

ORIGINAL ARTICLE

Stimulatory Autoantibodies to the PDGF Receptor in Systemic Sclerosis

Silvia Svegliati Baroni, Ph.D., Mariarosaria Santillo, Ph.D., Federica Bevilacqua, M.D., Michele Luchetti, M.D., Tatiana Spadoni, B.Sc., Matteo Mancini, B.Sc., Paolo Fraticelli, M.D., Paola Sambo, M.D., Ada Funaro, Ph.D., Andrius Kazlauskas, Ph.D., Enrico V. Avvedimento, M.D., Ph.D., and Armando Gabrielli, M.D.

ABSTRACT

BACKGROUND

Systemic sclerosis (scleroderma) is characterized by immunologic abnormalities, injury of endothelial cells, and tissue fibrosis. Abnormal oxidative stress has been documented in scleroderma and linked to fibroblast activation. Since platelet-derived growth factor (PDGF) stimulates the production of reactive oxygen species (ROS) and since IgG from patients with scleroderma reacts with human fibroblasts, we tested the hypothesis that patients with scleroderma have serum autoantibodies that stimulate the PDGF receptor (PDGFR), activating collagen-gene expression.

METHODS

We analyzed serum from 46 patients with scleroderma and 75 controls, including patients with other autoimmune diseases, for stimulatory autoantibodies to PDGFR by measuring the production of ROS produced by the incubation of purified IgG with mouse-embryo fibroblasts carrying inactive copies of PDGFR α or β chains or the same cells expressing PDGFR α or β . Generation of ROS was assayed with and without specific PDGFR inhibitors. Antibodies were characterized by immunoprecipitation, immunoblotting, and absorption experiments.

RESULTS

Stimulatory antibodies to the PDGFR were found in all the patients with scleroderma. The antibodies recognized native PDGFR, inducing tyrosine phosphorylation and ROS accumulation. Autoantibody activity was abolished by preincubation with cells expressing the PDGFR α chain or with recombinant PDGFR or by PDGFR tyrosine kinase inhibitors. Stimulatory PDGFR antibodies selectively induced the Ha-Ras-ERK1/2 and ROS cascades and stimulated type I collagen-gene expression and myofibroblast phenotype conversion in normal human primary fibroblasts.

CONCLUSIONS

Stimulatory autoantibodies against PDGFR appear to be a specific hallmark of scleroderma. Their biologic activity on fibroblasts strongly suggests that they have a causal role in the pathogenesis of the disease.

From the Dipartimento di Scienze Mediche e Chirurgiche, Sezione di Clinica Medica, Università Politecnica delle Marche, Ancona (S.S.B., F.B., M.L., T.S., M.M., P.F., P.S., A.G.); the Sezione di Fisiologia (M.S.) and the Dipartimento di Biologia e Patologia Molecolare e Cellulare, Centro di Endocrinologia ed Oncologia Sperimentale del CNR (E.V.A.), Università Federico II, Naples; the Dipartimento di Genetica, Biologia e Biochimica and the Research Center for Experimental Medicine, Università di Torino, Turin (A.F.) — all in Italy; and Schepens Eye Research Institute, Harvard Medical School, Boston (A.K.). Address reprint requests to Dr. Gabrielli at the Dipartimento di Scienze Mediche e Chirurgiche—Università Politecnica delle Marche, Polo Didattico, Via Tronto 10, 60020 Ancona, Italy, or at a.gabrielli@univpm.it, or to Dr. Avvedimento at the Dipartimento di Biologia e Patologia Molecolare e Cellulare, Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Università Federico II, Via Pansini 5, 80131 Napoli, Italy, or at avvedim@unina.it.

N Engl J Med 2006;354:2667-76.
Copyright © 2006 Massachusetts Medical Society.

SYSTEMIC SCLEROSIS (SCLERODERMA) IS A disorder characterized by fibrosis of the skin and visceral organs.¹ Although some features of the scleroderma phenotype are well established and represent the hallmarks of the disease, the primary cause has not been fully delineated; endothelial-cell damage, immunologic abnormalities, and excessive extracellular-matrix production are well documented associations.² The scleroderma phenotype at the cellular level is characterized by oxidative stress associated with the accumulation of large amounts of reactive oxygen species (ROS) in fibroblasts.³⁻⁶ ROS are key cell transducers of fibroblast proliferation⁷ and collagen-gene expression.^{7,8} We recently observed that a pathway linking the signaling proteins Ha-Ras, growth-factor-activated extracellular-signal-regulated kinases 1 and 2 (ERK1/2), and ROS is amplified in fibroblasts from patients with scleroderma.⁸

Since platelet-derived growth factor (PDGF) can induce ROS and Ras-ERK1/2 signaling^{8,9} and since IgG derived from patients with scleroderma reacts with human fibroblasts,¹⁰ we sought Ha-Ras, ERK1/2, and ROS stimulatory molecules in the serum of patients with scleroderma. We hypothesized that serum from patients with scleroderma may contain stimulatory IgG autoantibodies directed toward the PDGF receptor (PDGFR). These autoantibodies may trigger PDGFR, which induces ROS by way of Ha-Ras and ERK1/2 signaling. This cascade is thought to be ultimately responsible for fibroblast activation, which represents a distinctive feature of scleroderma.

METHODS

PATIENTS

Forty-six consecutive white patients with scleroderma (8 men and 38 women; median age, 58 years [range, 35 to 77]) were studied.¹¹ We also included 10 recipients of allogeneic bone marrow transplants who presented with graft-versus-host disease and scleroderma-like skin lesions. (Additional information is provided in the Supplementary Appendix, available with the full text of this article at www.nejm.org.) The patients were classified into subgroups according to whether they had diffuse scleroderma or limited scleroderma¹² and then were divided into those with early or late disease, as previously described.¹³ At the time of the current investigation, these patients had

not received any immunosuppressive treatment for the previous six weeks.

Control groups included 20 age-, sex-, and race-matched healthy volunteers, 14 patients with systemic lupus erythematosus, 15 patients with rheumatoid arthritis, 15 patients with primary Raynaud's phenomenon, and 10 patients with idiopathic pulmonary fibrosis. The diagnosis in each control group was made according to established criteria for each condition.¹⁴⁻¹⁷ The demographic and clinical characteristics of the study groups are presented in Table 1.

The study was approved by the institutional ethics committee (Università Politecnica delle Marche, Ancona, Italy). After oral and written informed consent had been obtained, a blood sample was taken from the patients and controls (after acclimatization at 21°C for 30 minutes), and the sample was spun in a refrigerated centrifuge after clot formation. The supernatants were collected and stored at -30°C until they were assayed, usually within four weeks.

BIOASSAY FOR ANTI-PDGFR AUTOANTIBODIES

The serum samples from patients with scleroderma and from controls were tested for the presence of PDGFR-activating autoantibodies in a functional bioassay. In brief, mouse-embryo fibroblasts expressing PDGFR α subunits, β subunits, or both¹⁸ (F α , F β , and F $\alpha\beta$) were exposed in vitro to immunopurified IgG. Mouse F-/- cells, derived from PDGFR-knockout embryos and devoid of PDGFR, were used as control cells (as described in the Supplementary Appendix). Cells were plated in duplicate at a density of 20,000 per 1.83-cm² well, cultured for 24 hours at 37°C in 0.2 percent fetal-calf serum, and incubated in the presence of 1 ml of immunopurified IgG (200 μ g per milliliter) from a patient or a normal control for 15 minutes at 37°C before ROS production was determined.

Fluorimetric determination of intracellular ROS generated by adherent fibroblasts was carried out after the cells had been loaded with 2',7'-dichlorofluorescein diacetate (DCF) (10 μ M, Molecular Probes) as previously described.⁸ Each IgG sample was tested in duplicate. The results were expressed as a stimulation index, which corresponds to $(S-C) \div (P-C)$, where S is the DCF fluorescence intensity of the test IgG, C is the DCF fluorescence intensity of a negative control obtained by culturing cells without IgG, and P is the DCF fluorescence intensity of a positive control

obtained by incubating cells with PDGF (15 ng per milliliter for 15 minutes). The intraplate variation was less than 3 percent. The samples were recorded as positive if the stimulation index was greater than the 95th percentile among the normal controls. In some experiments, ROS generation was evaluated in cells exposed to several inhibitors: the PDGFR tyrosine kinase inhibitor (AG 1296; 2 μ M for two hours), the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (AG 1478; 2 μ M for two hours), a chemical inhibitor of ERK1/2 signaling (PD 98059; 40 μ M for two hours), and a farnesyl transferase inhibitor, which inhibits Ras farnesylation (FTI-277; 20 μ M for two hours) (Calbiochem).

STATISTICAL ANALYSIS

The stimulation index is expressed as a median value and a range. Comparisons of the levels of anti-PDGFR autoantibodies between the group with scleroderma and the control groups were performed with the nonparametric Kruskal–Wallis test. Other data are expressed as means \pm SE. Mean values were compared with the use of Student’s paired t-test. All reported P values are two-sided. Data were analyzed with the use of SAS software (SAS Institute).

ies targeting PDGFR were present in patients with scleroderma. To test this hypothesis and to eliminate the confounding effect of PDGF or other cytokines present in serum, we purified total IgG from patients with scleroderma and determined its biologic activity. As target cells we used a mouse-embryo cell line carrying inactive copies of PDGFR α and β subunits as a reference, as well as the same line of cells expressing recombinant and functional PDGFR α or β subunits (F α , F β , and F $\alpha\beta$). These cells were exposed to the IgG fractions isolated from patients’ serum specimens. To determine ROS levels, cells were starved and incubated with the peroxide-sensitive fluorophore DCF before treatment with purified IgG.

IgG from patients with scleroderma stimulated ROS production in F α , F β , and F $\alpha\beta$ cells in a dose-dependent manner. ROS rapidly increased to the maximum level 15 minutes after IgG exposure and returned to baseline 40 to 120 minutes later. The best discrimination in ROS-stimulation activity between normal IgG and IgG from patients with systemic sclerosis was obtained in F α cells and with IgG at a concentration of 200 μ g per milliliter. These conditions were followed in all subsequent experiments unless otherwise specified.

RESULTS

Since PDGF induces the accumulation of ROS,^{8,9} we hypothesized that stimulatory serum antibody

IgG FROM PATIENTS WITH SCLERODERMA

Induction of ROS and Reaction with PDGF Receptor Figure 1A indicates that the levels of ROS induced by IgG from patients with scleroderma (at a con-

Table 1. Demographic Characteristics of the Patients and Controls.*

Characteristic	Systemic Sclerosis (N=46)†	Primary Raynaud’s Phenomenon (N=15)	Systemic Lupus Erythematosus (N=14)	Rheumatoid Arthritis (N=15)	Idiopathic Pulmonary Fibrosis (N=10)
Male sex (no.)	8	2	1	2	6
Age (yr)					
Median	58	42	36	65	64
Range	35–77	22–70	26–50	22–91	56–79
Duration of Raynaud’s phenomenon (yr)			NA	NA	NA
Median	14	6			
Range	2–50	2–25			
Duration of disease (yr)					
Median	7	6	7	11	0.5
Range	1–48	2–25	1–23	1–28	0–4

* NA denotes not applicable.

† Of the 46 patients with systemic sclerosis (scleroderma), 24 had limited disease and 22 diffuse disease. Nine of the patients with systemic sclerosis had anticentromere antibodies, and 26 had anti-Scl-70 antibodies.

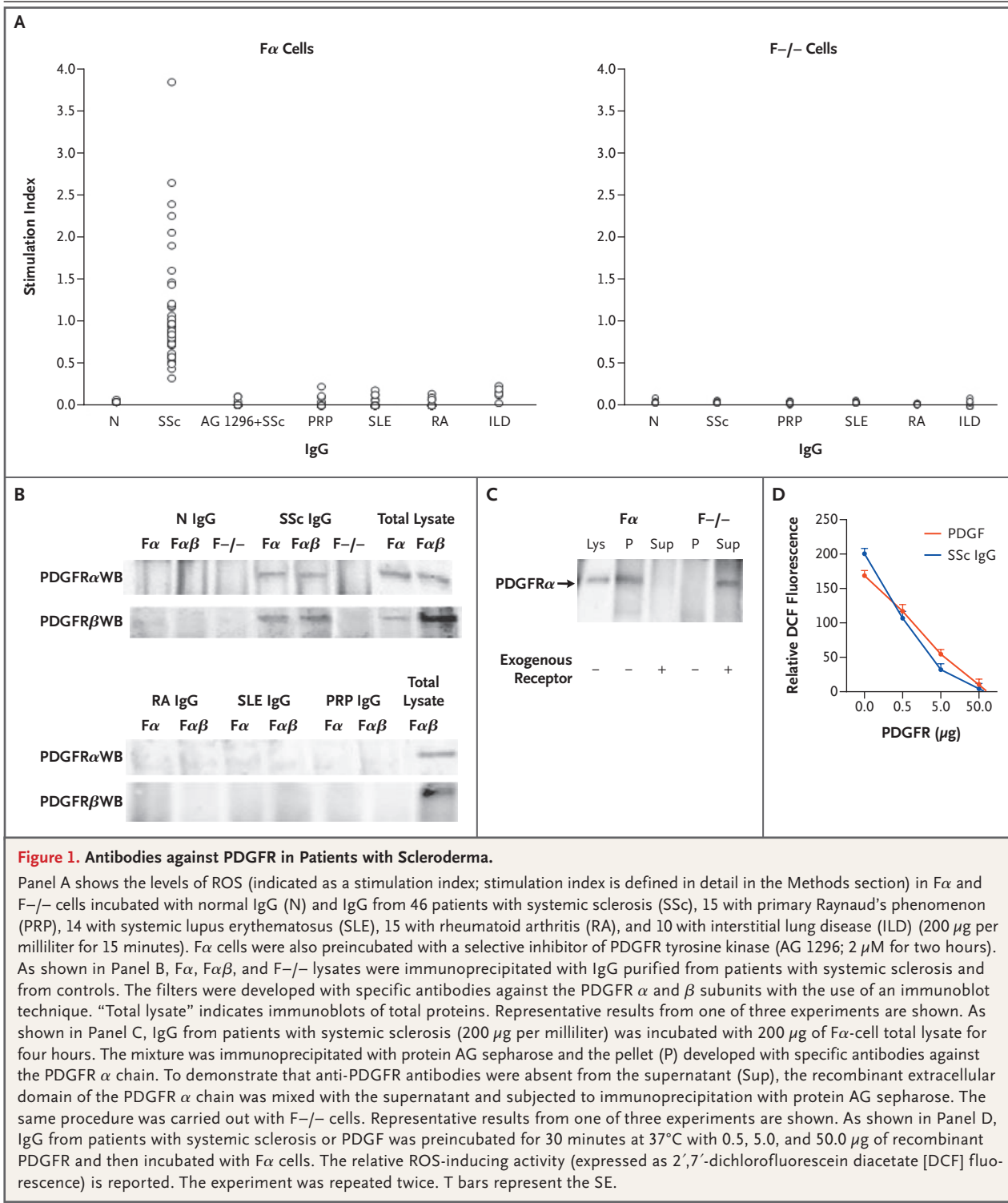


Figure 1. Antibodies against PDGFR in Patients with Scleroderma.

Panel A shows the levels of ROS (indicated as a stimulation index; stimulation index is defined in detail in the Methods section) in F α and F $-/-$ cells incubated with normal IgG (N) and IgG from 46 patients with systemic sclerosis (SSc), 15 with primary Raynaud's phenomenon (PRP), 14 with systemic lupus erythematosus (SLE), 15 with rheumatoid arthritis (RA), and 10 with interstitial lung disease (ILD) (200 μ g per milliliter for 15 minutes). F α cells were also preincubated with a selective inhibitor of PDGFR tyrosine kinase (AG 1296; 2 μ M for two hours). As shown in Panel B, F α , F $\alpha\beta$, and F $-/-$ lysates were immunoprecipitated with IgG purified from patients with systemic sclerosis and from controls. The filters were developed with specific antibodies against the PDGFR α and β subunits with the use of an immunoblot technique. "Total lysate" indicates immunoblots of total proteins. Representative results from one of three experiments are shown. As shown in Panel C, IgG from patients with systemic sclerosis (200 μ g per milliliter) was incubated with 200 μ g of F α -cell total lysate for four hours. The mixture was immunoprecipitated with protein AG sepharose and the pellet (P) developed with specific antibodies against the PDGFR α chain. To demonstrate that anti-PDGFR antibodies were absent from the supernatant (Sup), the recombinant extracellular domain of the PDGFR α chain was mixed with the supernatant and subjected to immunoprecipitation with protein AG sepharose. The same procedure was carried out with F $-/-$ cells. Representative results from one of three experiments are shown. As shown in Panel D, IgG from patients with systemic sclerosis or PDGF was preincubated for 30 minutes at 37°C with 0.5, 5.0, and 50.0 μ g of recombinant PDGFR and then incubated with F α cells. The relative ROS-inducing activity (expressed as 2',7'-dichlorofluorescein diacetate [DCF] fluorescence) is reported. The experiment was repeated twice. T bars represent the SE.

centration of 200 μ g per milliliter for 15 minutes per 20,000 cells), expressed as the stimulation index (median, 0.86; range, 0.31 to 3.82) were significantly higher than the levels of ROS generated by normal IgG (median, 0; range 0 to 0.02) or by IgG from patients with primary Raynaud's phenomenon (median, 0; range, 0 to 0.19), systemic lupus erythematosus (median, 0; range, 0 to

0.22), rheumatoid arthritis (median, 0; range, 0 to 0.13), or interstitial lung disease (median, 0; range, 0 to 0.17) ($P < 0.001$ for all comparisons). Using the 99th percentile as the upper limit of normal, antibodies stimulating ROS production were found in all patients with scleroderma and in none of the controls (Fig. 1A).

To prove that ROS-inducing activity was mediated by the activation of PDGFR, we performed several additional experiments. First, the ROS accumulation induced in $F\alpha$ cells exposed to sclerodermal IgG was inhibited by preincubating the cells with the PDGFR tyrosine kinase inhibitor AG 1296 (Fig. 1A). Sclerodermal IgG did not stimulate ROS in $F-/-$ cells (Fig. 1A). In addition, sclerodermal IgG, and not IgG from controls, immunoprecipitated the PDGFR α and β subunits (Fig. 1B). PDGFR-interacting antibodies in sclerodermal IgG were completely removed by preabsorption with $F\alpha$ cells but not $F-/-$ cells (Fig. 1C).

Moreover, the supernatant after absorption with $F\alpha$ cells did not stimulate ROS production (data not shown). Conversely, when IgG from patients with scleroderma was incubated with $F-/-$ cells, PDGFR-interacting antibodies were not removed (Fig. 1C). Recombinant PDGFR receptor, preincubated with sclerodermal IgG, inhibited its ROS-inducing activity completely and in a dose-dependent manner (Fig. 1D).

To purify these antibodies, we immortalized the lymphocytes derived from two randomly selected patients with scleroderma and screened isolated clones for the presence of ROS-inducing antibodies in the cell system shown in Figure 1A. Figure 2A shows the results of fluorescence-activated cell-sorting analysis of two of these antibodies purified from lymphocytes from the same patient. These antibodies reacted with $F\alpha$ cells but not with $F-/-$ cells (Fig. 2A). They also stimulated ROS production in $F\alpha$ cells but not in

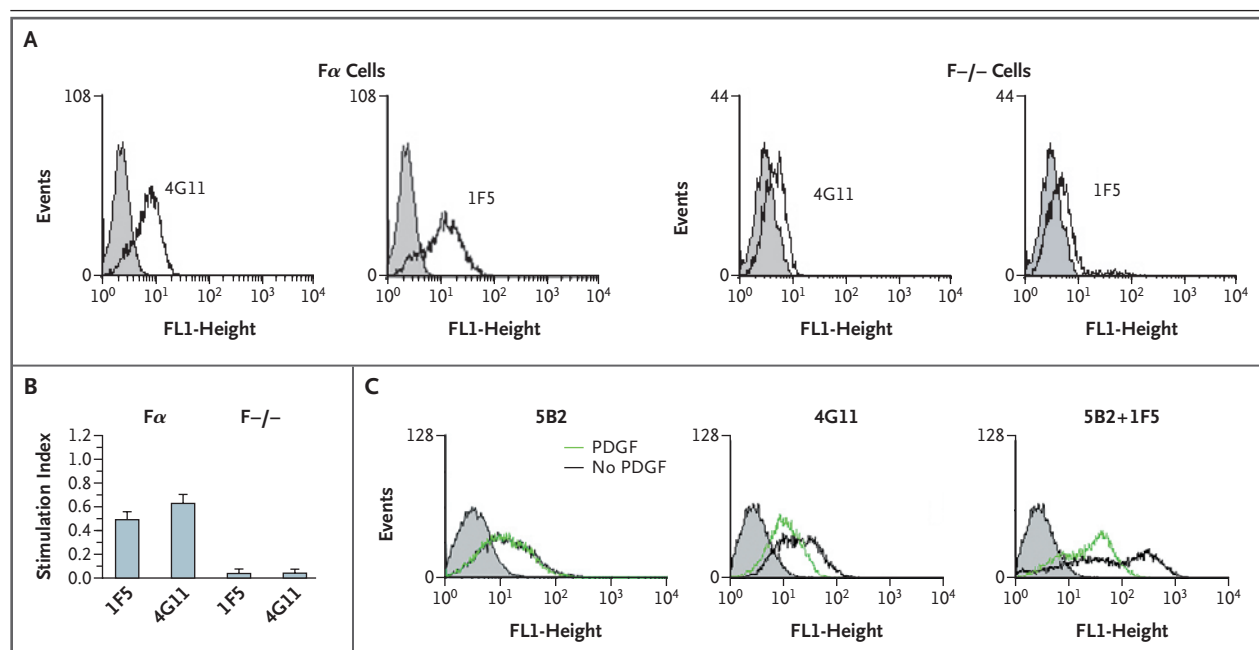


Figure 2. Isolation of Stimulatory Antibodies Derived from Patients with Scleroderma.

As shown in Panel A, $F\alpha$ and $F-/-$ cells were incubated with medium (0.1 ml) obtained from clones 4G11 and 1F5 before fluorescence-activated cell-sorting analysis. These clones were obtained by immortalizing lymphocytes from patients with scleroderma with Epstein-Barr virus. Shaded profiles are those of the isotype-negative control antibody. The results are representative of seven experiments with clones from two patients with scleroderma. The concentration of IgM was approximately 1 to 2 μg per milliliter. Panel B shows the levels of ROS (expressed as the stimulation index) in $F\alpha$ and $F-/-$ cells incubated with 1 ml of medium obtained from clones 4G11 and 1F5. Results are the means of the results of three experiments. T bars represent the SE. The concentration of IgM was approximately 1 to 2 μg per milliliter. As shown in Panel C, $F\alpha$ cells were starved for 48 hours in 0.2 percent fetal-calf serum, trypsinized, and incubated with PDGF (green) and without PDGF (black) (at 15 ng per milliliter for 30 minutes at 4°C) before incubation with 100 μl of medium from clone 5B2, which reacts with $F-/-$ cells and does not induce ROS, and from ROS-inducing clones (4G11 and 1F5). Shaded profiles are those indicated in Panel A. The concentration of IgM was approximately 1 to 2 μg per milliliter.

F-/- cells (Fig. 2B). PDGF could competitively inhibit the binding to F α cells (Fig. 2C).

With respect to the relation between the ROS-inducing activity of sclerodermal IgG and the patients' clinical features, we found that activity (measured as ROS induction) was higher in patients with early, limited scleroderma (duration, less than 5 years) than in patients with late disease (duration, more than 10 years) ($P < 0.01$). In the subgroup of patients with scleroderma whose disease was diffuse, there was a trend toward higher values among patients with early disease (less than three years) than among patients with disease of more than six years' duration. The difference, however, was not statistically significant, possibly because of the small number of patients (four) with early diffuse disease. No correlation was found with other clinical and serologic features. Recently, we found similar agonistic autoantibodies in 10 recipients of allogeneic bone marrow transplants who presented with graft-versus-host disease and had scleroderma-like skin lesions (unpublished data).

Triggering of the Ha-Ras-ERK1/2-ROS Cascade in Normal Fibroblasts

To dissect the signaling cascade triggered by sclerodermal IgG, we analyzed the ROS-generating activity of IgG from three patients with scleroderma as compared with three normal controls in the presence of specific inhibitors: inhibitors of EGFR and PDGFR signaling (AG 1478 and AG 1296, respectively); FTI-277, an inhibitor of farnesyl transferase, an enzyme required for Ras attachment to the plasma membrane; and a MEK inhibitor, PD 98059, which is a kinase located upstream of ERK1/2. Figure 3A shows that inhibitors of PDGFR (not EGFR), Ras, and MEK prevented ROS induction by sclerodermal IgG in normal fibroblasts ($P < 0.001$ for the comparison with sclerodermal IgG alone). Another feature of PDGFR activation is the stabilization of Ha-Ras protein. We recently found that Ha-Ras protein levels are regulated by PDGF, ROS, and ERK1/2 and that low levels are maintained by proteasome degradation.⁸ Sclerodermal IgG stimulated Ha-Ras levels, and this increase was abolished by inhibiting PDGFR, as demonstrated by immunofluorescence and immunoblot analysis (Fig. 3B). These data point to PDGFR as the primary target of scleroderma.

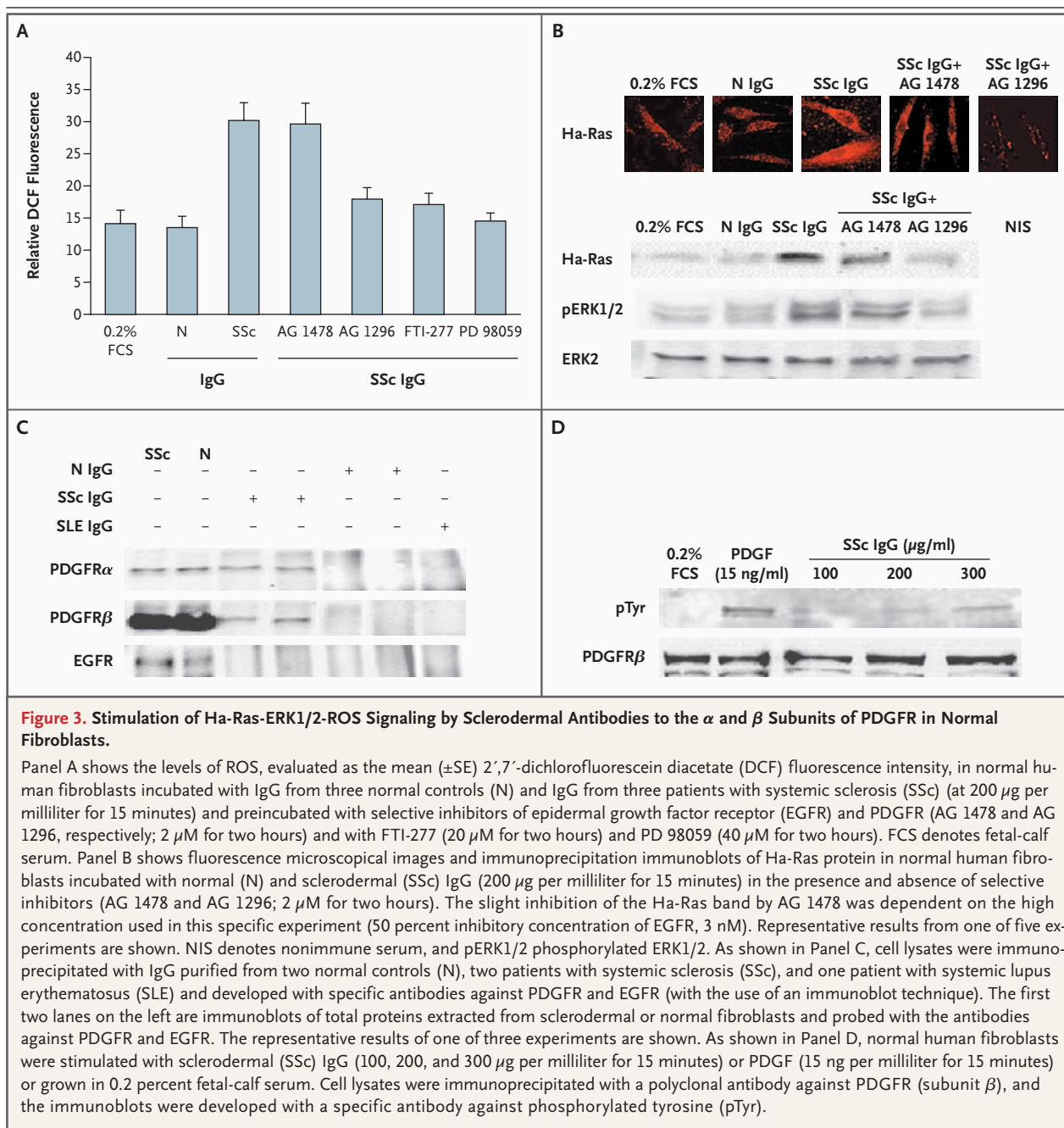
To determine more precisely the site of action of sclerodermal IgG, we immunoprecipitated to-

tal cellular proteins with IgG from two patients with scleroderma, one patient with systemic lupus erythematosus, and two normal controls at the same protein concentration (200 μ g per milliliter). Figure 3C shows the immunoblot with commercial anti-PDGFR α , anti-PDGFR β , or anti-EGFR antibodies. Sclerodermal IgG efficiently immunoprecipitated the PDGFR α and β subunits but not EGFR (Fig. 3C). We noticed that IgG isolated from the serum of 10 tested patients with scleroderma did not recognize recombinant α and β subunits of PDGFR on Western blotting, indicating that these antibodies recognize conformations present only in the native receptor (data not shown).

To delineate the stimulatory nature of the antibody-receptor interaction, we challenged normal fibroblasts for 15 minutes with increasing concentrations of IgG derived from a patient with scleroderma and evaluated the tyrosine phosphorylation of the PDGFR. Sclerodermal IgG induced tyrosine phosphorylation of the PDGFR in a dose-dependent manner (Fig. 3D). The time course of tyrosine phosphorylation was longer than that induced by PDGF (data not shown).

BIOLOGIC CONSEQUENCES OF ANTI-PDGFR AGONISTIC ANTIBODIES FROM PATIENTS WITH SCLERODERMA

To determine the biologic effects induced by sclerodermal IgG, we assayed the expression of two genes that characterize scleroderma fibroblasts — the genes encoding α -smooth-muscle actin and type I collagen — in normal human fibroblasts exposed to sclerodermal IgG. α -Smooth-muscle actin is a distinctive marker of myofibroblasts, which are mesenchymal cells that are derived from fibroblasts and that possess characteristics of both smooth-muscle cells and fibroblasts. α -Smooth-muscle actin was induced by sclerodermal IgG in normal human fibroblasts but not by normal IgG ($P < 0.001$), according to immunohistochemical assays and immunoblotting (Fig. 4A). Messenger ribonucleic acid encoding the type I collagen $\alpha 1$ and $\alpha 2$ chains was robustly induced by sclerodermal IgG and not by normal IgG, as shown by Northern blot analysis and real-time quantitative polymerase-chain-reaction analysis (Fig. 4B). In addition, the PDGFR tyrosine kinase inhibitor AG 1296 down-regulated expression of the type I collagen gene. To demonstrate that a single antibody was able to induce expression of the genes encoding collagen and α -smooth-



muscle actin, we tested purified clonal antibodies. These antibodies induced the expression of these genes with higher specific activity than did total sclerodermal IgG (1 to 2 μ g of protein per milliliter vs. 200 μ g per milliliter) (Fig. 1A and 1B of the Supplementary Appendix). Furthermore, these antibodies were long-lasting stimulators, since their induction of ROS persisted longer than ROS induction by PDGF (Fig. 2 of the Supplementary Appendix). A general scheme outlin-

ing the possible mechanism triggered by the autoantibodies to PDGFR is shown in Figure 5.

DISCUSSION

Five independent experiments in the current study document the presence of PDGFR stimulatory antibodies in the serum of patients with scleroderma. First, purified immunoglobulin fractions from patients with scleroderma induced ROS levels in cells

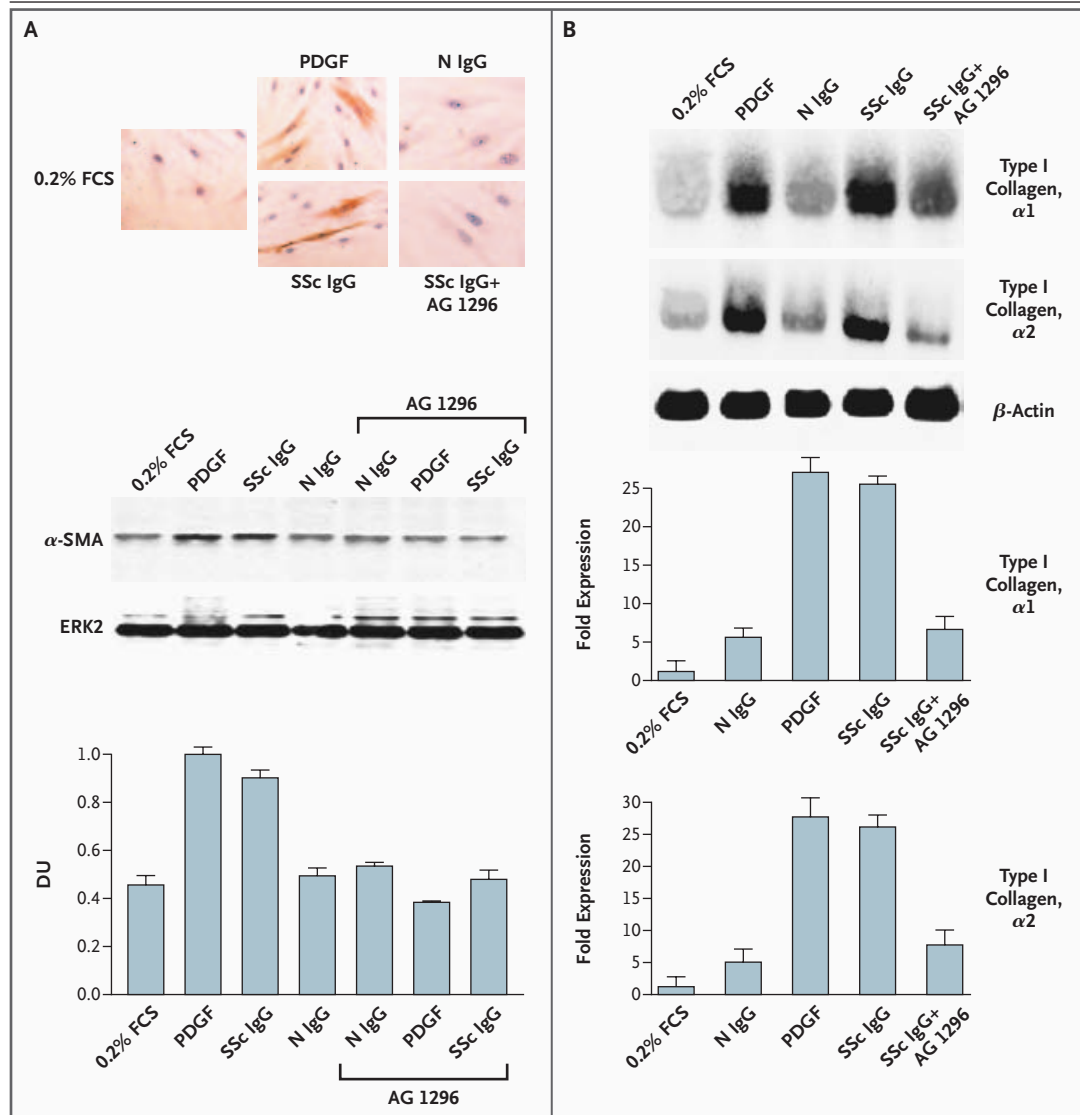


Figure 4. Induction of the Expression of Type I Collagen and α -Smooth-Muscle Actin by Autoantibodies against PDGFR.

Panel A (top) shows α -smooth-muscle actin (α -SMA) induction in normal human fibroblasts stimulated with PDGF (15 ng per milliliter for 30 minutes), normal (N) human IgG (200 μ g per milliliter for 30 minutes), and sclerodermal (SSc) IgG (200 μ g per milliliter for 30 minutes) in the presence and absence of AG 1296 (2 μ M for 1 hour before treatment). The experiment shown represents a typical blot obtained in three independent experiments. The results of densitometric analysis of three independent experiments are also shown in Panel A (bottom). The data shown represent the means (\pm SE) of the results of three independent experiments. FCS denotes fetal-calf serum. Panel B (top) shows type I collagen-gene expression by normal human fibroblasts after incubation with 0.2 percent fetal-calf serum (for 48 hours), PDGF (15 ng per milliliter for 15 minutes), normal (N) human IgG (200 μ g per milliliter for 15 minutes), and sclerodermal (SSc) IgG (200 μ g per milliliter for 15 minutes) in the presence and absence of AG 1296 (2 μ M for 1 hour before treatment). Northern blot analysis was used to detect messenger RNA encoding the α 1 and α 2 chains of type I collagen. Representative results from one of three experiments are shown. The results of real-time quantitative polymerase-chain-reaction analysis of the transcripts of genes encoding the α 1 and α 2 chains of type I collagen are also shown in Panel B (bottom).

expressing PDGFR but not in cells from which PDGFR was absent. Second, immunoglobulin fractions from patients with scleroderma recognized and immunoprecipitated PDGFR (α and β chains) in its native configuration. Third, PDGFR binding and the ROS-generating activities of sclerodermal IgG were removed by preadsorption to recombinant PDGFR or to PDGFR-expressing cells and not to cells without PDGF. Similarly, PDGF competitively inhibited the binding of purified antibodies to PDGFR-expressing cells. Fourth, immunoglobulin fractions or purified antibodies derived from patients with scleroderma induced myofibroblast conversion, type I collagen expression, and ROS production in normal fibroblasts. Finally, we detected these antibodies in all 46 tested patients with scleroderma and in none of the controls. The ROS-inducing activity of the sclerodermal immunoglobulins was inhibited by PDGFR inhibitors. These antibodies were not detected in patients with primary Raynaud's phenomenon, systemic lupus erythematosus, rheumatoid arthritis, or interstitial lung disease. The current *in vitro* data indicate that these antibodies recognize and activate PDGFR as a primary target. The data also might be taken to imply that fibrosis in scleroderma is triggered by the accumulation of these activating autoantibodies in the blood.

In dissecting the cascade initiated by PDGF *in vitro*, we recently observed that growth factors and ROS regulate Ras proteins in a seemingly novel manner.⁸ In normal primary cells, Ras proteins are maintained at low levels by continuous proteasomal degradation. In that setting, PDGF transiently induces ROS through ERK1/2 and ultimately prevents Ras degradation by proteasomes.⁸ Sclerodermal IgG activates PDGFR but replicates the action of PDGF only in part. We monitored the response of sclerodermal cells in culture under serum-deprived conditions. The cells maintained high ROS production, but after a day or two, the rate of ROS production slowly decreased. We hypothesize that antibodies to PDGFR remain in the membrane for a longer time than PDGF and generate a more persistent stimulus. Figure 5 is a schematic diagram of the pathway that we consider to be activated by antibodies by way of PDGFR.

We also analyzed the short-term and long-term biologic effects of sclerodermal IgG in normal cells — namely, ROS production and collagen-

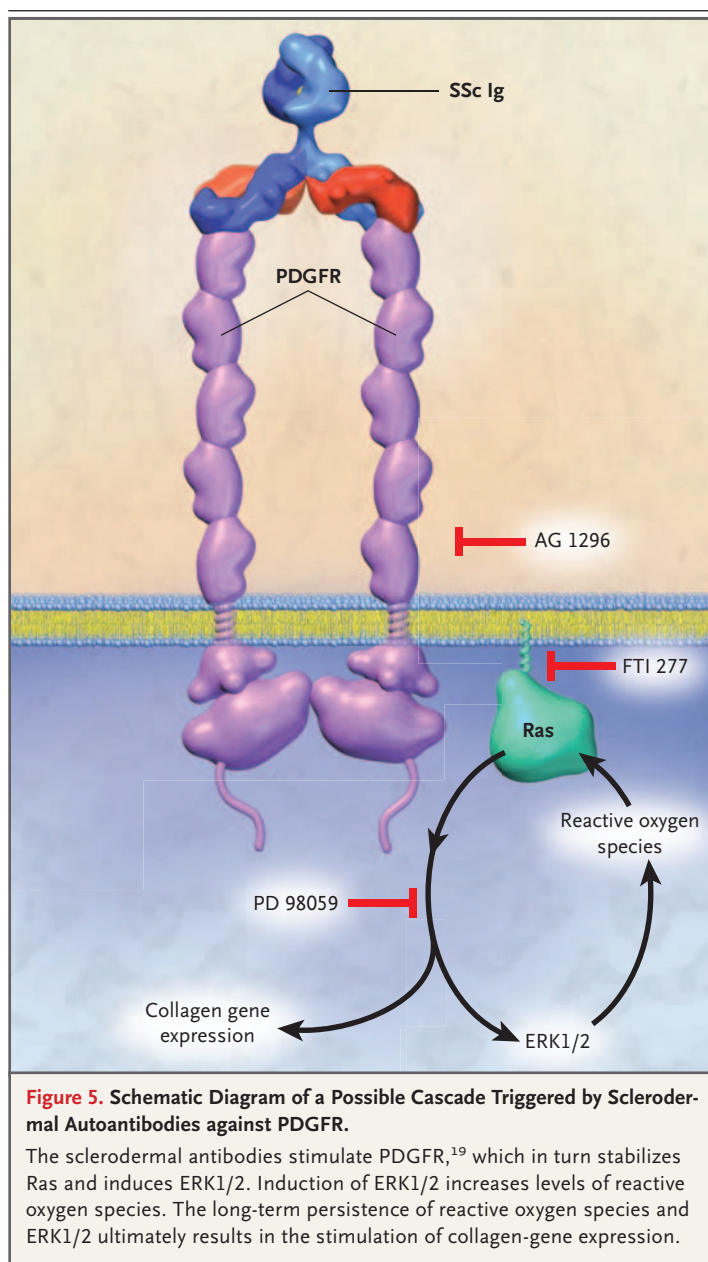


Figure 5. Schematic Diagram of a Possible Cascade Triggered by Sclerodermal Autoantibodies against PDGFR.

The sclerodermal antibodies stimulate PDGFR,¹⁹ which in turn stabilizes Ras and induces ERK1/2. Induction of ERK1/2 increases levels of reactive oxygen species. The long-term persistence of reactive oxygen species and ERK1/2 ultimately results in the stimulation of collagen-gene expression.

gene expression, respectively. The phenotypes we describe here closely replicate the features of sclerodermal fibroblasts. Activation of the transcription of collagen genes and the gene encoding α -smooth-muscle actin results in the appearance of myofibroblasts and fibrosis. We previously noted that fibroblasts derived from patients with scleroderma undergo rapid senescence and accumulate DNA and chromosomal aberrations⁸

— a process that may explain the loss of cells in chronic lesions. In addition, these data indicate that early, limited scleroderma (duration, less than 5 years) can be distinguished from late disease (duration, more than 10 years) on the basis of the ROS-inducing activity of antibodies. It remains to be seen whether ROS-inducing activity can be detected before the appearance of clinical symptoms.

In conclusion, we have identified agonistic antibodies against PDGFR in patients with scleroderma. These antibodies appear to trigger an intracellular loop that involves Ha-Ras, ERK1/2, and ROS and that leads to increased collagen-gene expression. The purification of anti-PDGFR ROS-stimulating antibodies and the results of tests of their biologic activity as purified clones strongly argue that these antibodies have a causal role in the pathogenesis of scleroderma. However, direct

proof awaits *in vivo* studies and validation in larger numbers of patients from other populations.

Supported in part by Società Italiana di Reumatologia, Associazione Italiana per la Lotta alla Sclerodermia, Ministero Italiano per l'Università e la Ricerca Scientifica (2003), Associazione Italiana Ricerca sul Cancro, and the Naples Oncogenomic Center, Fondazione Italiana Ricerca Cancro, Biotecnologie Avanzate, and by a grant (EY012509, to Dr. Kazlauskas) from the National Institutes of Health.

No potential conflict of interest relevant to this article was reported.

This article is dedicated to the memory of Dr. E. Carwile LeRoy and Stelio Varrone.

We are indebted to Dr. Marcello Melone (Dipartimento di Neuroscienze—Istituto di Fisiologia, Università Politecnica delle Marche, Ancona, Italy) for the help with confocal microscopy; to Prof. Pier Paolo Roggero (Dipartimento di Scienze Ambientali e delle Produzioni Vegetali—Università Politecnica delle Marche, Ancona) for help with statistical analysis; and to Drs. Stefano Gasparini and PierGiorgio Zitti (Dipartimento di Medicina Interna, Pneumologia ed Immunologia Clinica—Azienda Ospedali Riuniti, Ancona) for allowing us to study their patients with idiopathic pulmonary fibrosis.

REFERENCES

1. Black CM, Stephen C. Systemic sclerosis (scleroderma) and related disorders. In: Maddison PJ, Isemberg DA, Woo P, Glass DN, eds. Oxford textbook of rheumatology. Oxford, England: Oxford University Press, 1993:771-89.
2. Jimenez SA, Derk CT. Following the molecular pathways toward an understanding of the pathogenesis of systemic sclerosis. *Ann Intern Med* 2004;140:37-50.
3. Stein CM, Tanner SB, Awad JA, Roberts LJ II, Morrow JD. Evidence of free-radical-mediated injury (isoprostane overproduction) in scleroderma. *Arthritis Rheum* 1996;39:1146-50.
4. Bruckdorfer KR, Hillary JB, Bunce T, Vancheeswaran R, Black CM. Increased susceptibility to oxidation of low-density lipoproteins isolated from patients with systemic sclerosis. *Arthritis Rheum* 1995;38:1060-7.
5. Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implication for pathogenesis. *J Exp Med* 1997;185:71-9.
6. Sambo P, Jannino L, Candela M, et al. Monocytes of patients with systemic sclerosis (scleroderma) spontaneously release *in vitro* increased amounts of superoxide anion. *J Invest Dermatol* 1999;112:78-84.
7. Sambo P, Baroni SS, Paroncini P, Dusi S, Orlandini G, Gabrielli A. Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive upregulation of free radical generation through the NADPH oxidase complex pathway. *Arthritis Rheum* 2001;44:2653-64.
8. Svegliati S, Cancellò R, Sambo P, et al. Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2: amplification of ROS and Ras in systemic sclerosis fibroblasts. *J Biol Chem* 2005;280:36474-82.
9. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction. *Science* 1995;270:296-9.
10. Chizzolini C, Raschi E, Rezzonico R, et al. Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. *Arthritis Rheum* 2002;46:1602-13.
11. Masi AT, Rodnan GP, Medsger TA, et al. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980;23:581-90.
12. LeRoy EC, Black CM, Fleischmajer R, et al. Scleroderma (systemic sclerosis) classification, subsets and pathogenesis. *J Rheumatol* 1988;15:202-5.
13. Medsger TA Jr, Steen VD. Classification, prognosis. In: Clements PJ, Furst DE, eds. Systemic sclerosis. Baltimore: Williams & Wilkins, 1996:51-64.
14. LeRoy EC, Medsger TA. Raynaud's phenomenon: a proposal for classification. *Clin Exp Rheumatol* 1992;10:485-8.
15. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:1725-34.
16. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
17. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. *Am J Respir Crit Care Med* 2000;161:646-64.
18. Andrews A, Balciunaite E, Leong FL, et al. Platelet-derived growth factor plays a key role in proliferative vitreoretinopathy. *Invest Ophthalmol Vis Sci* 1999;40:2683-9.
19. Pollard TD, Earnshaw WC. Cell biology. Philadelphia: Saunders Elsevier, 2004:394.

Copyright © 2006 Massachusetts Medical Society.