Article

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Abstract

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Reference

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Autoantibodies to Fibroblasts Induce a Proadhesive and Proinflammatory Fibroblast Phenotype in Patients With Systemic Sclerosis

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Objective. **Fibroblasts play a major role in the development of systemic sclerosis (SSc), and the occurrence of serum autoantibodies reacting with fibroblast plasma membrane antigens in SSc has been reported. This study was undertaken to investigate whether IgG from SSc sera that react with human fibroblasts modulates the fibroblasts' function.**

Methods. **Sera from 69 patients with SSc (28 with limited cutaneous SSc [lcSSc] and 41 with diffuse cutaneous SSc [dcSSc]), 30 patients with sarcoidosis, and 50 matched healthy controls were examined. We evaluated antibody binding to human skin and lung fibroblasts by cell-based enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, and flow cytometry. We further investigated the ability of puri-fied IgG to modulate 1) intercellular adhesion molecule 1 (ICAM-1) expression, 2) U937 cell adhesion to fibroblasts, and 3) fibroblast steady-state messenger RNA (mRNA) levels of interleukin-1α (IL-1α), IL-β, and IL-6, and IL-6 protein production.**

Results. **Of 69 SSc sera tested by cell-based ELISA, 58% bound to normal skin and lung fibroblasts. The prevalence of binding was significantly higher in dcSSc than in lcSSc ($P < 0.05$). Only IgG from SSc sera that were positive for antifibroblast antibody (AFA) induced a dose-dependent up-regulation of ICAM-1 expression and IL-6 production, enhancement of U937 cell adhesion, and increased levels of IL-1α, IL-1β, and IL-6 mRNA in fibroblasts. Up-regulation of ICAM-1 mediated by AFA IgG was inhibited by the addition of IL-1 receptor antagonist, indicating an autocrine activation loop.**

Conclusion. **Our findings confirm the presence of AFAs in SSc sera and demonstrate, for the first time, that autoantibodies reacting with fibroblast surface molecules act as an extrinsic stimulus inducing fibroblast activation in vitro.**

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Systemic sclerosis (SSc) is a disease that is clinically dominated by fibrosis involving the skin and internal organs, including (but not exclusively) the lung, the gastrointestinal tract, and the heart. SSc is also characterized by vasculopathy, with clinical manifestations ranging from Raynaud's phenomenon and pulmonary hypertension to life-threatening renal hypertensive crisis. Autoantibodies to nuclear components are characteristically associated with SSc and segregate differentially in distinct clinical subsets. Thus, anticentromere antibodies are more often found in SSc with limited skin involvement (lcSSc), while anti–DNA topoisomerase I

and anti–RNA polymerase I antibodies are preferentially associated with SSc with diffuse skin involvement (dcSSc) (1).

In addition to antibodies to nuclear components, several other autoantibody specificities have been found in the sera of SSc patients. They may recognize cytoplasmic structures including mitochondria, protein complexes expressed at the surface of cells such as Fcγ receptor II (FcγRII) (CD32) and FcγRIII (CD16) (2), and released products such as fibrillin 1, a component of the extracellular matrix (ECM) (3–5), or IgE produced by B cells (6). Fibroblasts, lymphocytes, and endothelial cells (among other cells) have been found to be targets of antibodies in SSc (4,7–9). Except for the cytolytic capacity of IgM directed against CD4+ T cells (9), the functional role of antibodies to the cell surface in SSc has received little attention.

Fibroblasts are thought to play a major role in the pathogenesis of SSc. Indeed, they are directly involved in the synthesis and continuous renewal of many ECM components, and dysregulated ECM turnover is central to fibrosis development in SSc (10–12). In addition, fibroblasts contribute to the inflammation that precedes and accompanies fibrosis, by producing soluble mediators. For instance, SSc fibroblasts have been shown to release high levels of interleukin-6 (IL-6), a characteristic related to high ECM production (13,14). More recently, high levels of intracellular IL-1α and intracellular IL-1 receptor antagonist (IL-1Ra) have been associated with the SSc fibroblast phenotype, and endogenous IL-1α has been shown to induce production of high levels of IL-6 and platelet-derived growth factor (15–17). IL-1α also induces fibroblast production of IL-1β, IL-8, tumor necrosis factor α (TNFα), and granulocyte–macrophage colony-stimulating factor (18–20). In addition, fibroblasts release chemokines such as monocyte chemotactic protein 1 (MCP-1), whose serum levels are increased in SSc (21), and stimulate the transendothelial migration of mononuclear cells (22,23). Migrating cells may then bind to SSc fibroblasts that express high levels of adhesion molecules, particularly intercellular adhesion molecule 1 (ICAM-1), whose serum levels of the soluble isoform appear to correlate with disease activity (24–28).

The aim of the present investigation was to assess the role of antifibroblast IgG purified from SSc sera and its capacity to induce a proinflammatory and proadhesive phenotype on dermal and lung fibroblasts. Our data indicate that a large proportion of SSc sera contain IgG that binds to fibroblast plasma membranes and induces fibroblast activation.

PATIENTS AND METHODS

Patient population. Sixty-nine SSc patients (58 women and 11 men; mean $\pm$ SD age 51.4 $\pm$ 14.8 years, mean $\pm$ SD disease duration 9.8 $\pm$ 3.2 years) were studied. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for SSc (29). They were divided into 2 groups according to type of cutaneous involvement, i.e., lcSSc (n = 28) or dcSSc (n = 41) (30). Lung involvement was determined by pulmonary function tests, carbon monoxide diffusing capacity (DL$_{CO}$), and high-resolution computed tomography (HRCT). Pulmonary artery pressure was evaluated indirectly by echo–color Doppler. Control sera were from 50 healthy individuals, matched for sex and age, and from 30 patients with sarcoidosis with lung involvement, 10 of whom had established lung fibrosis.

Reagents. Protein G–Sepharose was from Pharmacia-Biotech (Uppsala, Sweden), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, penicillin, and streptomycin from Gibco BRL (Paisley, UK), fetal calf serum (FCS) and Hanks' balanced salt solution from Flow (Irvine, UK), alkaline phosphatase–conjugated goat anti-human IgG and IgM, phosphatase-conjugated goat anti-mouse IgG, polymyxin B sulfate, p-nitrophenylphosphate, and bovine serum albumin from Sigma (St. Louis, MO), fluorescein isothiocyanate (FITC)–conjugated rabbit anti-human IgG from Dako (Glostrup, Denmark), peroxidase-conjugated γ chain–specific goat anti-human IgG from Cappel (Cochranville, PA), anti–ICAM-1 monoclonal antibody (mAb) and recombinant human IL-1Ra (rHuIL-1Ra) from Serotec (Oxford, UK), enhanced chemiluminescence reagents and ^{51}CrNa from Amersham Life Science, Amersham International (Little Chalfont, UK), XAR x-ray film from Eastman Kodak (Rochester, NY), rHuIL-1β from British BioTechnology (Oxford, UK), and rHuTNF and anti–IL-6 blocking mAb from R&D Systems (Minneapolis, MN). Murine monoclonal anti–human HLA class I, anti-CD14, anti-CD19, and anti-CD3 IgG were kindly provided by Prof. F. Malavasi (University of Turin).

Fibroblasts. Human fibroblasts were obtained by the standard explant technique from 1) foreskin of a healthy individual, 2) normal skin (3 individuals), 3) involved skin from the forearms of a patient with SSc, and 4) normal lung parenchyma from 3 individuals undergoing surgery for lung cancer (31). Fibroblasts were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin and used between the fourth and seventh passages.

Antifibroblast antibody detection. IgG fractions from SSc sera that were positive for antifibroblast antibody (AFA), from SSc sera that were negative for AFA, and from normal sera were affinity purified by protein G column chromatography (32). The final concentration of IgG was evaluated by nephelometry. Cell-based ELISA, flow cytometry, and indirect immunofluorescence were used to detect AFAs.

Cell-based ELISA was performed on living, unfixed fibroblasts as previously described (32), with minor modifications. Briefly, the indicated dilutions of serum samples or purified IgG were applied for 2 hours to confluent fibroblast monolayers on 96-well plates. After washing, the plates were incubated for 90 minutes with alkaline phosphatase–
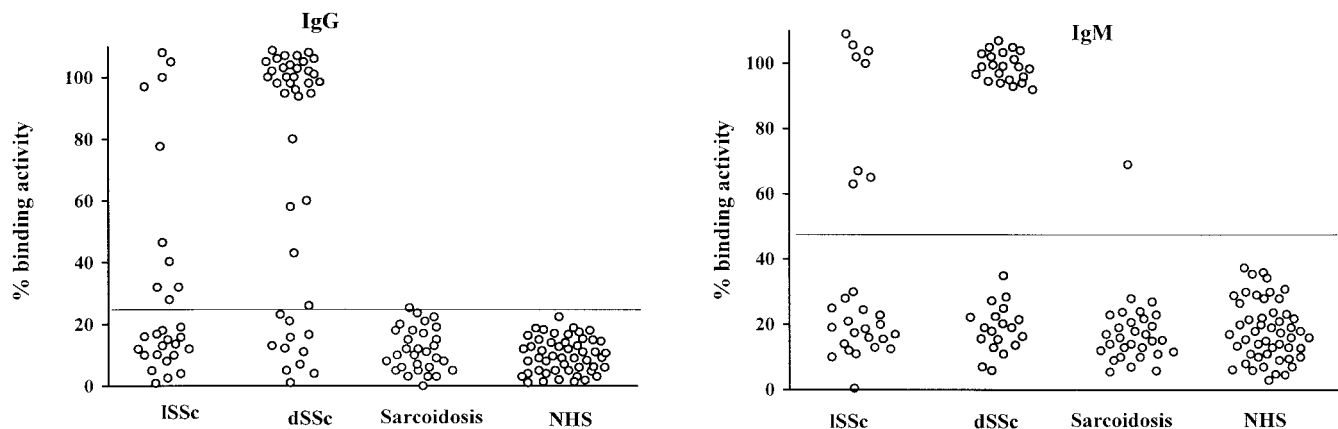
Figure 1. Prevalence of fibroblast cell binding activity in sera from patients with systemic sclerosis (SSc), sera from patients with sarcoidosis, and normal human sera (NHS). Values are expressed as the percentage of binding activity, as described in Patients and Methods. Horizontal lines represent the cutoff, calculated as 3 SD above the mean percentage of binding activity in 50 normal sera. lSSc = limited SSc; dSSc = diffuse SSc.

conjugated goat anti-human IgG or IgM. The reaction was detected by adding p-nitrophenylphosphate in $0.05M$ Mg-carbonate buffer (pH 9.8), and the optical density (OD) at 405 nm was recorded (Titertek Multiskan MCC/340; Lab Systems, Helsinki, Finland). Cell number per well was determined before and after washes to detect cell detachment, as described (33). No significant cell loss was found under the different experimental conditions (data not shown). Samples were tested in duplicate. The OD value of a positive reference serum at standard 1:25 dilution was arbitrarily defined as 100% of fibroblast binding activity. The results of tested samples were expressed as a percentage of this positive reference value, to adjust for interassay variability. An OD more than 3 SD above the mean OD obtained with sera from 50 healthy individuals (i.e., 22.2% for AFA IgG and 47.3% for AFA IgM) was considered positive.

In some experiments, human skin fibroblasts were preincubated with affinity-purified rabbit IgG. After 2 hours of incubation at room temperature, the monolayers were extensively washed with tissue culture medium and then tested for binding of serial protein concentrations of AFA-positive or AFA-negative SSc serum IgG as described above.

For flow cytometry, adherent fibroblasts were incubated for 30 minutes at 4°C with or without AFA-positive or AFA-negative human IgG at a final concentration of 100 μg/ml, then detached using 30 μM EDTA–phosphate buffered saline, washed twice, stained with FITC-conjugated rabbit anti-human IgG, and analyzed on a Facscalibur cytometer (BD Biosciences, San Jose, CA). CELLQuest software (BD Biosciences) was used to generate the analysis plots.

For indirect immunofluorescence, primary cultures of human dermal fibroblasts were detached by gentle scraping and incubated with purified IgG (10 μg/200,000 cells) for 30 minutes. Fibroblasts were photographed with an IM 35 fluorescence microscope (Zeiss, Milan, Italy) upon staining with FITC-conjugated anti-human immunoglobulins. To evaluate the purity of the cell preparations, the expression of several surface markers (HLA class I, ICAM-1, CD14, CD19, CD3) on the fibroblasts was investigated by cytofluorimetry. All of the preparations were found to be HLA class I positive, ICAM-1 positive, and CD14, CD19, and CD3 negative, ruling out any monocyte or T or B lymphocyte contamination.

ICAM-1 detection on fibroblast cell surface. Cell-based ELISA was used to detect ICAM-1 expression on confluent human dermal fibroblast monolayers. Fibroblasts in 96-well plates were incubated for 20 hours with various amounts (1.5–100 μg/ml) of IgG from SSc patients or normal subjects, positive control IL-1β (50 units/ml), or medium alone, in a final volume of 100 μl. In some blocking experiments, IL-1Ra (0.015–1 μg/ml) or anti-IL-6 mAb (0.015–1 μg/ml) was added before addition of IgG or IL-1β. The cells were then washed twice and incubated for 60 minutes with anti–ICAM-1 mAb. The reaction was revealed by adding phosphatase-conjugated goat anti-mouse IgG and the appropriate substrate. OD values at 405 nm were determined using a semiautomatic reader (Titertek Multiskan).

U937 cell functional adhesion assay. Fibroblast monolayers were incubated with SSc or control IgG at a final protein concentration of 100 μg/ml for 24 hours in 96-well plates. They were then washed extensively, and 3×10^5 ^{51}Cr-labeled U937 cells (30 μCi/10^6 cells) were added to each well for 1 hour at 37°C. Nonadherent cells were removed by washing, and remaining cells were lysed with 0.1% sodium dodecyl sulfate/$0.025M$ NaOH. Sample counts per minute were recorded, and adhesion was expressed as a percentage of adhered cells in relation to total ^{51}Cr-labeled U937 cells seeded to fibroblast monolayers (i.e., 100%) (34,35).

IL-1α, IL-1β, and IL-6 production by cultured fibroblasts. Fibroblasts were grown to confluence in 96-well plates and incubated for 24 hours at 37°C with SSc or normal IgG (1.5–100 μg/ml) at a final volume of 200 μl/well. Control cultures contained medium alone or rHuIL-1β (50 units/ml) for IL-6 detection or rHuTNFα (10 ng/ml) for IL-1α and IL-1β evaluation. At the end of the culture period, the plates were centrifuged (800 revolutions per minute; 4°C for 10 minutes), and the cell-free supernatant was removed for cytokine determinations. Content of IL-1α, IL-1β, and IL-6 was determined by commercial assays (from Immunotech [Marseille, France] for IL-1α; from Amersham International for IL-1β and IL-6).
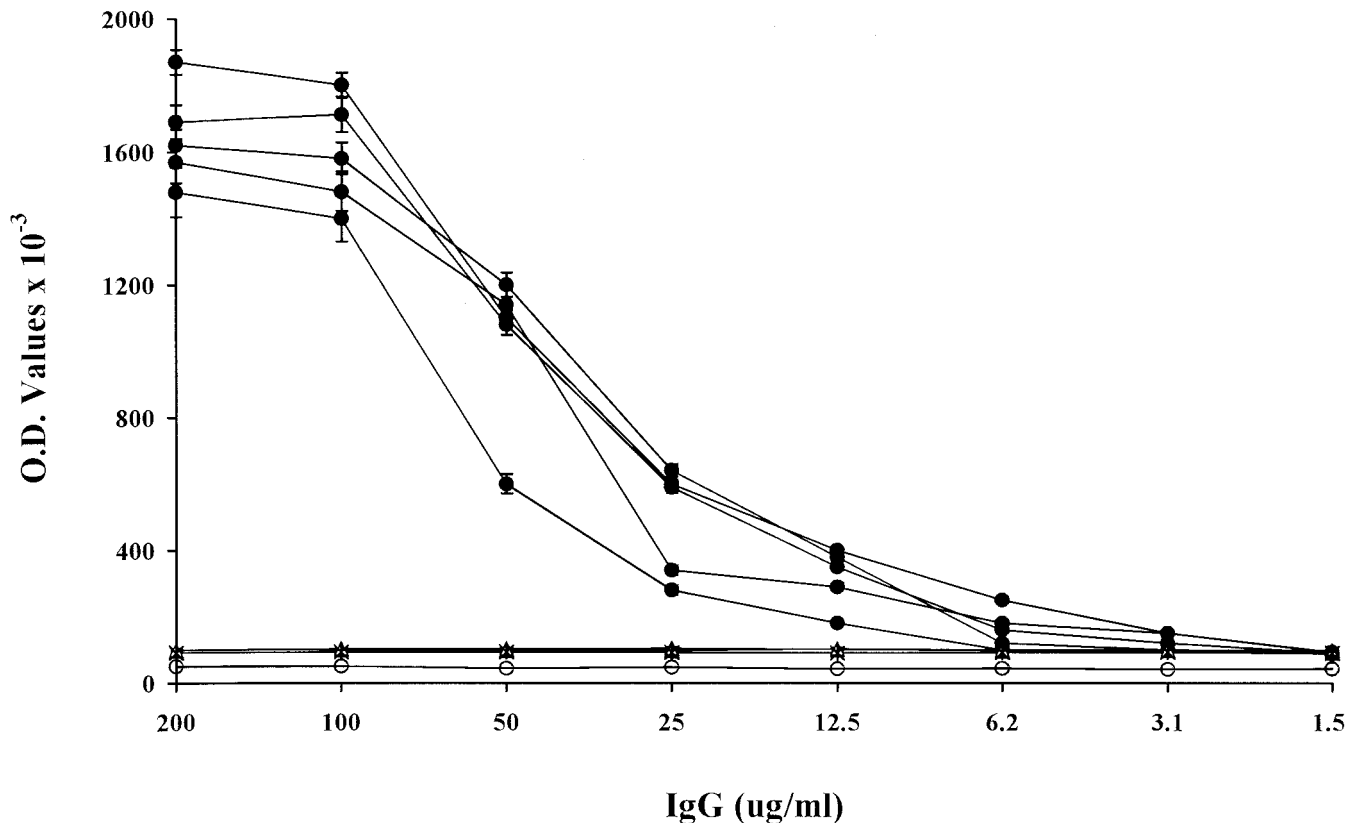
Figure 2. Binding to unfixed human dermal fibroblasts by SSc antifibroblast antibody (AFA)–positive sera (●) (n = 5), SSc AFA-negative serum (△) (n = 1), NHS IgG (×) (n = 1), or medium alone (○). Values are the mean ± SD optical density (OD) from triplicate experiments. See Figure 1 for other definitions.

RNA extraction and RNase protection assay (RPA) analysis. Lung fibroblasts were cultured in the presence of 100 μg/ml of control IgG or AFA-positive SSc IgG for 4 hours or 24 hours, and then total RNA was isolated by lysing fibroblasts with TRIzol reagent according to the instructions of the manufacturer (Gibco BRL) and analyzed for levels of expression of IL-1α, IL-1β, IL-6, macrophage colony-stimulating factor (CSF), L-32, and GAPDH messenger RNA (mRNA) by RiboQuant RPA using a multiprobe template set (PharMingen, San Diego, CA). Briefly, riboprobes were ^{32}P-labeled and hybridized overnight in solution with 10 μg of the RNA samples. The hybridized RNA was digested with RNase and the remaining "RNase-protected" probes purified, resolved on denaturing polyacrylamide gels, and imaged by autoradiography according to the RiboQuant protocol. Autoradiography results were quantified using Image-Quant Software (Amersham Biosciences, Buckinghamshire, UK). Absolute density values of cytokine mRNA were normalized using GAPDH mRNA density values. The ratio of relative mRNA levels in fibroblasts treated with IgG to mRNA levels in fibroblasts treated with medium alone was assessed. Ratios ≥1.5 or ≤0.5 were arbitrarily considered to represent significant modulation.

Statistical analysis. Descriptive statistics were expressed as the mean ± SD. Statistical analysis was performed using one-way analysis of variance for multiple comparisons. P values less than 0.05 were considered significant.

RESULTS

Prevalence and clinical associations of AFAs in SSc patients and controls. Serum samples from SSc patients, healthy individuals, and patients with pulmonary sarcoidosis were tested for the presence of AFAs using a cell-based ELISA. In this assay, the threshold for positivity was defined as 3 SD above the mean value for binding to skin fibroblasts by 50 normal human sera (NHS) (Figure 1). Among the 69 SSc sera, AFA IgG above this threshold was detected in 40 (58%) and AFA IgM in 33 (48%). Interestingly, we observed a significant difference ($P < 0.05$) in the prevalence of AFA positivity between patients with dcSSc (72% for IgG and 53% for IgM) and those with lcSSc (37% for IgG and 30% for IgM). The sera were tested for binding to both skin and lung fibroblasts. No significant difference in reactivity was observed between skin and lung fibroblasts (data not

Table 1. Lack of effect of preincubation with rabbit IgG on AFA+ SSc serum IgG binding to human skin fibroblast monolayers*

| Quantity of rabbit IgG added	AFA+ serum IgG sample 1		AFA+ serum IgG sample 2		AFA+ serum IgG sample 3		AFA− serum IgG	
	200 μg/ml	100 μg/ml	200 μg/ml	100 μg/ml	200 μg/ml	100 μg/ml	200 μg/ml	100 μg/ml
0	1,412 ± 202	1,150 ± 215	1,304 ± 182	1,125 ± 190	1,051 ± 139	850 ± 98	220 ± 20	215 ± 33
250 μg/ml	1,447 ± 148	1,067 ± 96	1,134 ± 110	1,074 ± 136	1,110 ± 141	701 ± 75	231 ± 31	221 ± 25
500 μg/ml	1,571 ± 193	1,193 ± 142	1,104 ± 178	1,125 ± 190	910 ± 98	654 ± 86	228 ± 27	222 ± 12

* Values are the mean ± SD optical density from triplicate experiments. AFA = antifibroblast antibody; SSc = systemic sclerosis.

shown). Furthermore, no significant correlation was found between the presence of AFAs and pulmonary function results (including DLCO), lung fibrosis evaluated by HRCT, and pulmonary artery pressures. Of interest, only 1 of the 30 sarcoidosis sera tested was positive for AFA IgG, and 1 for AFA IgM. Thus, AFAs are frequently and specifically found in SSc sera, and their prevalence is related to the clinical subset of the disease.

Characterization of AFA IgG. To investigate the binding characteristics of AFAs, we purified the IgG fractions from 5 AFA-positive and 2 AFA-negative SSc sera. All 5 AFA-positive IgG preparations showed dose-dependent AFA binding activity, with half-maximal binding at concentrations ranging from 25 μg/ml to 50 μg/ml as detected by cell-based ELISA (Figure 2). In contrast, IgG from AFA-negative SSc patients and healthy controls did not display any significant cell
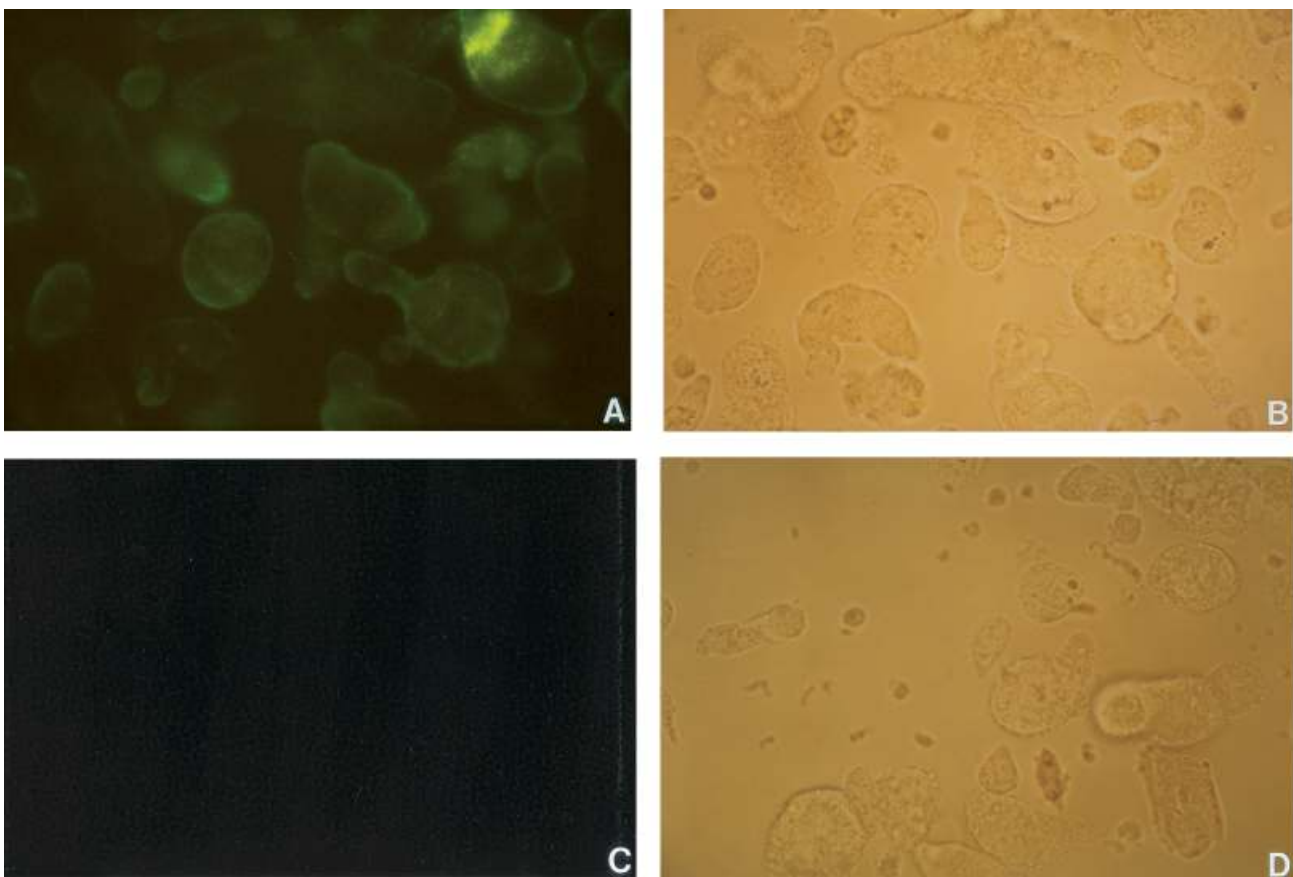
Figure 3. Immunofluorescence staining of dermal fibroblasts by antifibroblast antibody (AFA) IgG. **A,** Indirect immunofluorescence (IIF) on unfixed human dermal fibroblasts preincubated with systemic sclerosis AFA-positive IgG. **B,** Phase-contrast photomicrograph of the same fibroblast cell preparation as in **A. C,** IIF on fibroblasts incubated with AFA-negative normal human serum IgG. **D,** Phase-contrast photomicrograph of the same fibroblast cell preparation as in **C.** (Original magnification × 600.)
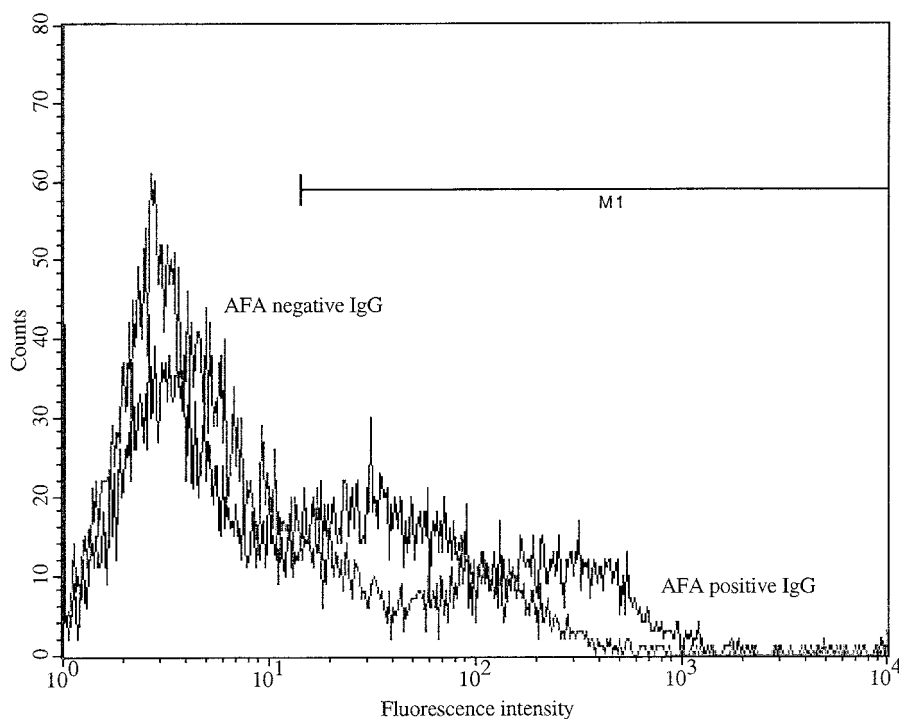
Figure 4. Flow cytometric analysis of fibroblasts incubated with antifibroblast antibody (AFA)–positive and AFA-negative fractions. IgG were used at a final protein concentration of 100 μg/ml. Data are presented as positive events versus fluorescence intensity. Representative results from 1 of 3 separate experiments are shown.

binding in this assay (Figure 2). Additional experiments performed with the same AFA-positive or AFA-negative IgG fractions on SSc skin fibroblasts revealed a binding pattern comparable with that found with normal fibroblasts (data not shown).

Although receptors for the Fc fragment of IgG (FcγR) have not been described to occur in significant amounts on fibroblast cell membrane (36), we assessed whether FcγR could play a role in SSc serum IgG binding to fibroblasts. Since preincubation of fibroblasts with high amounts of rabbit IgG did not affect AFA-positive SSc IgG binding (Table 1), we concluded that the cell-based ELISA results were not affected by FcγR.

Binding of the IgG preparations from AFA-positive sera was further evaluated by indirect immunofluorescence on unfixed human skin fibroblasts. As shown in Figure 3A, AFA-positive serum IgG displayed significant reactivity, which was confined to the plasma cell membrane; no reactivity was observed with AFA-negative IgG (Figure 3C).

A more complex pattern of reactivity was observed when SSc IgG was analyzed for its capacity to recognize unfixed fibroblasts by flow cytometry (Figure 4). Both AFA-positive and AFA-negative SSc IgG exhibited binding above background. However, the percentage of positive cells and mean fluorescence intensity were higher when SSc AFA-positive IgG was compared with SSc AFA-negative IgG in cell-based ELISA (Figure 4). Incubation of fibroblasts with NHS IgG yielded results comparable with those obtained with AFA-negative SSc IgG (data not shown).

Overall, these studies demonstrated that fibroblast binding of SSc sera is due to IgG reacting with components expressed at the surface of skin fibroblasts.

Induction of proadhesive phenotype by AFAs in fibroblasts. The detection of specific binding of SSc IgG to fibroblast plasma cell membrane prompted us to examine whether this binding resulted in fibroblast activation. First, we investigated whether mononuclear cell adhesion to fibroblasts was enhanced by previous exposure to AFA-positive SSc serum IgG. Incubation of dermal fibroblast monolayers with 3 AFA-positive SSc IgG resulted in enhanced adhesion
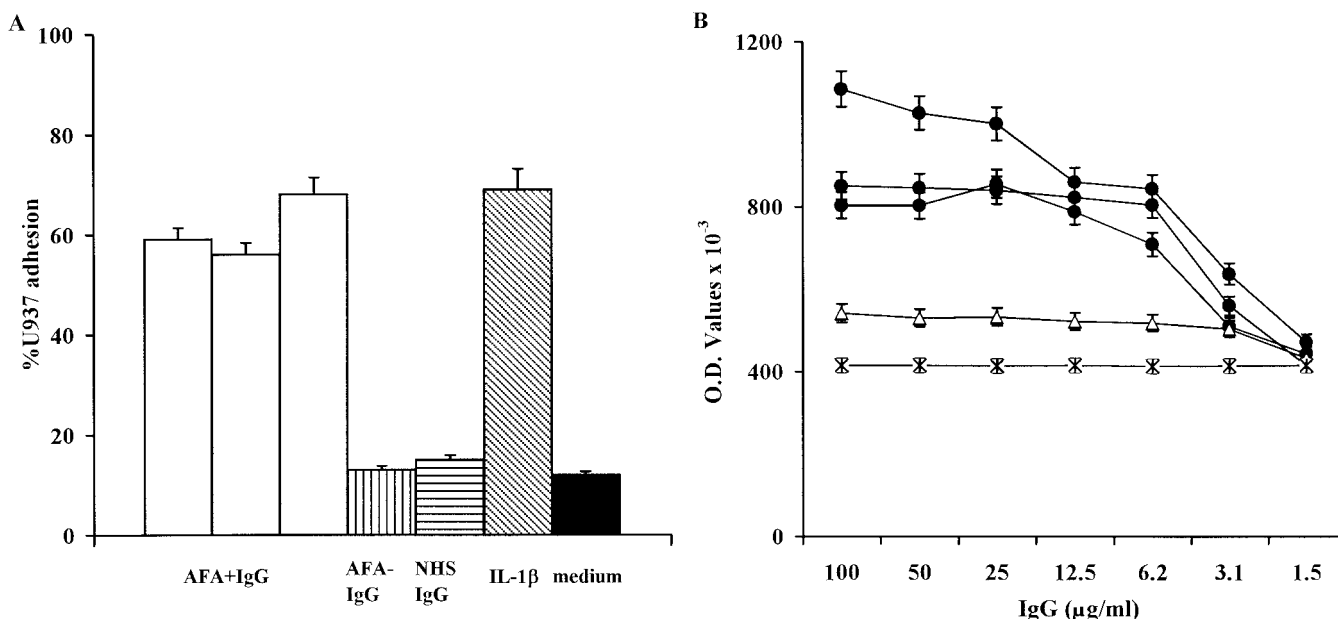
Figure 5. Induction of a proadhesive fibroblast phenotype. **A,** Percentage of U937 cells adhering to fibroblast monolayers incubated with different IgG preparations (100 μg/ml) from systemic sclerosis (SSc) patients or controls. **B,** Dose-dependent up-regulation of intercellular adhesion molecule 1 (ICAM-1) induced by serial protein concentrations of SSc antifibroblast antibody (AFA)–positive serum (●) (n = 3), AFA-negative serum (△) (n = 1), and normal human serum (NHS) (*) (n = 1) IgG fractions. As a positive control, fibroblast monolayers incubated with 50 units/ml of recombinant human interleukin-1β (IL-1β) gave a mean ± SD optical density (OD) value of 1.058 ± 167 for ICAM-1 expression; monolayers incubated with medium alone served as negative controls and gave background values. ICAM-1 expression was evaluated by the cell-based enzyme-linked immunosorbent assay, as described in Patients and Methods. Values are the mean ± SD from triplicate experiments.

of U937 monocytic cells with all 3 IgG (Figure 5A). The enhanced adhesion was specific, since AFA-negative or control IgG failed to induce this phenomenon, and it was powerful, since it was comparable in magnitude with that induced by saturating amounts of IL-1 (Figure 5A).

ICAM-1 plays a major role in adhesion, serving as a ligand for β2 integrins expressed at the surface of leukocytes (expression which is increased on SSc fibroblasts) (25). We therefore tested skin fibroblasts for ICAM-1 expression and its modulation by SSc IgG. A dose-dependent up-regulation of ICAM-1 was observed in the presence of AFA-positive, but not AFA-negative or control, IgG (Figure 5B). Similar experiments performed in the presence of polymyxin B yielded comparable results, ruling out endotoxin contamination of the IgG preparations. In addition, IL-1β, TNFα, and IL-6 (known to enhance ICAM-1 expression on fibroblasts) were not detected by ELISA in the IgG preparations used (data not shown). The notion that fibroblast activation was related to the binding of the antibodies and not to the contamination of IgG fractions by cytokines (i.e., IL-1) was also supported by the inability of IL-1Ra to inhibit AFA-positive IgG cell internalization, which was in turn found to be closely associated with

fibroblast activation (37). Thus, these data indicate that AFA-positive IgG specifically induce ICAM-1 expression on fibroblasts and enhance mononuclear cell adhesion to fibroblasts.

Induction of proinflammatory cytokines by AFAs in fibroblasts. The enhanced ICAM-1 expression on fibroblasts treated with AFA-positive serum IgG suggested enhanced production of various mediators involved in inflammation. To test this hypothesis, fibroblast monolayers were incubated in the presence of SSc AFA-positive or control IgG, and levels of mRNA for IL-1α, IL-1β, and IL-6 were determined by RPA. Compared with steady-state mRNA levels, measured in medium alone, up-regulation of IL-1α, IL-1β, and IL-6 mRNA was observed when fibroblasts were treated with AFA-positive SSc IgG, but not with control IgG (Figure 6). Maximal up-regulation was observed when fibroblasts were treated with IgG for 4 hours; values returned to baseline at 24 hours. Interestingly, M-CSF mRNA, detectable at baseline, was unaffected after both 4 hours and 24 hours of treatment with SSc IgG (Figure 6). Thus, SSc IgG selectively and specifically enhanced levels of IL-1α, IL-1β, and IL-6 mRNA.

To further document the potential of SSc IgG to
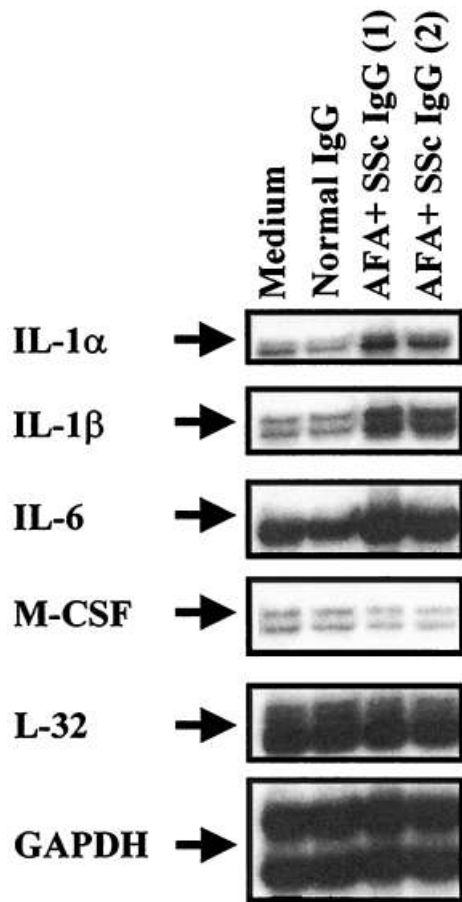
Figure 6. Up-regulation of steady-state levels of mRNA for proinflammatory cytokines induced in fibroblasts by AFA-positive SSc IgG. Lung fibroblasts were cultured in the presence of 100 μg/ml of IgG or medium alone for 4 hours. The RNase protection assay was performed as described in Patients and Methods. AFA-positive SSc IgG samples from 2 different patients were tested. M-CSF = macrophage colony-stimulating factor (see Figure 5 for other definitions).

up-regulate proinflammatory mediators, we tested their effect on IL-1α, IL-1β, and IL-6 production. When cultured in the presence of AFA-positive SSc IgG, human dermal fibroblasts released IL-6 in a dose-dependent manner (Figure 7). Half-maximal activity was observed at SSc IgG concentrations ranging from 3.1 μg/ml to 12.5 μg/ml for the 3 IgG preparations tested. Background levels were obtained in the presence of both NHS and AFA-negative SSc IgG. Under the same culture conditions, IL-1α and IL-1β proteins were consistently not detected above the sensitivity threshold (15.6 pg/ml).

Inhibition of AFA-induced up-regulation of ICAM-1 on fibroblasts by IL-1Ra but not by anti–IL-6. Finally, we tested whether autocrine effects of cytokines produced by SSc IgG–activated fibroblasts would account for ICAM-1 up-regulation or whether this was due to a direct effect of SSc IgG. When IL-1Ra was added to fibroblast cultures, ICAM-1 up-regulation induced by SSc AFA-positive IgG was completely reversed, in a dose-dependent manner (Figure 8). In contrast, the use of anti–IL-6 mAb as a neutralizing reagent in similar experiments resulted in marginal inhibition (27%; data not shown).

These results indicate that SSc IgG binding to the surface of fibroblasts induces the production of cytokines that, in an autocrine manner, up-regulate ICAM-1. IL-1 appears to play a major role in this autocrine up-regulation.

DISCUSSION

In systemic sclerosis and related conditions, the role of autoantibodies directed against fibroblasts has received little study, although fibroblasts play a major role in initiating and/or maintaining excessive ECM deposition. The findings of the present study demonstrate that antibodies reacting with the cell membrane of normal dermal and lung fibroblasts are present in a large proportion of sera from SSc patients. In addition, upon binding, these autoantibodies induce a proadhesive and proinflammatory function in normal fibroblasts.

To define the specificity in autoimmune settings, we tested sera from sarcoidosis patients with lung involvement, 10 of whom had established lung fibrosis. We found only 1 sarcoidosis serum to be AFA IgG positive, thus providing evidence that AFAs are not per se associated with pathologic conditions leading to fibrosis. In addition, cell-based ELISA revealed a higher prevalence of AFAs in patients with diffuse, compared with limited, SSc. This implies that the presence of AFAs is related to the severity of skin disease and suggests a possible role of these antibodies in SSc pathogenesis.

The presence in SSc sera of autoantibodies to membrane preparations of dermal fibroblasts has previously been documented to show differences in molecular species recognized, when fibroblasts were compared with endothelial cells (4). In a study using rat fibroblasts, all 33 SSc sera tested were found to be positive for surface staining, with differences in staining intensity when compared with controls (7). Another report mentions autoantibodies to a cell surface glycoprotein of 50 kd shared by fibroblasts and lymphocytes in 10% of
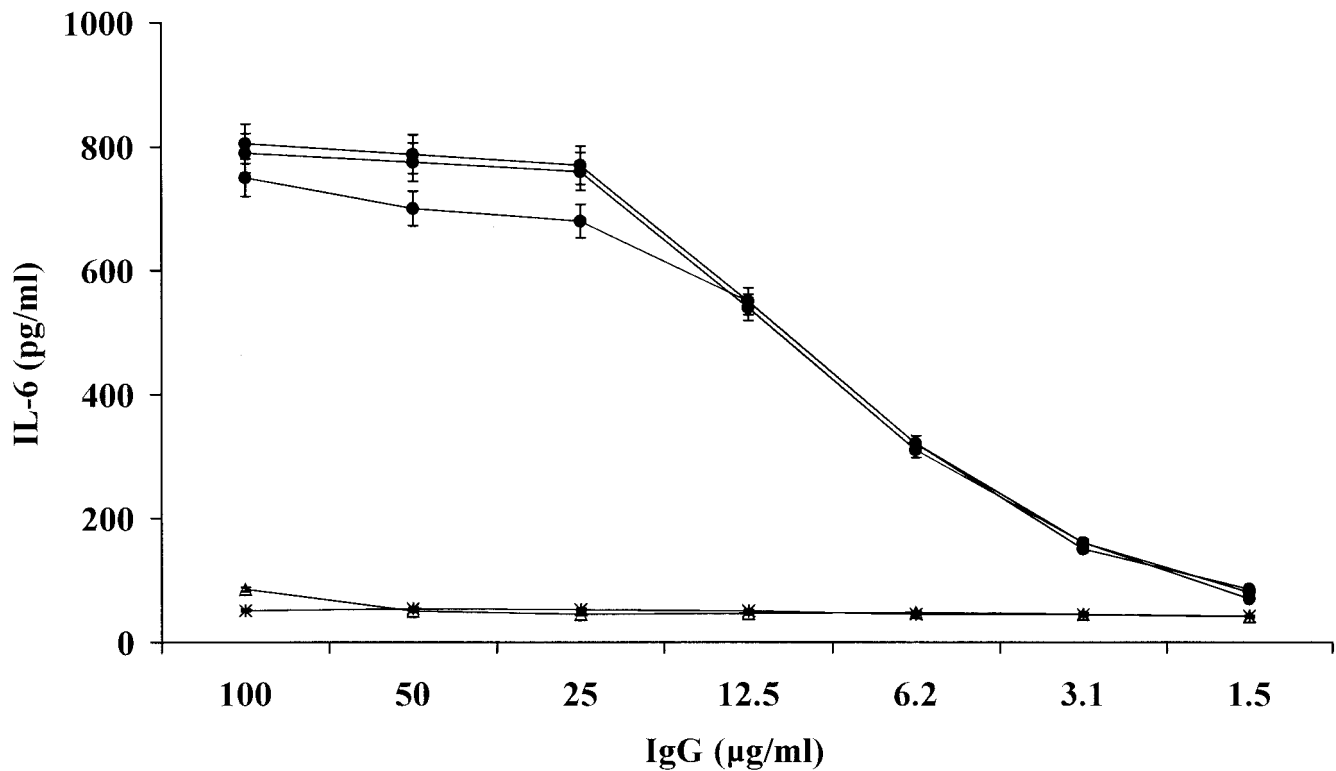
Figure 7. Induction of a proinflammatory phenotype (IL-6 production) in fibroblasts by AFA-positive SSc IgG (●) (n = 3). Negative controls were represented by AFA-negative (△) (n = 1) and NHS (*) (n = 1) IgG fractions. As a positive control, fibroblast monolayers incubated with 50 units/ml of recombinant human IL-1β secreted 815 ± 12 pg/ml of IL-6 (mean ± SD), while cells in medium alone gave background values (2 ± 0.3 pg/ml). Values are the mean ± SD from triplicate experiments. See Figure 5 for definitions.

patients with SSc; information on disease subset distribution was not provided (8). In our cross-sectional study, 58% of the sera were found to be positive for IgG AFA. Differences in the prevalence of positive sera between studies may be explained by differences in patient selection and/or in techniques used. It is quite possible that our cell-based ELISA may have selected for sera that had relatively high titers of autoantibodies. Indeed, consistent with a previous report (7), flow cytometry revealed positive binding of AFA-negative IgG that stained fibroblasts with lower intensity when compared with AFA-positive SSc IgG. Further, by confocal laser scanning microscopy, both control and AFA-positive SSc IgG stained the cell surface, but only AFA-positive SSc IgG were internalized (37).

We have not yet attempted to formally identify the antigens recognized by AFA-positive IgG. However, several lines of evidence suggest that the antigens recognized by these autoantibodies are indeed expressed on the surface of fibroblasts rather than in the nucleus. First, fluorescence microscopy examination findings

were indicative of surface staining. Second, for flow cytometric analysis, fibroblasts were not permeabilized and were kept at 4°C, making it unlikely that IgG had entered the cell and stained the nucleus. Third, some of the SSc sera that were positive for AFAs tested negative for the presence of antinuclear antibodies. However, as suggested by some authors, nuclear antigens could be exported to the fibroblast surface by exocytosis, ectocytosis, or during apoptotic processes and then recognized by autoantibodies (38,39). Nevertheless, antigen recognition by AFAs resulted in the up-regulation of mRNA for several cytokines as well as ICAM-1 expression and IL-6 production, all events that require metabolically active fibroblasts.

Interestingly, AFA-positive IgG up-regulated ICAM-1 expression, as well as IL-6 production, at protein concentrations lower than those resulting in maximal antibody binding to fibroblasts as detected by cell-based ELISA. Half-maximal binding ranged between 25 μg/ml and 50 μg/ml, while half-maximal IL-6 induction was observed at concentrations ranging from
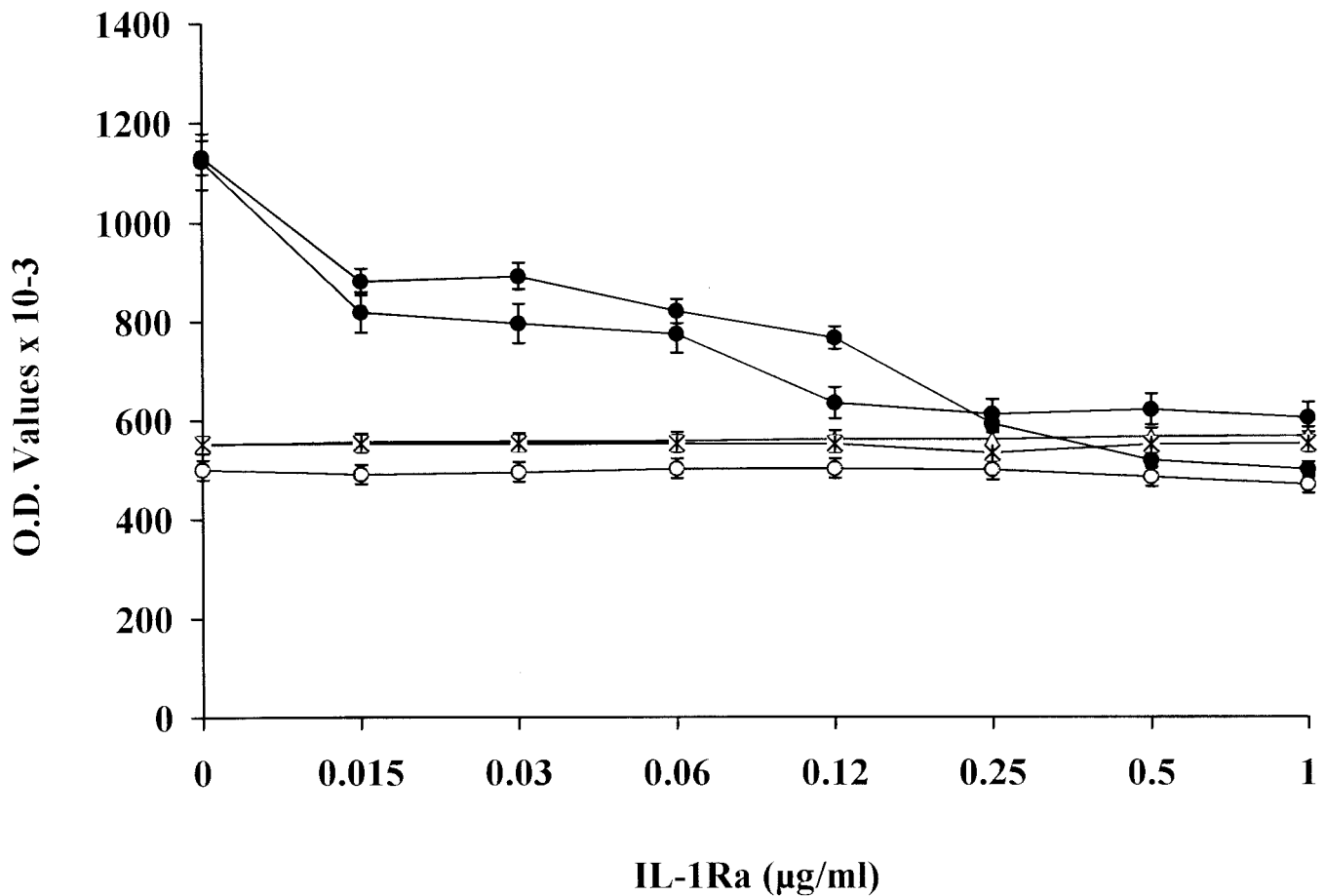
Figure 8. Dose-dependent abrogation, by IL-1 receptor antagonist (IL-1Ra), of ICAM-1 up-regulation induced by AFA-positive (●) (n = 2) SSc IgG. As negative controls, fibroblasts were incubated with AFA-negative (△) (n = 1) or NHS (*) (n = 1) IgG fractions or medium alone (○). Values are the mean ± SD from triplicate experiments. See Figure 5 for other definitions.

3.1 μg/ml to 12.5 μg/ml of IgG (see Figures 2, 5B, and 7). A difference in sensitivity between the 2 assays may account for the different results. Alternatively, fibroblast activation may require minute amounts of agonistic antibody, lower than those usually detected by immunohistochemical techniques. Further, relatively little is known about the actual concentration of IgG in interstitial fluids, particularly in tissues undergoing fibrosis. However, it can be estimated that under dynamic conditions, interstitial IgG may reach concentrations at least 50% of those present in the intravascular compartment (40,41). If this were the case, the concentration of IgG we found to be able to induce fibroblast activation would fall within the physiologic range of IgG diffusing in the interstitium.

In response to AFA-positive SSc IgG, but not to control IgG or AFA-negative SSc IgG, normal fibroblasts up-regulated ICAM-1 expression and bound higher numbers of U937, a monocytic cell line which expresses lymphocyte function–associated antigen 1, the counterligand of ICAM-1 (35). In SSc, particularly in the early inflammatory phases of the disease, mononuclear cells, i.e., both T cells and monocyte/macrophages, are found in perivascular areas and mixed with fibroblasts (23,42,43). Interestingly, fibroblasts showing enhanced collagen synthesis are surrounded by and in close contact with mononuclear cells, a finding consistent with the hypothesis that immune cells may be crucial to the initial activation of connective tissue metabolism in fibrosis (44,45). Thus, by enhancing the expression of adhesion molecules, AFAs may contribute to events leading to increased collagen synthesis.

In addition to ICAM-1 up-regulation, AFA-positive SSc IgG induced up-regulation of the steady-

state levels of mRNA for IL-1α, IL-1β, and IL-6, while levels of mRNA for M-CSF were unaffected. This indicates that the signaling pathways triggered in fibroblasts by AFA recognition have the capacity for broad, although selective, mRNA up-regulation. This is also consistent with the possibility that initially a single or limited number of mediators may be induced, which then activate secondary pathways. Indeed, as discussed below, IL-1 appears to play a dominant autocrine role in fibroblast activation by AFAs. However, the fact that levels of mRNA for IL-1α, IL-1β, and IL-6 were synchronously up-regulated within 4 hours upon AFA binding and returned to basal levels by 24 hours is consistent with the hypothesis that AFA initiated signaling pathways, leading to the simultaneous activation of several genes. This is reminiscent of the capacity of antiendothelial antibody to similarly activate, in vitro, endothelial cells in SSc and other autoimmune diseases (32,35,46).

A further analogy is that in our experiments with fibroblasts, as well as previous experiments with endothelial cells, IL-1 appears to be a relevant endogenous mediator acting in an autocrine manner (46). Indeed, IL-1Ra (but not anti–IL-6) inhibited, in a dose-dependent manner, the capacity of AFA-positive SSc IgG to up-regulate ICAM-1 expression. While we were unable to consistently detect IL-1α and IL-1β protein in fibroblast supernatants cultured in the presence of AFA-positive SSc IgG, the up-regulation of IL-1α and IL-1β steady-state mRNA levels observed in parallel cultures would account for the inhibitory activity of IL-1Ra. Thus, in parallel with a recent report showing that IL-1α acts in an autocrine loop to mediate human fibroblast activation induced by MCP-1 (47), our findings indicate that IL-1 is involved in ICAM-1 up-regulation induced by AFA-positive SSc IgG.

In conclusion, this is the first reported description of antifibroblast autoantibodies that, upon binding to the cell surface, induce fibroblast activation, leading to enhanced production of proinflammatory cytokines and up-regulation of ICAM-1. Whether these properties play an important role by triggering or maintaining the recruitment of inflammatory cells in tissues undergoing fibrosis, and whether they promote, directly or indirectly, a profibrotic phenotype on fibroblasts remains to be documented. Longitudinal clinical and serologic studies as well as in vitro studies aimed at elucidating the impact of these autoantibodies on the synthesis and digestion of extracellular matrix components will provide answers to these important questions.

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