells were harvested and [^{3}H]-thymidine incorporation was measured by liquid scintillation counting. All cultures were performed in triplicate.

Analysis of procollagen type I mRNA expression. Fibroblasts were cultured in 75 cm^2 culture flasks with DMEM plus 10% FBS. Confluent monolayer fibroblasts were cultured with serum-free medium for 48 h. The cells were harvested and total RNA was extracted as described above. Total RNA (15 µg) was size-fractioned by electrophoresis through 1% agarose–7% formaldehyde gels and transferred to nitrocellulose membranes. A fragment of the α1(I) procollagen cDNA (1.8 kb, Hf677) was used for the hybridization probe[23]. The cDNA probe was radiolabeled to a high specific activity (10^9 cpm/µg DNA) with [α-^{32}P]dCTP by the random primer method (Amersham). The membranes were hybridized with the ^{32}P-labeled probe for 16 h. After washing, the blots were exposed to x-ray film for 72 h and the radioactivity on the blots was quantified using an image acquisition and analysis system (PhosphorImager SI; Molecular Dynamics). Normalization of RNA loading was achieved by subsequent hybridization of the membranes with a radiolabeled cDNA probe encoding aldolase.

RESULTS

Constitutive expression of IL-1α in SSc-affected fibroblasts. The expression of IL-1α mRNA in primary fibroblast cultures from 7 Caucasian patients with SSc was analyzed by RT-PCR using total RNA. As shown in Figure 1, IL-1α mRNA was detectable in all fibroblast cultures derived from clinically affected ("A") SSc skin, but was not detected in fibroblasts from clinically unaffected ("U") skin from SSc patients or skin obtained from 4 healthy controls ("NC").

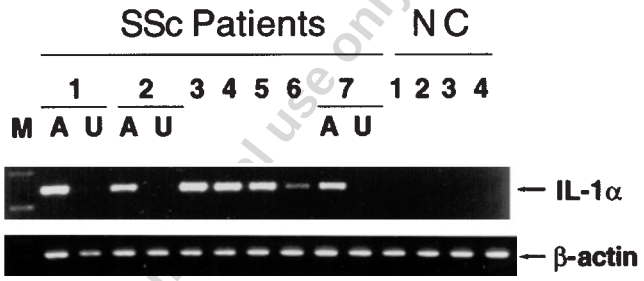

Figure 1. Analysis of interleukin 1α (IL-1α) expression in fibroblast cultures by reverse transcriptase-polymerase chain reaction (RT-PCR). Lane M contains DNA size markers (100 bp ladder). Arrow indicates the expected IL-1α product size (421 bp). Patients 1, 2, and 7 had paired biopsies from clinically affected (A) and unaffected (U) skin. Other SSc patients had a single biopsy of affected skin. NC: fibroblast samples from 4 healthy controls. Lower panel shows agarose gel analysis of RT-PCR products generated from the same RNA samples as above, amplified using ß-actin primers. Arrow indicates the expected ß-actin product size (218 bp).

Similar results were obtained by northern hybridization, confirming the absence of IL-1α mRNA in healthy control fibroblasts and the constitutive expression of IL-1α mRNA in affected SSc skin fibroblasts (data not shown).

To evaluate the expression of IL-1α protein and to determine the proportion of cells positive in the cultures, IL-1α expression was analyzed by flow cytometry. We detected no surface expression of IL-1α in cultures of fibroblasts derived from affected SSc skin and fibroblasts from clinically unaffected skin or from a healthy control (data not shown). Using the same procedure, there was positive staining for other surface proteins including MHC class I antigens. When cells were incubated with anti-IL-1α antibody after fixing with PFA and permeabilized with Triton X-100 prior to staining, there was uniformly bright staining of SSc-affected fibroblasts and consistently no staining of unaffected or control fibroblasts (data not shown). These results indicated that there was relatively homogenous IL-1α protein expression in SSc-affected fibroblasts and suggested that IL-1α protein expression in these cells was intracellular in location.

Subcellular distribution of IL-1α protein. As shown in Table 1, IL-1α protein detected by ELISA was present predominantly in the nuclear fraction of affected SSc fibroblasts. By contrast, IL-1α protein was undetectable in culture supernatants from these cells (data not shown) and in subcellular fractions from fibroblasts derived from unaffected skin of SSc patients and healthy controls (Table 1).

For immunohistochemical analysis, fibroblasts grown on chamber slides were processed in 2 ways: (1) fixation with

Table 1. Localization of IL-1α in cultured fibroblasts. Confluent fibroblasts were cultured in serum-free medium, and the amount of IL-1α in 3 different subcellular fractions was measured by ELISA, as described in Methods.

	IL-1α, pg/10^6 cells		
	Membrane	Cytosol	Nucleus
Affected fibroblasts			
A1	79.3	20.6	122.5
A2	2.8	5.3	61.4
A3	18.5	28.1	65.8
A4	104.9	79.6	161.8
A5	30.0	6.1	71.5
A6	9.7	2.4	56.3
A7	57.7	86.0	105.3
A8	20.8	14.5	112.4
A9	12.8	10.5	88.2
A10	44.1	32.9	79.6
A11	8.6	7.5	62.4
A12	5.7	8.1	47.6
Mean ± SD	32.9 ± 32.5	25.1 ± 28.5	86.2 ± 33.5
Unaffected fibroblasts			
U1, U2, U7	< 1.8	< 1.8	< 1.8
Normal fibroblasts			
N1–N7	< 1.8	< 1.8	< 1.8

2% PFA alone; or (2) fixation with 2% PFA in combination with permeabilization using 0.1% Triton X-100. As controls, we performed staining for known cytoplasmic (i.e., prolyl-4-hydroxylase) and nuclear (histones) antigens. Our results indicated that staining after fixing in PFA resulted in strong cytoplasmic staining of prolyl-4-hydroxylase, and no staining for histones. Detection of nuclear antigens (histones) was entirely dependent upon permeabilization with Triton X-100 (data not shown). As presented in Figure 2, affected SSc fibroblasts stained after fixing alone (i.e., without permeabilization) showed patchy cytoplasmic staining for IL-1α that excluded the nucleus. When cells were both fixed and permeabilized, there was prominent staining for IL-1α in SSc fibroblasts that was primarily nuclear in location. The identical pattern of IL-1α staining was found in affected fibroblasts from all 12 SSc patients (data not shown). Fibroblasts from unaffected skin and healthy control skin were negative for IL-1α staining (Figure 2 and data not shown).

Confocal microscopy was performed to further characterize the nuclear distribution (perinuclear vs intranuclear) of IL-1α in affected SSc fibroblasts. As shown in Figure 2D, IL-1α present in SSc fibroblasts was predominantly intranuclear and was homogeneous in its distribution throughout

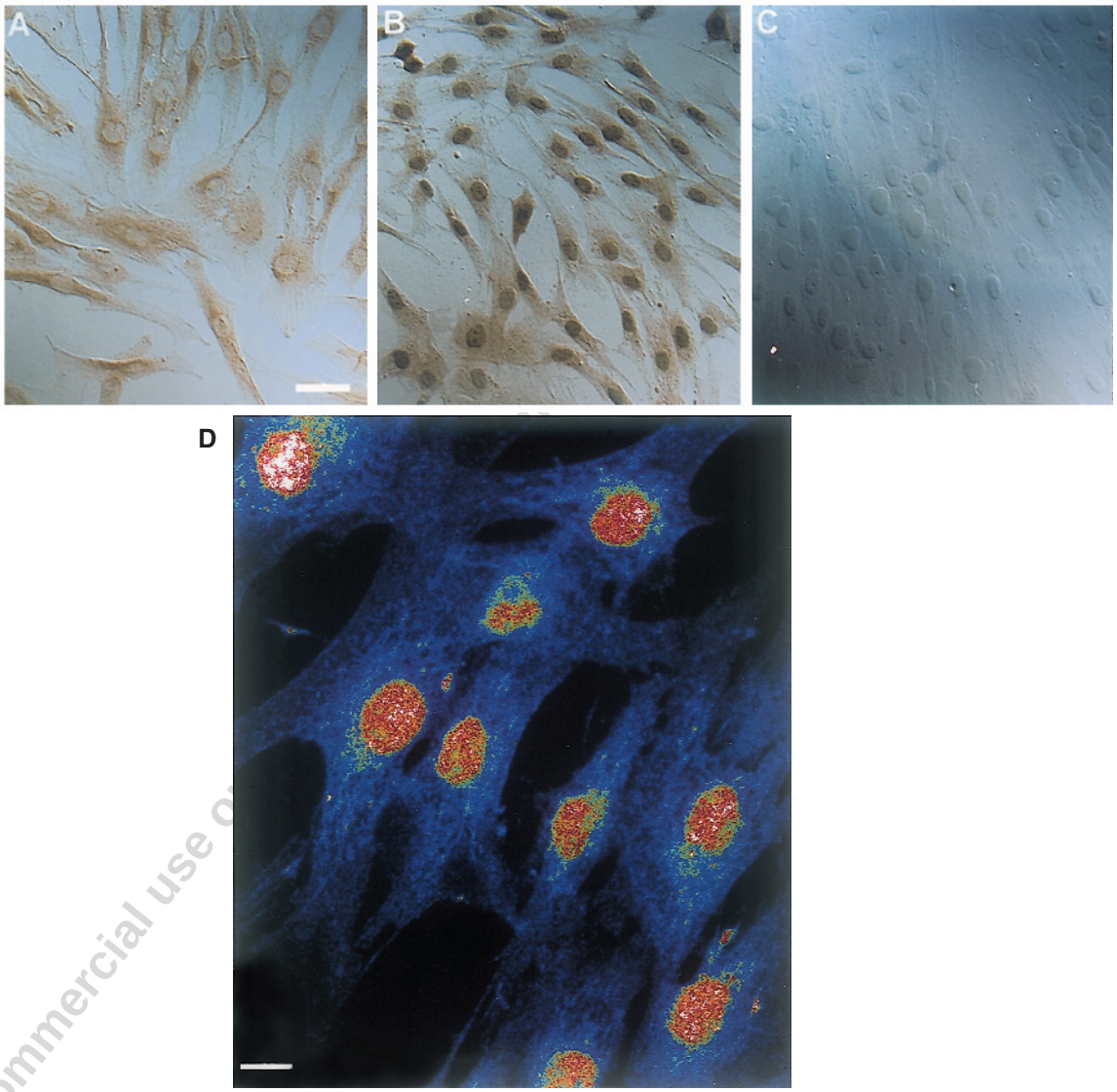

Figure 2. Immunohistochemical and immunofluorescence analysis of interleukin 1α (IL-1α) protein expression in cultured fibroblasts. Fibroblasts derived from affected skin (Panels A and B) and unaffected skin (Panel C) from an SSc patient were cultured on a 4-well chamber slide and fixed with 2% PFA (A) or 2% PFA plus Triton X-100 (B, C). All samples were stained with anti-IL-1α antibody. In Panel C the cells were visualized using differential interference microscopy. Similar results were obtained with an additional 6 SSc fibroblast lines (all positive), 2 SSc-unaffected, and 3 normal fibroblast lines (all negative). Bar = 25 µm. Panel D shows a digitized image of a high power confocal microscopic view of SSc fibroblasts stained with anti-IL-1α antibody (blue = negative; green/yellow = intermediate; red/white = maximal intensity). Bar = 10 µm.

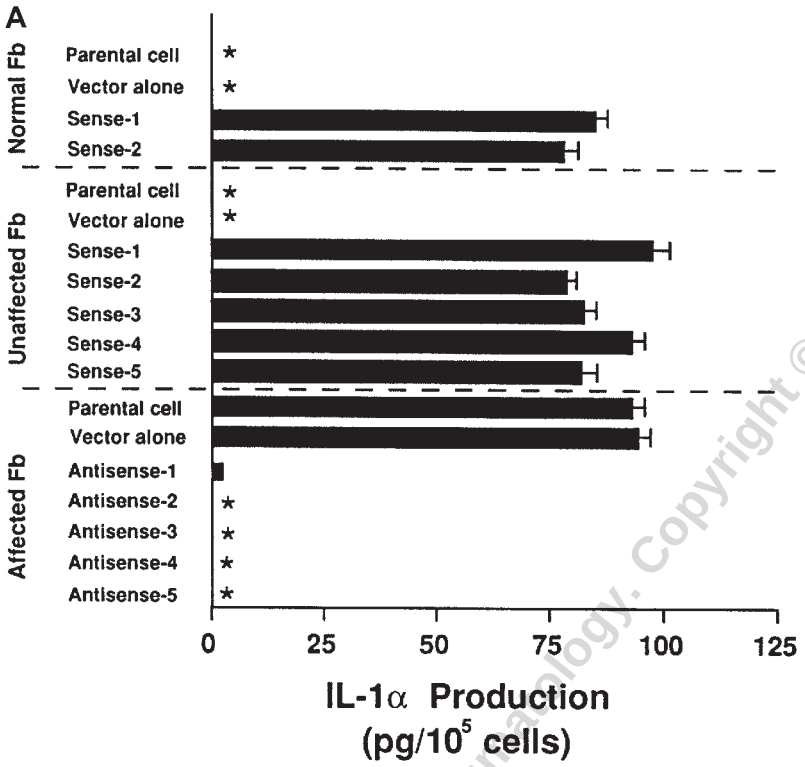

Figure 3. Analysis of IL-1α and IL-6 production and [^{3}H]-thymidine incorporation in stable transfectants. Fibroblasts (SSc-affected, SSc-unaffected, and healthy control) were stably transfected with antisense (SSc-affected) or sense (SSc-unaffected and control) IL-1α mRNA-encoding constructs or vector (pcDNA3) and stably transfected lines were selected in G418. A. Cell lysates were analyzed for IL-1α by ELISA; values less than the limit of sensitivity (1.8 pg) are indicated by an asterisk.

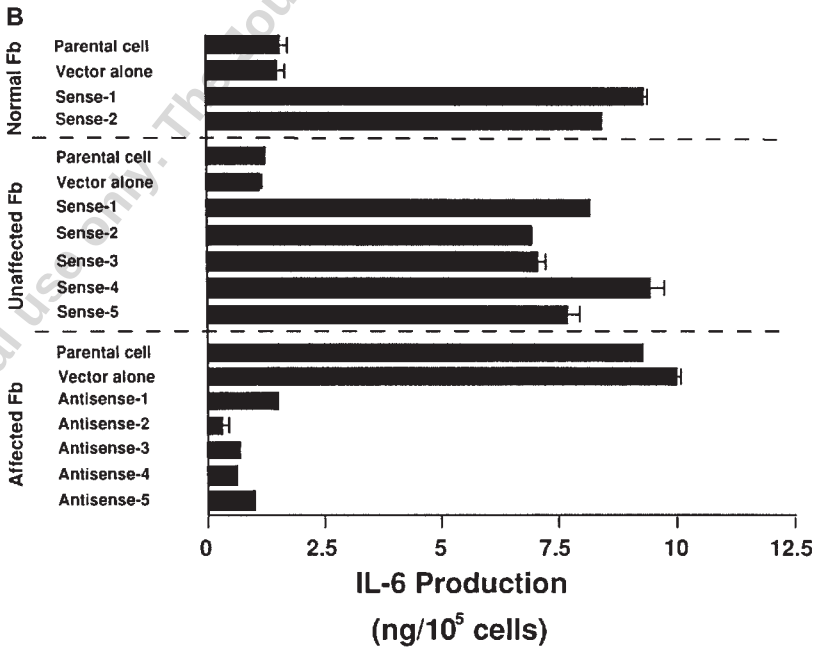

Figure 3B. Interleukin 6 production in culture supernatants of the stable transfectants measured by ELISA.

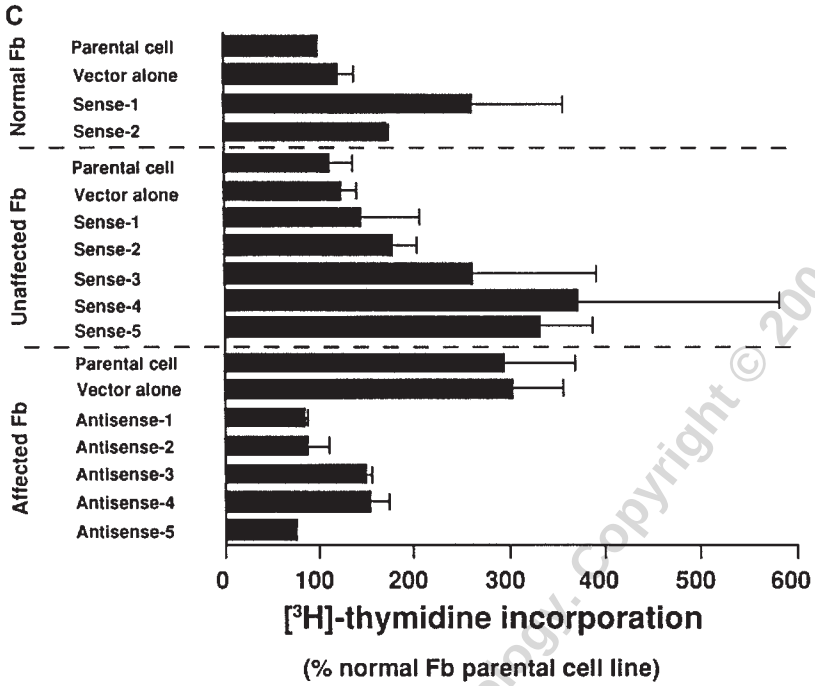

Figure 3C. Fibroblast proliferation measured by [^{3}H]thymidine incorporation. Similar results were obtained using fibroblast lines from 4 additional SSc patients. Results shown are the means of triplicate values in panels A, B, and C.

the nucleus. Also evident from the confocal micrographs was a relatively small amount of IL-1α staining in perinuclear vesicles.

Effect of stable expression of antisense or sense IL-1α mRNA. To investigate the role of IL-1α in the SSc fibroblast phenotype, we examined the effect of inhibiting endogenous IL-1α expression in affected SSc fibroblasts using a plasmid construct encoding antisense IL-1α mRNA. Stably transfected fibroblast clones (5 fibroblast lines derived from 2 Caucasian and 3 Japanese patients with SSc) were prepared and analyzed for IL-1α protein expression. As controls, affected SSc fibroblasts were stably transfected with vector without insert, and both normal and unaffected fibroblasts were stably transfected with the sense-encoding construct or vector control. As shown in Figure 3A, stable transfection of an affected SSc fibroblast line with the IL-1α antisense-expressing construct resulted in the reduction of constitutive IL-1α expression to undetectable levels. Conversely, stable transfection of unaffected or healthy control fibroblasts with the sense-encoding construct resulted in IL-1α protein expression comparable to the affected SSc parental line. The results of Western blot analysis of the IL-1α protein expressed in these cells are shown in Figure 4. Only the 31 kDa precursor form of IL-1α protein was detected both in the parental-affected SSc line and in the sense-transfected clones. Consistent with the ELISA analysis of cell lysates (Figure 3A), Western blot analysis revealed no detectable IL-1α protein in the antisense-transfected affected SSc clones (Figure 4).

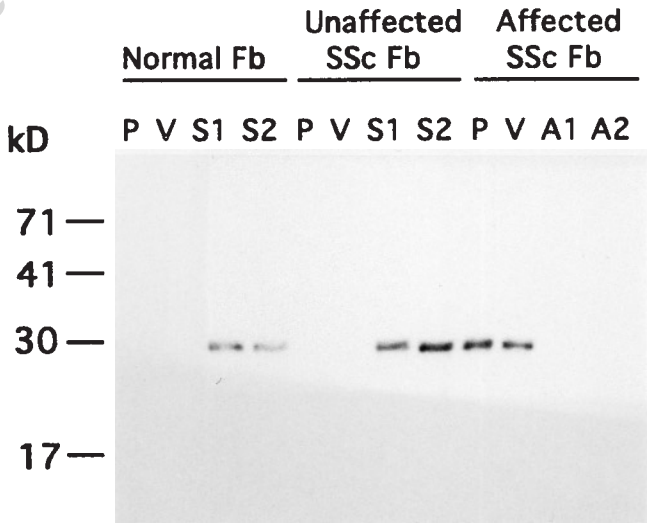

Figure 4. Western blot analysis of interleukin 1α (IL-1α) protein expression in stably transfected healthy control fibroblasts (sense construct and vector control) and SSc-affected fibroblasts (antisense-encoding plasmid construct and vector control). P: parental line (not transfected); V: control vector without insert; S: IL-1α antisense-encoding construct.

The activation of normal dermal fibroblasts with exogenous IL-1 is reported to stimulate IL-6 secretion[24] and proliferation[25] and the synthesis of collagen[26]. These features are characteristic of the fibrogenic phenotype of the affected SSc fibroblasts[12-15]. Therefore the effect of inhibiting IL-1α expression in affected SSc fibroblasts and overexpressing IL-1α in normal and unaffected SSc fibroblasts on these

aspects of fibroblast function was examined. As shown in Figure 3B, SSc-affected fibroblasts stably transfected with the antisense IL-1α-encoding construct showed a reduction in IL-6 secretion to levels found in normal and unaffected fibroblasts. These antisense IL-1α mRNA-expressing clones also had reduced proliferation compared to the parental line (Figure 3C). In contrast, both control and non-lesional fibroblasts transfected with the IL-1α sense mRNA-encoding construct showed increased IL-6 secretion (Figure 3B) and increased proliferation (Figure 3C).

Since the fibrosis in SSc is due largely to increased elaboration of type I collagen, it was necessary to determine the effect of modulating IL-1α expression on this process. The synthesis of α1(I) procollagen mRNA and protein was analyzed by Northern blot hybridization and C-terminal peptide ELISA, respectively. These results showed that antisense-transfected affected SSc fibroblast clones had significant reduction in procollagen mRNA (Figure 5) and protein levels (Figure 6), while the IL-1α-overexpressing sense-transfected clones expressed elevated levels of α1(I) procollagen mRNA and protein similar to the affected SSc fibroblast parental line.

DISCUSSION

This report provides evidence for a direct link between pro-

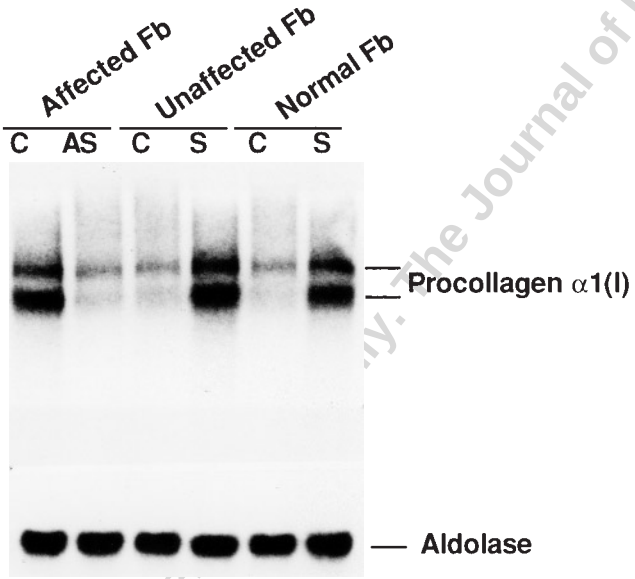

Figure 5. Analysis of α1(I) procollagen mRNA in parental fibroblast lines and antisense and sense interleukin 1α (IL-1α) transfected fibroblast clones. Levels of pro-α1(I) collagen mRNA expression were analyzed by Northern blot analysis of total RNA isolated from fibroblast cultures (parental lines and stable transfectants) that were grown to confluence and incubated in serum-free medium for 48 h. Shown is a representative Northern blot from 3 independent experiments. C: control line (parental line, not transfected); AS: IL-1α antisense-encoding construct; S: IL-1α sense-encoding construct. After stripping, the filters were hybridized with a radiolabeled probe prepared from the cDNA encoding aldolase to assess RNA loading (lower panel). Fb: fibroblast.

IL-1α expression and the pathogenesis of SSc. IL-1α is a member of a growing family of cytokines that includes IL-1ß, IL-18, multiple isoforms of the IL-1 receptor antagonist, and several recently described homologs IL-1H1, IL-1H2, IL-1H3 and IL-1H4 (also known as IL-1F7)[27-29]. Both IL-1α and IL-1ß are synthesized as 31 kDa precursors. The 31 kDa immature and the 17 kDa mature forms of IL-1α are biologically active[30], whereas IL-1ß must be cleaved to its 17 kDa form by the IL-1-converting enzyme (ICE or caspase 1) to be active[31].

Whether IL-1 is basally expressed in fibroblasts appears to depend on the tissue of origin. Studies have described that IL-1α and IL-1ß proteins were not expressed in normal skin fibroblasts[32,33]. Consistent with these reports, we detected no IL-1α or IL-1ß protein in the culture supernatants from normal or SSc skin fibroblasts (reference[16] and data not shown), but we did detect constitutive expression of IL-1ß mRNA by RT-PCR in these cells (data not shown). IL-1 protein expression has been reported in cultures of primary renal fibroblasts[34] has been linked to the expression of IL-6 and IL-8 in these cells[35]. IL-1α expression has also been observed during the senescence (> 30 passages) of human dermal fibroblast cultures *in vitro*[32]. In contrast to its effect on early passage fibroblasts, the constitutive expression of IL-1α in aging fibroblasts was associated with a markedly reduced proliferation rate and a limited response to mitogenic stimuli (including exogenous IL-1α).

The data reported here show that endogenous IL-1α plays a crucial role in procollagen production by fibroblasts derived from patients with SSc. The inhibition of IL-1α expression in stable lines derived from SSc-affected skin by expression of IL-1α antisense construct was associated with a reversion of the phenotype of these cells to that of normal skin fibroblasts (Figures 3, 5, and 6), with a decrease in IL-6 and collagen production and proliferation. These studies extend and confirm our previous findings of the inhibitory effect of IL-1α antisense oligonucleotides on platelet derived growth factor and IL-6 mRNA levels in SSc-affected fibroblasts using antisense oligodeoxynucleotides[17]. In contrast, exogenous IL-1α did not affect the expression of procollagen type I by normal fibroblasts at the mRNA and protein levels; nor did the culture with neutralizing anti-IL-1α antibodies or IL-1 receptor antagonist affect the elevated expression of IL-6 and procollagen type I by SSc-affected fibroblasts (unpublished data). We infer from these observations that endogenous IL-1α, potentially acting at the nucleus, is involved in the establishment of a fibrogenic phenotype in SSc-affected fibroblasts.

In this regard, there is increasing evidence that nuclear localization of IL-1α may be important in eliciting certain cellular responses. Wessendorf, *et al* identified a functional nuclear localization sequence (NLS) consisting of 8 amino acids (KVLKKRRL) in the amino-terminal portion of the 31 kDa IL-1α precursor[36]. The NLS-containing 31 kDa IL-1α

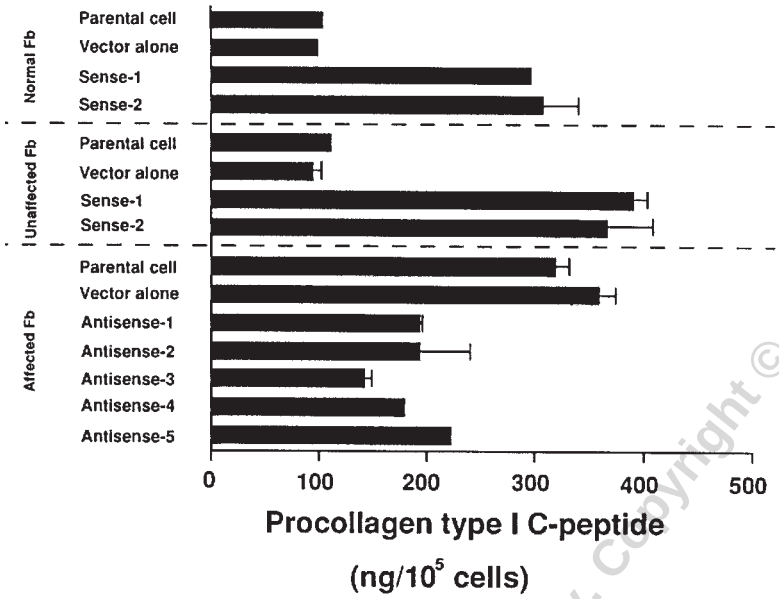

Figure 6. Analysis of type I collagen protein synthesis by stably transfected fibroblast clones and parental lines determined by ELISA measurement of pro-α1(I) collagen C-terminal peptide. Fb: fibroblast.

precursor, and not the mature 17 kDa form, was active in inhibiting endothelial cell growth[37] and migration[38]. Stevenson, *et al* reported that expression of the 16 kDa amino-terminal fragment (propiece) of IL-1α in rat glomerular mesangial cells resulted in malignant transformation[39].

Our findings indicate that in human dermal fibroblasts, similar to human endothelial cells, nuclear localization of the IL-1α 31 kDa precursor is associated with profound effects on cell phenotype. These studies suggest that the propiece of the IL-1α protein may contain a domain responsible for a nuclear signal capable of regulating cell growth and gene expression. Using a yeast two-hybrid approach, we recently found that IL-1α binds a growth and differentiation regulatory protein known as necdin. This interaction occurs in the nucleus and increases cell growth and collagen production[40]. Yin, *et al* also showed that the N-terminus of the IL-1 precursor binds HAX-1[41].

The role of IL-1α in the development of tissue fibrosis in SSc *in vivo* is supported by the observations that increased levels of IL-1α have been observed in SSc skin[17] and in bronchoalveolar lavage fluids from SSc patients with pulmonary fibrosis[42,43]. Recently, Yamamoto, *et al* showed that IL-1α plays a role in the autocrine upregulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of MMP-1 in normal human dermal fibroblasts stimulated by monocyte chemoattractant protein-1[44]. Together with our observations, these findings identify IL-1α as an important factor in the pathogenesis of SSc and a potential therapeutic target for SSc and other fibrotic disorders.

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