

Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis

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Short running title: AOPP-Mediated Oxidative Stress in Scleroderma

ABSTRACT

Objectives: To investigate the role of reactive oxygen species (ROS) in the development of the various patterns of systemic sclerosis (SSc) and the mechanisms of ROS production by endothelial cells and fibroblasts.

Methods: Production of hydrogen peroxide (H_2O_2), nitric oxide (NO) and cellular proliferation were determined upon incubation of endothelial cells and fibroblasts with 56 SSc and 30 healthy sera. Correlations were established between those markers, the type and the severity of the clinical involvements, and the response to treatment. The factors leading to ROS production were determined.

Results: H_2O_2 production by endothelial cells and fibroblasts was higher after incubation with SSc sera than with normal sera ($p<0.001$) and with sera from SSc patients with severe complications than sera from other patients ($p<0.05$). Sera from patients with lung fibrosis triggered the proliferation of fibroblasts more than other SSc sera ($p<0.001$), whereas sera from patients with vascular complications exerted no proliferative effect on fibroblasts, but inhibited endothelial cell growth ($p<0.05$) and induced NO overproduction ($p<0.05$). Bosentan reduced NO release by 32% whereas N-acetylcystein potentiated 5-fluorouracil (5FU) to inhibit fibroblast proliferation by 78%. Those serum-mediated effects did not involve antibodies but advanced oxidation protein products that selectively triggered cells to produce H_2O_2 or NO.

Conclusions: SSc sera induce the production of different types of ROS that selectively activate endothelial cells or fibroblasts, leading to vascular or fibrotic complications. Assaying serum-induced ROS production allows to follow clinical activity of the disease and to select appropriate treatments.

Abbreviations: AOPP, advanced oxidation protein products; DAF2-DA, 4,5-diaminofluorescein diacetate; DI, digital ischemia; 5FU, 5-fluorouracil; H2-DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HC, healthy control(s); HUVEC, human umbilical venous endothelial cell; NAC, N-acetyl-L-cysteine; NO, nitric oxide; PAH, pulmonary arterial hypertension; ROS, reactive oxygen species; RS, restrictive syndrome; SSc, systemic sclerosis; VA, vascular abnormalities

Systemic sclerosis (SSc) is a connective tissue disorder of unknown etiology whose main features are vascular hyperreactivity and fibrosis of skin and visceral organs.^{1,2}

Actually, SSc is characterized by a large variety of clinical patterns. Although genetic and environmental factors are known to play a role in the initiation and progression of the disease, the mechanisms that determine the clinical manifestations remain unclear.³⁻⁵

Recent reports have suggested that an oxidative stress could be involved in the pathogenesis of SSc⁶⁻⁹, and a spontaneous production of reactive oxygen species (ROS) by skin fibroblasts from SSc patients has been observed.^{6,10}

Our aim was to determine whether serum factors can mediate an oxidative stress that activates endothelial cells and fibroblasts in SSc patients. The involved ROS and their respective effects on both types of cells have been characterized in order to determine the mechanisms that lead to a vascular or a fibrotic form of the disease. In addition, various drugs and antioxidant molecules have been tested for their counteracting effects on the oxidative stress.

PATIENTS AND METHODS

Patients and sera

Fifty-six SSc patients were enrolled in the study. The clinical and serological characteristics of these SSc patients are summarized in table 1.

Table 1 Clinical and serological characteristics of the 56 SSc patients	
	SSc Patients
Age-years	53 ± 15.9
Duration of SSc- years	8 ± 7.4
Duration of Raynaud syndrome- years	14 ± 14
Female sex-no. (%)	47 (83.9)
Diffuse SSc-no. (%)	39 (69.6)
Restrictive syndrome (RS)-no. (%)	11 (19.6)
Digital ischemia-no. (%)	12 (21)
Renal crisis-no. (%)	6 (10.7)
Pulmonary arterial hypertension (PAH)-no. (%):	14 (25)
Associated with restrictive syndrome (RS)-no. (%)	8 (14.3)
Without restrictive syndrome (RS)-no. (%)	6 (10.7)
Specific autoantibodies-no. (%):	15 (26.8)
Anti-centromere antibodies-no. (%)	8 (14.3)
Anti-DNA topoisomerase I antibodies-no. (%)	7 (12.5)
Immunosuppressant at the time of serum collection-no. (%)	7 (12.5)
ACE inhibitors at the time of serum collection -no. (%)	7 (12.5)
Plus-minus values are Means ± SEM. No difference was observed between these groups of patients by one way ANOVA and multivariate analysis in terms of age, duration of disease or duration of Raynaud's phenomenon.	

The onset of the disease was defined as the time of the first non Raynaud's phenomenon. To be eligible for the study, patients had to fulfill the American College of Rheumatology criteria and/or the Leroy & Medsger criteria for SSc.¹¹⁻¹³ Limited cutaneous SSc was defined by skin thickening in areas solely distal to the elbows and knees and diffuse cutaneous SSc was defined by the presence of skin thickening proximal, as well as distal, to the elbows and knees.¹²

Interstitial lung disease was assessed by chest high-resolution computed tomodensitometry and pulmonary function test values. A restrictive syndrome (RS) was defined

by a reduction (<75%) as compared with values obtained from a well-defined population of normal subjects matched for gender, age, height and ethnic origin using similar test protocols in both forced expired volume in one second (FEV₁) and forced vital capacity (FVC), and a FEV₁/FVC ratio >75%.

Vascular abnormalities (VA) were defined as digital ulcers or scars of ulcers related to digital ischemia or pulmonary arterial hypertension (PAH) defined by systolic pulmonary arterial pressure >40 mmHg upon echocardiography and/or mean pulmonary arterial pressure >25 mmHg upon right heart catheterization in the absence of restrictive syndrome. Twelve patients presenting with active digital ulcers, and/or scars of digital ulcers, and six patients with PAH in the absence of RS were classified as patients with VA. One patient had both digital ischemia (DI) and PAH and was taken onto account in both groups. The eight patients with PAH associated with a RS were not included in the group of patients with vascular primitive PAH in order to form a homogenous group of patients with primitive vascular abnormalities. Only 1 of 56 patients had both RS and VA and was taken onto account in both groups.

Since no sample contemporary of a renal crisis was available, this complication was not used to classify patients in this study. Two patients had tendon friction rubs. None of the 56 patients had another connective tissue disease or cancer.

Normal sera were collected from 30 healthy adults (22 females and 8 males, mean age 44 ± 17 years). All patients and controls gave their written informed consent.

Cells and chemicals

HUVEC were obtained by digestion of umbilical cords with 0.1% collagenase. NIH 3T3 fibroblasts were obtained from American Type Culture Collection (Manassas, VA). HEp-2 cells were obtained from EuroBio (Les Ulis, France). Primary normal human dermal fibroblasts were a gift from A Munnich, (INSERM 781, Necker hospital, Paris, France). All cells were cultured as previously reported.^{14, 15}

Measurement of hydrogen peroxide (H₂O₂) and nitric oxide (NO) production

Cells (8x10³ per well) were seeded in 96-well plates (Costar, Corning, Inc, NY) and incubated with their respective growth medium alone for 12 hours at 37°C in 5% CO₂. Culture media were then removed and cells pre-incubated with 50 µl 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA) diluted 1:1000 in PBS to test cellular H₂O₂ production or with 50 µl 4,5-diaminofluorescein diacetate (DAF2-DA) diluted 1:375 in PBS to test NO release. After 30 minutes, 50 µl of SSc or control sera were added and H₂O₂ and NO productions were assayed spectrofluorimetrically after 6 hours (Fusion, PerkinElmer, Wellesley, MA, USA). Results were expressed in arbitrary units (A.U.) per minute and per million cells.

***In vitro* cell proliferation assays**

HUVEC, NIH 3T3, human primary fibroblasts or HEp-2 cells (4x10³ per well) were seeded in 96-well plates (Costar) and incubated with 50 µl of SSc or control serum and 150 µl of culture medium without fetal calf serum at 37°C in 5% CO₂ for 48 hours. Cell proliferation was determined by pulsing the cells with [³H]thymidine (1µCi/well) during the last 16 hours of culture. Results were expressed as absolute numbers of counts per minute.

Assay of serum anti-endothelial and anti-fibroblast antibodies

NIH 3T3 fibroblasts and HUVEC (4×10^4 cells per well) were incubated with 1:5 dilution of SSc or control sera for one hour at 4°C, then washed in PBS and incubated with 1:200 FITC-rabbit polyclonal anti-human IgG, A, M antibody (Dako, Glostrup, Denmark) for one hour at 4°C. Fluorescence intensity was determined spectrofluorometrically and expressed as A.U.

Assay of advanced oxidation protein products (AOPP) in sera

AOPP were measured by spectrophotometry as previously described.¹⁶ The assay was calibrated using chloramine-T. The absorbance was read at 340 nm on a microplate reader (Fusion, PerkinElmer, Wellesley, MA, USA). AOPP concentrations were expressed as $\mu\text{mol/l}$ of chloramine-T equivalents.

ROS production by cellular AOPP.

HUVEC protein extracts were obtained by incubation of HUVEC with 1% NP40 and protease inhibitors in PBS buffer. Proteins were oxidized by 1mM HOCl for one hour or 1mM peroxyntrites for 18 hours at room temperature, then dialyzed overnight against PBS and tested for AOPP content. Endothelial cells and NIH 3T3 (8×10^3 per well) were incubated with 0.5 mg per well of either unoxidized or oxidized HUVEC extract. Production of H_2O_2 and NO was assessed spectrofluorometrically using H2-DCFDA and DAF2-DA as described above.

Effects of drugs on serum-induced ROS production

SSc sera were incubated with either 50 μM bosentan (Actelion, Allschwil, Switzerland) or 10 μM nifedipine (Bayer Pharma, Leverkusen, Germany) or 50 μM D-penicillamine (Dexo, Saint Cloud, France), or 10 μg etanercept (Wyeth, Madison, USA) in PBS for 90 minutes at 37°C. PBS alone was used as control. HUVEC (8×10^3 per well) seeded in 96-well plates were incubated with 50 μl of H2-DCFDA or DAF2-DA. After 30 minutes, the sera with drugs were added and H_2O_2 and NO productions assessed as above. In other experiments, HUVEC and NIH 3T3 (8×10^3 per well) were incubated with 1600 μM N-acetyl-L-cysteine (NAC), or 400 μM reduced glutathione (GSH), or 10 U PEG-catalase for 18 hours at 37°C in 5% CO_2 . Medium and chemicals were removed and replaced by fifty μl of H2-DCFDA (for HUVEC and NIH 3T3) or DAF2-DA (for HUVEC). After 30 minutes, 50 μl serum diluted 1:2 were added and ROS production assessed as above. Results were expressed as percentages of ROS production versus untreated cells (100%).

Effects of drugs on serum-induced HUVEC and fibroblast proliferation.

NIH 3T3 cells or HUVEC (4×10^3 per well) were incubated with SSc or control serum (1:8 v:v) in culture medium without fetal calf serum at 37°C in 5% CO_2 for 48 hours. For NIH 3T3 cells, one of the following molecules was added: 1600 μM NAC, 400 μM reduced GSH, 10 μM nifedipine, 50 μM D-penicillamine, 50 μM bosentan, 10 μg etanercept, 10 U PEG-catalase, or 25 μM 5FU (Dakota Pharm, Le Plessis Robinson, France) with or without 1600 μM NAC. For HUVEC, 600 μM NAC, or 400 μM reduced GSH, or 10 U PEG-catalase were added. Cell proliferation was assessed as described above. Results were expressed as percentages of cellular proliferation versus untreated cells (100%).

Longitudinal study of *in vitro* ROS production by HUVEC and of NIH 3T3 fibroblast proliferation

A longitudinal study was performed with the sera from 5 patients. Productions of H₂O₂ and NO, cell proliferation and serum AOPP concentration were measured as previously described.

Statistical analysis.

Data were compared with the Student's *t* unpaired, paired tests and Fisher's exact test. When analysis included > 2 groups, one way analysis of variance was used. Pearson's *r* correlation coefficient was used to analyze the relationships between quantitative variables. *p* value < 0.05 was considered significant. Quantitative values higher than 2 SD above the mean obtained with control sera were considered as positive.

RESULTS

Effects of SSc sera on ROS production and proliferation of fibroblasts, HUVEC and HEp-2 cells

HUVEC, NIH 3T3 fibroblasts or HEp-2 cells exposed to SSc sera released higher amounts of H₂O₂ than when exposed to normal sera (*p*<0.001 in all cases; fig 1A). In contrast, mean NO release was similar whether HUVEC, NIH 3T3 fibroblasts or HEp-2 cells had been incubated with sera from SSc patients or with normal sera (fig 1B).

Since ROS can modulate cell proliferation, we tested the effects of SSc sera on the proliferation of NIH 3T3 and human fibroblasts, HUVEC, and HEp-2 cells as control. SSc sera exerted a higher pro-proliferative effect on NIH 3T3 fibroblasts (fig 1C) and on human fibroblasts (fig 1D) than normal sera (*p*=0.03 and *p*=0.001, respectively) and exerted an anti-proliferative effect on HUVEC when compared with normal sera (*p*<0.001). No difference between SSc sera and control sera was observed on HEp-2 cells (fig 1C). A strong correlation was observed between the pro-proliferative effect exerted by SSc sera on normal human fibroblasts and on NIH 3T3 cells. (*r* = 0.85; *p*<0.0001).

H₂O₂ production by NIH 3T3 fibroblasts was correlated with SSc serum-induced proliferation (*r* = 0.31, *p* = 0.02). The antiproliferative effect observed on HUVEC was also correlated with the amounts of H₂O₂ produced by these cells (*r* = -0.30, *p* = 0.03).

Correlations between clinical involvement of the disease and the serum-induced ROS production and cellular proliferation

Sera from the 11 patients with a restrictive syndrome induced higher levels of H₂O₂ than sera from the 28 SSc patients with no restrictive syndrome and no vascular abnormality and from healthy subjects (*p* = 0.02 and *p* < 0.0001; fig 2A). Conversely, sera from these 11 patients did not induce higher release of NO as compared with sera from the two other groups (fig 2B). These 11 sera exerted no higher pro-proliferative effect on HUVEC than sera from patients with digital ischemia or vascular PAH or from SSc patients with no restrictive syndrome and no vascular abnormality (fig 2C and data not shown). In contrast, these 11 sera exerted a significant higher pro-proliferative effect on NIH 3T3 fibroblasts as compared with sera from patients with no restrictive syndrome and no vascular abnormality, from patients with digital ischemia, with vascular PAH and from healthy subjects (*p* = 0.04; *p* = 0.04; *p* = 0.02; *p* = 0.008; fig 2D and data not shown).

Sera from the 18 patients with vascular abnormality (11 patients with digital ischemia, 5 with PAH and 1 with both involvements) induced a higher release of NO by HUVEC than sera from the 11 patients with a restrictive syndrome and from the 28 SSc patients with no restrictive

syndrome and no vascular abnormality ($p = 0.01$ and $p = 0.03$ respectively; fig 2B). These 18 sera exerted no effect on the growth of NIH 3T3 fibroblasts when compared with sera from the 28 SSc patients with no restrictive syndrome and no vascular abnormality and with sera from healthy controls (fig 2D) but abrogated the proliferation of HUVEC more strongly than the sera from these two other groups of individuals ($p = 0.02$ and $p = 0.0004$ respectively; fig 2C). Among these 18 patients with vascular abnormality, no difference was observed between patients with digital ischemia and those with PAH either in H_2O_2 and NO induction or in modulation of cell proliferation. Nevertheless, the sera from the 12 patients with digital ischemia abrogated the proliferation of HUVEC more strongly than the sera from the 28 SSc patients with no restrictive syndrome and no vascular abnormality ($p = 0.02$ and $p = 0.18$ respectively, data not shown). This significant difference was not observed with sera from the six patients with PAH.

Conversely, 50% of the 12 patients with significant serum-induced fibroblast proliferation *versus* controls, presented with a restrictive syndrome that was detected in only 11.4 % of the other SSc patients ($p = 0.007$). Fifty-five percent of patients with significant serum-induced antiproliferative effect on HUVEC suffered from digital ischemia that was present in only 13 % of the other SSc patients ($p = 0.007$).

No difference was observed in NO and H_2O_2 release and on cell proliferation between patients with diffuse or limited cutaneous SSc.

Serum AOPP are responsible for ROS production and modulate fibroblast proliferation

We then characterized the serum factors responsible for the oxidative stress. We observed no correlations between anti-centromere, anti-topoisomerase 1 (Table 1), anti endothelial cell (24% SSc sera), or anti-fibroblast antibodies (28% SSc sera), and H_2O_2 or NO production by endothelial cells or fibroblasts ($p \geq 0.13$ in each case). In addition, the depletion of serum IgG by protein G (assessed by nephelometric assay) affected neither serum-induced ROS production nor fibroblast proliferation (data not shown).

The serum concentration of AOPP was higher in SSc patients than in healthy controls ($p = 0.02$; fig 3A) and was correlated with the induction of H_2O_2 production by HUVEC ($r = 0.46$, $p < 0.001$), NIH 3T3 ($r = 0.35$, $p = 0.01$), and HEp-2 cells ($r = 0.31$, $p = 0.02$). HOCl-induced AOPP activated endothelial cells and, at a lesser extent, fibroblasts while ONOO⁻-induced AOPP activated endothelial cells only. HOCl-AOPP induced more H_2O_2 than NO, while ONOO⁻-AOPP mainly induced NO (fig 3B and 3C). Furthermore, a dose-dependent proliferative response was observed with with HOCl-AOPP and, at a lesser extent, with ONOO⁻-AOPP (data not shown).

Disease-modifying agents modulate ROS generation and cellular proliferation induced by SSc sera

The ROS production and the fibroblast proliferation induced by the sera from seven patients (12.5%) who received immunosuppressive therapy at the time of serum collection was similar to those induced by SSc sera from non treated patients. However, the sera from patients with immunosuppression abolished NO release by HUVEC (mean NO release: 10.14 ± 2.3 versus 17.4 ± 1.3 ; $p = 0.049$). Seven other patients (12.5%) received angiotensin-converting enzyme inhibitors. Their sera exerted the same effects as sera from SSc patients who did not receive this type of drugs.

In vitro, no significant effect on H_2O_2 generation by HUVEC was observed in the presence of nifedipine, D-penicillamine, or etanercept. Catalase, NAC and GSH decreased H_2O_2

levels by 23.4% ($p = 0.006$), 24.9% ($p = 0.048$) and 14.2% ($p = 0.05$), respectively (fig 4A). GSH increased HUVEC proliferation by 5% ($p = 0.29$). NAC by 22.2% ($p = 0.07$) and catalase significantly improved the proliferation of HUVEC by 53% ($p = 0.047$) (data not shown).

Bosentan inhibited NO release by 32.7% ($p < 0.001$) on HUVEC whereas other molecules did not (fig 4B). GSH and catalase decreased SSc serum-induced production of H_2O_2 by NIH 3T3 by 19.8% ($p = 0.048$) and by 27.8% ($p = 0.044$) respectively, whereas NAC did not (data not shown). GSH, D-penicillamine and 5FU inhibited fibroblast proliferation by 45.15% ($p = 0.047$), 71.8% ($p < 0.001$) and 78.4% ($p < 0.001$), respectively, whereas NAC alone could ($p = 0.15$) (fig 4C). However, NAC potentiated the anti-proliferative activity of 5FU on fibroblasts by 10% ($p = 0.02$). Nifedipine, bosentan, etanercept and catalase had no or little effect on fibroblast proliferation (fig 4C).

Longitudinal study of *in vitro* ROS production by HUVEC and NIH 3T3 fibroblast proliferation

In four patients (patients A,B,C,D), clinical involvement worsened and immunosuppressive drug(s) was(were) given (fig 5A, 5B and 5C). AOPP concentrations and serum-induced H_2O_2 production decreased when the clinical status stabilized or improved. In patient E, no visceral involvement was detected in the course of the disease and serum properties were not modified when assayed longitudinally (fig 5C).

DISCUSSION

This report highlights the involvement of the oxidative stress in SSc and describes new serum markers in order to assess the activity of the disease and predict the response to treatment.

A common feature of the SSc sera tested in this study is their ability to induce the production of H_2O_2 especially by endothelial cells. Since ROS can modulate cell activation and proliferation^{17, 18}, and since fibroblasts and endothelial cells are selectively targeted in SSc, we tested the effects of SSc sera on the proliferation of murine and human fibroblasts and HUVEC. SSc sera exert a cell-specific pro-proliferative effect, since the same sera that stimulate fibroblasts to proliferate, inhibit HUVEC proliferation and have no effect on HEp-2 cells. The difference observed between fibroblasts and endothelial cells could be related to the level of ROS produced since a reverse correlation between the rate of proliferation and that of H_2O_2 production is observed in both types of cells. The high levels of H_2O_2 generated in endothelial cells probably reaches the threshold of toxicity and inhibits cell proliferation, whereas the proliferation of fibroblasts from SSc patients is directly correlated with intrinsic production of H_2O_2 as previously shown.^{6, 19}

We then observed that the sera from patients with a restrictive syndrome induce a higher production of H_2O_2 and a higher fibroblast proliferation than sera from other SSc patients. Those data are in agreement with the previous finding that the synthesis of collagen by fibroblasts from SSc patients is correlated with the oxidative stress.^{6, 19}

On the other hand, serum-induced NO release is associated with digital ischemia or pulmonary arterial hypertension, although beneficial vasodilating effects could have been expected from a high rate of NO production.²⁰⁻²⁴ The deleterious effect of NO might be related to

its combination with other ROS to form highly toxic peroxynitrites resulting in cell injuries and cell death.^{25, 26}

We next characterized the serum factors responsible for the oxidative stress. In our hands, the prevalence of auto-antibodies to endothelial cells and fibroblasts is that found usually in SSc²⁷⁻³⁰, but those antibodies are not correlated with serum-induced ROS production or cell proliferation. Therefore, we turned to AOPP whose concentration has been found elevated in SSc sera by us and others^{8,31} and that can trigger a respiratory burst in monocytes.¹⁶ In our hands, AOPP activate endothelial cells and, at a lesser extent, fibroblasts to generate ROS. Furthermore, AOPP generated by different oxidation patterns lead to the production of either NO or H₂O₂, suggesting AOPP may be involved in the generation of different types of ROS in SSc patients. However, the role of autoantibodies cannot be totally ruled out. For instance, anti-PDGF receptor antibodies³² and possibly environmental factors^{4, 33-35} could initiate a secondary self-maintained process³ linked to the production of AOPP and ROS that perpetuate the disease.

We finally investigated whether drugs used in SSc and antioxidant molecules can modulate the pro-activating effects of SSc sera. NAC and GSH decrease the generation of H₂O₂ by HUVEC and the proliferation of fibroblasts upon incubation with the majority of SSc sera. This is in accordance with the observation that NAC can decrease ROS production in SSc fibroblasts, inhibit their proliferation and collagen synthesis,⁶ and abrogate ONOO⁻ synthesis by activated lung macrophages from SSc patients *in vitro*.³⁶ In addition, we observed that NAC potentiates the antiproliferative effect of 5FU, which is in agreement with the beneficial effect of NAC in association with immunosuppressive therapies observed in patients with lung idiopathic interstitial fibrosis.³⁷

Bosentan, an antagonist of endothelin A and B receptors that exerts beneficial effects in SSc patients with pulmonary arterial hypertension³⁸⁻⁴⁰, abrogates NO overproduction by HUVEC stimulated by sera from patients with vascular complications. Consequently, this drug could be preferentially used in SSc patients whose sera trigger NO release, prior to the development of severe vascular complications. Prospective therapeutic trials have to be performed in SSc patients to confirm these results.

In conclusion, our data underline a pivotal role of serum factors as ROS and AOPP in the pathogenesis of the different patterns observed in SSc. Those serum markers could help in assessing the activity of SSc and selecting the most appropriate treatments according to the fibrotic or vascular pattern.

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FIGURE LEGENDS

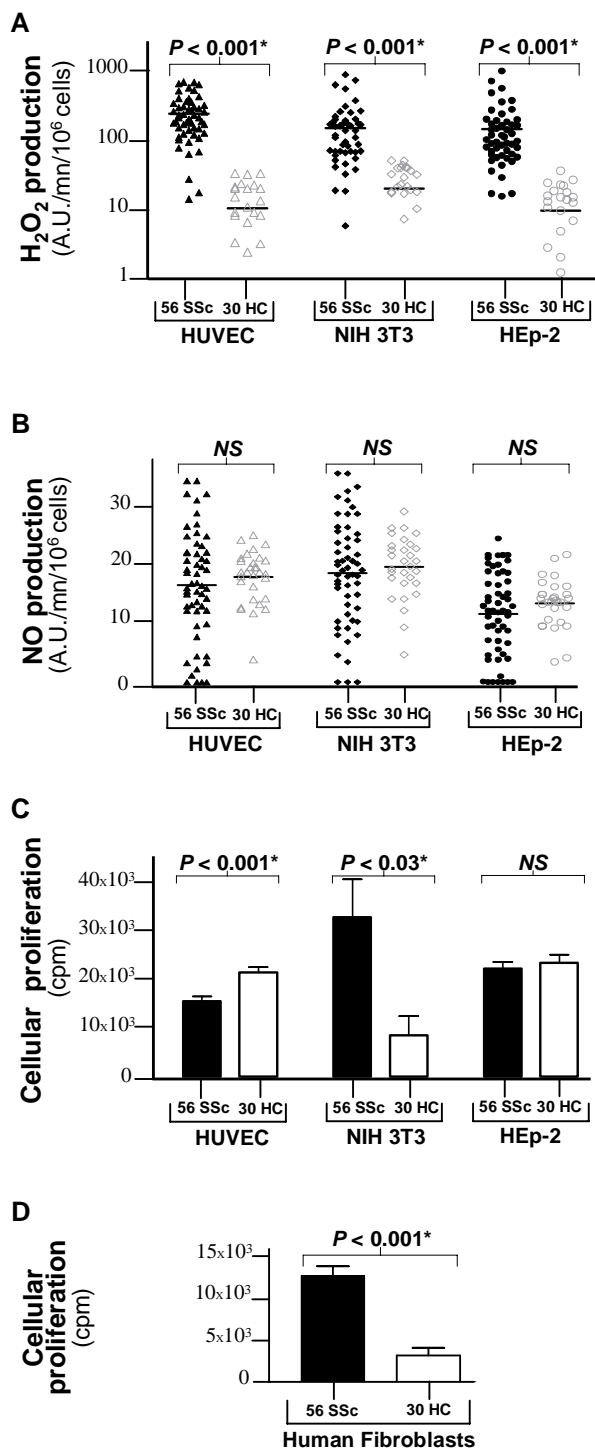
Figure 1 Effects of 56 sera from SSc patients (solid symbols) and 30 sera from healthy controls (HC) (open symbols) on ROS generation and cell proliferation. (A) Production of H_2O_2 and (B) Production of NO by HUVEC, NIH 3T3 fibroblasts, and HEp-2 cells. (C) Cell proliferation determined by [3H]thymidine incorporation. (D) Primary normal human fibroblast proliferation. Error bars represent SEM.

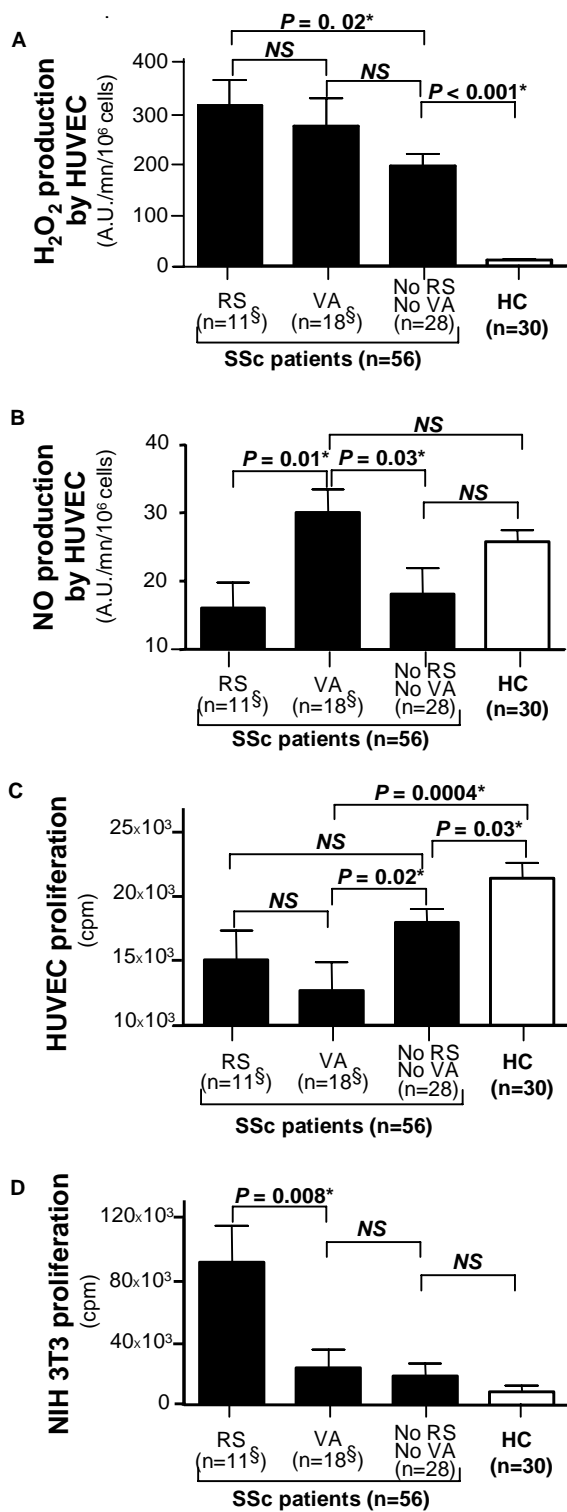
Figure 2 ROS production and cell proliferation induced by sera from SSc patients according to their clinical pattern: 11 patients had a restrictive syndrome (RS); 18 had vascular abnormalities (VA); 28 had none of these complications; 30 sera were collected from healthy controls (HC). (A) Serum-induced production of H_2O_2 by HUVEC. (B) Serum-induced production of NO production by HUVEC. (C) Serum-induced proliferation of HUVEC. (D) Serum-induced proliferation of NIH 3T3 fibroblasts. Error bars represent SEM. [§]One patient had both a RS and VA.

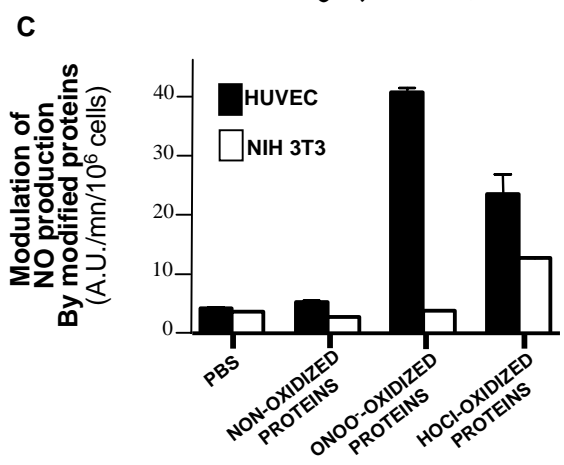
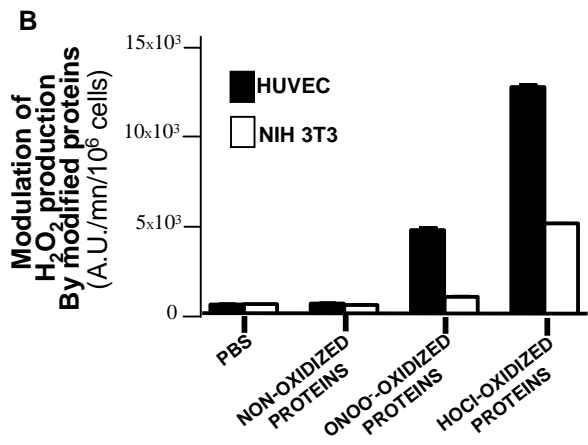
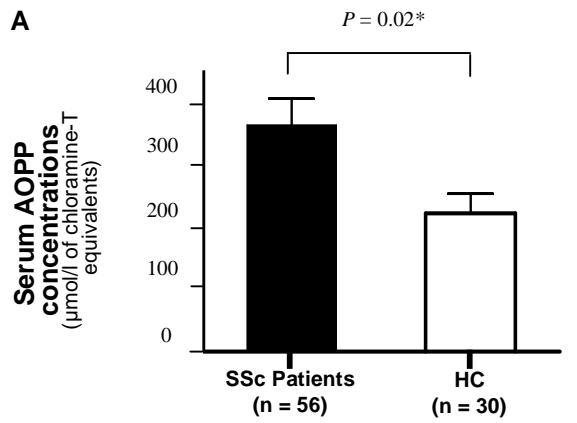
Figure 3 Role of AOPP in serum-induced ROS production. (A) Serum AOPP concentration in 56 SSc patients and in 30 healthy controls (HC). Data are means \pm SEM. (B) Production of H_2O_2 induced by HOCl-AOPP, peroxyntrites-AOPP, or non oxidized proteins by endothelial cells and fibroblasts. (C) Production of NO induced by HOCl-AOPP or peroxyntrites-AOPP or non oxidized proteins in endothelial cells and fibroblasts. Data are means \pm SEM.

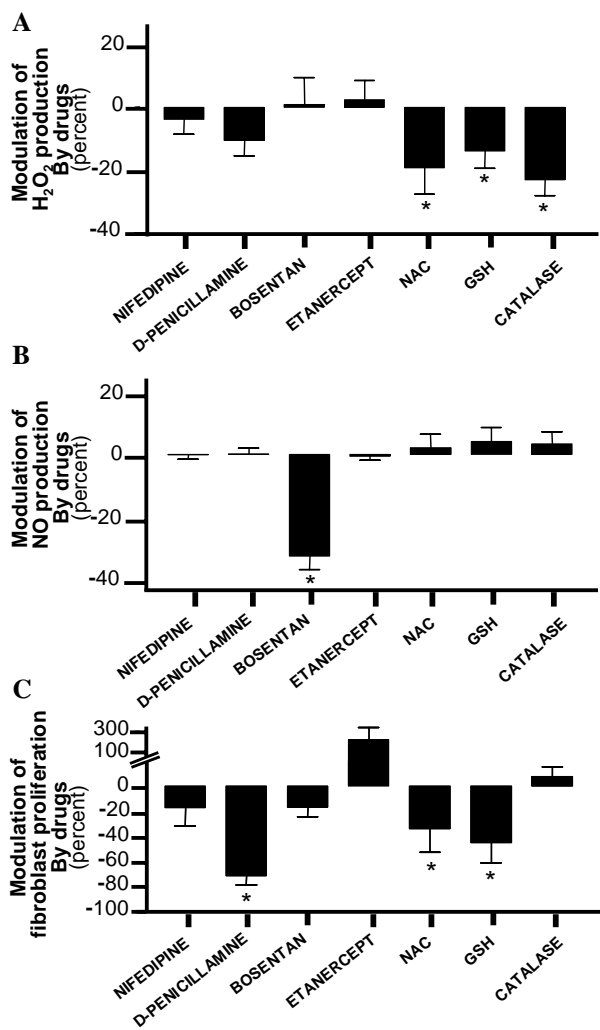
Figure 4 Modulation of serum-induced ROS generation and cell proliferation by antioxidative drugs. (A) Modulation of H_2O_2 production by HUVEC. (B) Modulation of NO production by HUVEC. (C) Modulation of NIH 3T3 fibroblast proliferation. In A, B, and C, HUVEC and fibroblasts were incubated with SSc sera and one of the following molecules: 10 μ M nifedipine, 50 μ M D-penicillamine, 50 μ M bosentan, 10 μ g etanercept, 1600 μ M NAC, 400 μ M reduced GSH, 10 U PEG-catalase. Results are expressed as percentages of ROS production or fibroblast proliferation *versus* untreated cells (100%). Error bars represent SEM.

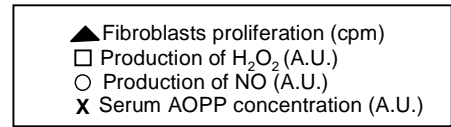
Figure 5 Longitudinal study of *ex vivo* serum-induced ROS production by HUVEC, proliferation of NIH 3T3 fibroblast proliferation, and serum AOPP concentrations in five patients. (A) Patient A had progressive RS with decreased DLCO/VA and biopsy-proven inflammatory myositis. He received mycophenolate mofetyl and intravenous immunoglobulins. Test values from three successive sera obtained every six-months are presented. (B) Patient B had diffuse SSc with progressive deterioration of the Rodnan's score. Test values from three successive sera are presented. Azathioprin was given at the time of the collection of the first serum, then suspended and resumed at the time of collection of the third serum sample. (C) Patient C had a RS related to lung fibrosis. (D) Patient D had RS and digital ulcers. Both patients C and D had been receiving immunosuppressive drugs at the time of the second serum collection. Patient E had SSc without complication and received no immunosuppressant. AZA, Azathioprin; CYC, cyclophosphamide; IVIg, intravenous immunoglobulins; MMF, mycophenolate mofetyl.



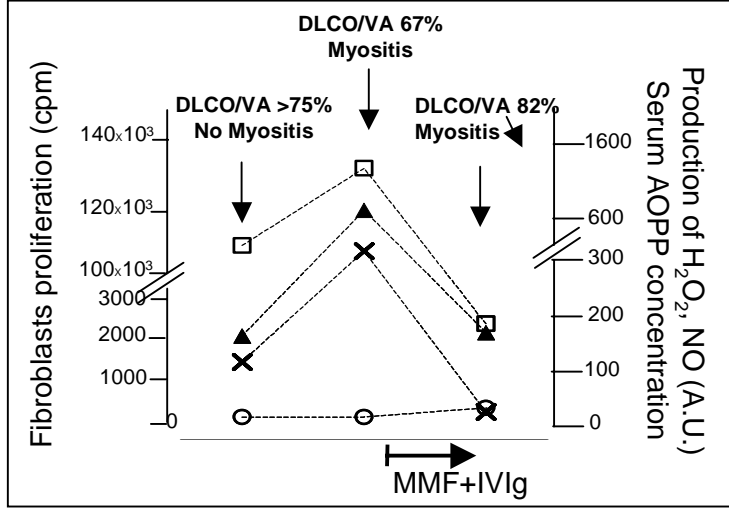




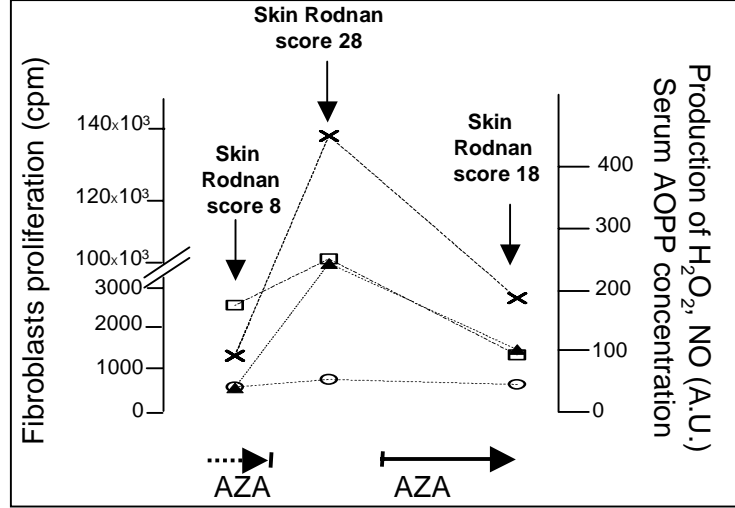




A: Patient A



B: Patient B



C: Patients C, D, E

SSc patients	Initial evaluation						Second evaluation					
	Drugs	Clinical characteristics	serological evaluation				Drugs name (Duration)	Clinical characteristics	serological evaluation			
			AOPP (A.U.)	H ₂ O ₂ (A.U.)	NO (A.U.)	Fibroblast proliferation (cpm)			AOPP (A.U.)	H ₂ O ₂ (A.U.)	NO (A.U.)	Fibroblast proliferation (cpm)
C	none	Progressive lung fibrosis CPT 73%	87.1	297	19.7	89806	CYC (6) AZA (24)	Stabilization of lung fibrosis CPT 73%	32.33	49.1	12.5	2241
D	none	Digital ulcers Interstitial syndrome DLCO/VA : 70%	141	259	44.5	100674	Bosentan (14) MMF (24)	Decrease in number of ulcers Interstitial syndrome DLCO/VA : 69%	46	40	14	22756
E	none	Telangiectasia No interstitial syndrome DLCO/VA >80%	117	113	21.4	163	none	Telangiectasia No interstitial syndrome DLCO/VA >80%	21.36	54.8	21.9	393