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Anti-fibroblast antibodies detected by cell-based ELISA in systemic sclerosis enhance the collagenolytic activity and matrix metalloproteinase-1 production in dermal fibroblasts

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Objectives. Antibodies binding to the surface of fibroblasts (anti-fibroblast antibodies: AFA) have been described in systemic sclerosis (SSc). We aimed to assess the effect of AFA on extracellular matrix (ECM) turnover and whether AFA were associated with anti-topoisomerase-I antibody.

Methods. IgG were purified from AFA-positive and AFA-negative sera selected within 20 SSc and 20 healthy individuals, and tested on normal dermal fibroblasts, at protein and mRNA level, for their capacity to induce collagen deposition or degradation.

Results. Fibroblasts stimulated with AFA-positive but not with AFA-negative and control IgG showed an increased capacity to digest collagen matrix and produce metalloproteinase-1 (MMP-1) while their production of total collagen, type I collagen and tissue inhibitor of metalloproteinase-1 (TIMP-1) was unaffected. The steady-state mRNA levels of MMP-1, COL1A1 and TIMP-1 paralleled the protein levels. AFA-positive IgG did not induce Smad 2/3 phosphorylation, indicating that this transforming growth factor-β signalling pathway was not involved. IL-1 and tumour necrosis factor (TNF) neutralization did not reverse the enhanced production of MMP-1, suggesting a direct effect of AFA on fibroblasts. Finally, anti-topoisomerase-I antibodies were present in 11 of 12 AFA-negative IgG, and an anti-topoisomerase-I monoclonal antibody failed to enhance MMP-1 production, thus indicating a lack of correlation between AFA and anti-topoisomerase-I antibody.

Conclusions. These results indicate that SSc antibodies binding to fibroblasts enhance matrix degradation and MMP production events that may favour inflammation but do not directly impact on fibrosis development.

Key words: Anti-fibroblast antibodies, Collagen, Matrix metalloproteinase, Smad2/3, Fibrosis.

Introduction

Systemic sclerosis (SSc) is a disorder of unknown origin characterized by the presence of autoantibodies, fibroproliferative vasculopathy and excessive extracellular matrix (ECM) deposition, leading to fibrosis of the skin and internal organs [1]. Fibroblasts play a central role in ECM production and degradation [2]. They synthesize ECM molecules, including several collagens (particularly type I collagen, the most abundant in the skin), express degrading enzymes such as matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs) [3, 4]. SSc fibroblasts differ from normal fibroblasts in many respects as they produce more collagen [5], constitutively overexpress connective tissue growth factor (CTGF) which promotes fibroblast proliferation and ECM production [6], as well as α-smooth muscle actin (α-SMA) [7]. Transforming growth factor-β (TGF-β) stimulates matrix production and induces its own synthesis and that of CTGF [8]. Smads are among the intracellular effectors of TGF-β, the principal mediator of tissue fibrosis [9]. In scleroderma fibroblasts, Smad3 and Smad4 show nuclear localization and elevated levels of phosphorylated Smad2/3 in the absence of exogenous TGF-β, suggesting a constitutive activation of the Smad pathway [9].

Antibodies against ubiquitous cellular self-components are characteristically present in more than 90% of SSc patients and are associated with different clinical subsets [10]. In addition, autoantibodies that specifically bind to fibroblasts have been described. We reported the presence of anti-fibroblast antibodies (AFA), capable of binding the fibroblast cell surface and induce a pro-adhesive and pro-inflammatory phenotype, up-regulating the expression of intercellular adhesion molecule 1 (ICAM-1) and IL-6 [11] in more than 40% of SSc patients. Others have found AFA in SSc patients with anti-topoisomerase-I antibodies, mostly affected by the diffuse form of the disease, and shown that they strongly correlated with anti-topo-I Ab [12, 13]. Of interest, IgG of SSc individuals with pulmonary artery hypertension (PAH) were shown to recognize fibroblast components distinct from those with primary PAH [14]. In addition, non-ubiquitous antigen target of autoantibodies with the potential to modify ECM turnover include fibrillin-1, a non-structural matrix component and platelet-derived growth factor (PDGF) receptor [15, 16]. Stimulatory antibodies to PDGF-R are not restricted to SSc but have also been found in chronic graft *vs* host disease [17]. Furthermore, fibroblast activation has also been associated with antibodies which cross-recognize the human CMV-derived protein UL94 and the adhesion self-molecule NAG-2 [18, 19].

The contribution of AFA to pathogenic events leading to fibrosis in SSc is still debated. In the present study, we asked the question whether AFA could impact on ECM turnover. Our findings indicate that SSc AFA-positive IgG preferentially induce ECM degradation rather than deposition, in a tumour necrosis factor (TNF) and IL-1-independent manner. Thus, a subset of SSc patients harbours autoantibodies that bind to fibroblasts and restrain their potential of ECM deposition.

Materials and methods

Patients and controls

Twenty SSc patients (17 women and 3 men, mean ± S.D. age 49 ± 11 yrs) and 20 age- and sex-matched healthy blood donors were enrolled in the study. All patients fulfilled the American

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College of Rheumatology criteria for SSc classification [20], and all but one were anti-topoisomerase-I antibodies positive. The only anti-topo-I-negative had a nucleolar ANA aspect. None had anti-centromere antibodies. Peripheral blood and skin biopsies were obtained from patients and controls after informed written consent according to the Declaration of Helsinki. The study was approved by the Ethics Committee of the Geneva University Hospital.

Reagents

Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids solution 100×, sodium pyruvate, penicillin and streptomycin were obtained from GIBCO (Invitrogen); α-keto-glutaric acid, β-amino propionitrile and fetal calf serum (FCS) from Sigma (St Louis, MO, USA); recombinant human TGF-β1 (rhTGF-β_1), recombinant human interleukin-1-β (rhIL-1β), anti-intercellular adhesion molecule 1 (ICAM-1) monoclonal antibody, recombinant human TNF-α (rhTNF-α) from R&D System (Minneapolis, MN, USA); mouse anti-topo-I monoclonal antibody from Immunovision, (Springdale, AR, USA); recombinant human interleukin-1 receptor antagonist-1 (rhIL-1Ra) and soluble TNF receptor p75 (anti-TNF-α) from Amgen (Boulder, CO, USA); Limulus Amebocyte Lysate Endochrome, multitest vials for endotoxin from Charles River Endosafe (Charleston, SC, USA).

Culture conditions

Skin biopsies were taken from the forearm of normal individuals under local anaesthesia. Fibroblasts were grown in 100 mm culture dishes at 37°C in 5% CO_2 humidified atmosphere in DMEM with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1% non-essential amino acids and 1% sodium pyruvate supplemented with 10% FCS, and used from passages 3 to 10.

Enzyme-linked immunosorbent assay (ELISA)

SSc sera were screened for the presence of anti-topo-I Ab by ELISA (QuantaLite™ Scl-70, Inova Diagnostic, San Diego, USA). Total IgG were purified from 12 SSc sera (eight AFA-positive and four AFA-negative) and eight normal human sera (NHS) by protein G-sepharose chromatography (Pharmacia, Uppsala, Sweden). To detect AFA and ICAM-1 expression on the surface of fibroblasts, we performed a cell-based ELISA as described [11]. Sera and IgG were tested in triplicates. As positive control, fibroblasts were stimulated by TNF (10 ng/ml) and IL-1β (50 ng/ml).

Type I collagen, MMP-1 and TIMP-1 protein production assay

Fibroblasts were plated in 96-well trays at 2×10^4 cells/well and serum-starved overnight before being cultured with IgG, or cytokines, or medium alone for an additional 48 h in medium supplemented with 1% FCS, 25 μg/ml L-ascorbic acid, 3.4 μg/ml α-ketoglutaric acid and 50 μg/ml β-amino propionitrile in order to favour normal collagen fibrillogenesis [21]. When required, IL-1Ra (2 μg/ml) and anti-TNF (10^{-8} M) were added 1 h before IL1-β (10 ng/ml), TNF (10 ng/ml) or IgG (300 μg/ml). The supernatants were collected and stored at −20°C until protein determination. All experimental conditions were made in triplicate. The ELISA for TIMP-1 (R&D) and pro-MMP-1 (Binding Site, Birmingham, UK) and the RIA for the determination of N-terminal propeptide of type I procollagen (PINP-1) (Orion Diagnostica, Espoo, Finland) were performed according to the manufacturer's instructions. Total collagens was assessed on supernatants of fibroblasts cultured for 24, 48 and 72 h in 100 mm dishes with DMEM supplemented with 25 μg/ml vitamin C, in the absence of FCS, by the Sircol TM assay (Biocolor Ltd, New Townabbey, UK).

Collagenase assay

Collagenase activity was assessed by ELISA on type I calf skin collagen (Sigma) coated for 7 days on to 96-well plates, as described [22]. Samples were activated with trypsin 100 μg/ml for 20 min at 37°C, to convert latent collagenase into its active form, followed by a 5-fold excess addition of soybean trypsin inhibitor (Worthington, Freehold, NJ, USA) and tested in triplicate. The undigested collagen was detected with an anti-type I collagen mouse monoclonal antibody (Calbiochem, La Jolla, CA, USA) followed by alkaline phosphatase-labeled anti-mouse IgG sheep antibody (Cappel, West Chester, PA, USA). One unit of collagenase was defined as the activity that solubilizes 1 μg of collagen in 1 min.

Western blotting

Fibroblasts cultured in serum-free medium for the indicated times under different experimental conditions were lysed in lysis buffer containing protease inhibitors. Twenty micrograms of total protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electophoresis under reducing conditions and electroblotted on to nitrocellulose membrane (Hybond™ ECL™ Amersham Pharmacia Biotech, UK). Blots were incubated with antibodies against phospho Smad 2/3 (ser 465/467) (Cell Signaling, Beverly, MA, USA) and β-tubulin (Sigma). HRP-conjugated antisera were used to reveal primary binding and detected by chemiluminescence using the ECL system (Amersham).

Indirect immunoflurescence (IIF)

Dermal fibroblasts were grown on glass coverslips to 80% confluence, in serum-free conditions. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, made permeable or not in 0.1% saponin, 1% BSA in PBS for 30 min and subsequently incubated with mouse anti-topo-I mAb (20 μg/ml) or AFA-positive IgG, AFA-negative IgG or NHS IgG (300 μg/ml) for 1 h, followed by anti-human IgG FITC (Inova Diagnostic, San Diego, CA, USA) or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probe, Eugene, OR, USA). The nonspecific binding was assessed by testing the binding of the secondary antibody alone (anti-human and anti-mouse). Slides were mounted in Vectashield fluorescence medium (Vector Laboratories, Burlingame, CA, USA), and images were acquired using a Zeiss fluorescence microscope equipped with AxioCam Color CCD Camera.

Determination of mRNA levels of COL1A1, MMP-1, MMP-9, TIMP-1, CTGF and α-SMA

Total RNA of fibroblasts, starved in serum-free medium overnight and cultured for 24, 48 and 72 h with IgG and cytokines, was isolated by RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Samples were homogenized on QIA shredder columns (Qiagen) before RNA isolation, and treated with RNAse-free DNAse set (Qiagen) to remove DNA during RNA purification. The quality of RNA was checked by the Agilent Technologies 2100 Bioanalyzer (version A02.12 SI 292). The mRNA levels were determined by real-time quantitative RT-PCR. The TaqMan assay reagents (Universal PCR Master Mix Buffer) and primers for human MMP-1, MMP-9, TIMP-1, CTGF, α-SMA, COLA1A, GADPH and 18S rRNA were from by Applied Biosystem (Foster City, CA, USA). The primers and probes for human eukaryotic translation elongation factor 1-α1 (EEF1A1) (forward, 5′-AGCAAAAATG ACCCA CCAATG-3′ and reverse, 5′-GGCCTGGATGGTTCA GGATA-3′), and human transferrin receptor (TFRC) (forward, 5′-CATTTGTGAGGGATCTGAACCA-3′ and reverse, 5′-CGA GCAGAATACAGCCACTGTAA-3′) were provided by the

Genomic Platform, NCCR Frontiers in Genetics, University of Geneva. The GeNorm program was used to select the two best housekeeping genes among GAPDH, 18S, EEF1A1, TFRC and TBC, based on stability in response to experimental treatments [23]. The results were normalized to the geometric mean of two housekeeping genes (EEF1A1 and TFRC). PCR was performed in triplicate using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystem) in a final volume of 10 μl. The thermal cycler conditions were the following: 50°C for 2 min, 95°C for 10 m, then 50 cycles of 95°C for 15 s and 60°C for 1 m. The cDNA synthesis reaction without RNA or cDNA synthesis reaction without reverse transcriptase were used as controls and were negative for amplification products.

Statistical analysis

Student's *t*-test was used to analyse differences between the two groups. A one-way ANOVA test (Dunnett method) was used to assess the differences among groups. A *P*-value ≤ 0.05 was considered statistically significant.

Results

Antifibroblast antibody identification

Serum samples from 20 SSc patients whose clinical characteristics are reported in Table 1 and 20 healthy individuals (NHS) were tested for their capacity to bind human dermal fibroblasts in a cell-based assay [11]. Binding to fibroblasts was observed in 8 of 20 SSc and in none of the 20 healthy control sera (Table 1). AFA-positive had less immunosuppressive therapy than AFA-negative individuals. Two of eight AFA-positive had low-dose prednisone (<10 mg/day) for 6 months, while nine of twelve AFA-negative patients had low-dose prednisone from 6 months to 7 yrs (Table 1). IgG purified from SSc patients was defined AFA-positive when binding to fibroblasts was observed, and AFA-negative when it was not. Total IgG was purified by protein G-sepharose chromatography from eight AFA-positive, four AFA-negative and eight NHS. All eight AFA-positive IgG showed a dose-dependent binding to fibroblasts, with half-maximal binding at the concentration of 59.7 ± 6.4 μg/ml; in contrast AFA-negative IgG, NHS IgG and one preparation of commercial human IgG (Redimune, Behring AG) did not show significant binding. In all cases, IgG binding resulted in ICAM-1 up-regulation, while undetectable binding to fibroblasts resulted in the lack of ICAM-1 up-regulation, as reported previously [11]. Experiments performed in the presence of polymyxin B gave comparable results, and in all IgG preparations endotoxin levels were below the threshold of detection (<0.25 EU/ml) (data not shown).

AFA-positive IgG enhance MMP-1 production and collagenolytic activity in dermal fibroblasts, but not type I collagen and TIMP-1

We then assessed whether IgG could affect the balance between ECM production and degradation. While total collagen production was not modified, the collagenolytic activity of fibroblasts cultured in the presence of AFA-positive but not AFA-negative IgG was markedly enhanced ($P < 0.05$) (Fig. 1A and B). Notably, AFA-positive IgG was as potent as 10 ng/ml of TNF in inducing fibroblast collagenolytic activity (Fig. 1B). To substantiate these findings, we determined the amount of MMP-1 and its inhibitor TIMP-1 in addition to type-I collagen (PINP-1) produced by fibroblasts exposed to IgG preparations. Consistently, AFA-positive but not AFA-negative nor NHS IgG enhanced in a dose-dependent manner the production of MMP-1, while TIMP-1 and PINP-1 were not affected (Fig. 1C–H). At 300 μg/ml, AFA-positive IgG increased by 3-fold MMP-1 production ($P < 0.05$) (Fig. 1F). Of interest, in the same culture conditions, TGF-β used as pro-fibrotic control enhanced PINP-1 and TIMP-1 and decreased MMP-1 production (Fig. 1C, E and G). Thus, AFA-positive IgG specifically favour ECM degradation.

Table 1. Clinical characteristics of SSc patients and autoantibodies

N	Age	Gender	Disease duration (months)	Form of SSc	Lung involvement	Anti-topo-I	Therapy	AFA
1	30	F	4	dSSc	No	+	None	+
2	24	F	43	lSSc	No	+	Iloprost	+
3	61	F	240	dSSc	Yes	+	PRED	+
4	38	F	36	dSSc	Yes	+	None	+
5	42	F	48	dSSc	Yes	+	None	+
6	52	F	12	lSSc	Yes	+	Iloprost	+
7	43	F	6	lSSc	No	+	Iloprost	+
8	50	F	72	dSSc	Yes	+	PRED, Iloprost	+
9	57	M	9	dSSc	Yes	+	PRED, Iloprost	−
10	58	F	4	dSSc	No	+	PRED, MTX	−
11	43	F	12	dSSc	Yes	+	None	−
12	58	F	36	dSSc	No	+	PRED	−
13	50	F	60	lSSc	Yes	+	None	−
14	47	F	48	dSSc	No	−	PE, D-Penicillamine	−
15	68	M	6	dSSc	Yes	+	MMF, PRED, CYC	−
16	63	M	11	dSSc	Yes	+	PRED, Iloprost	−
17	54	F	63	lSSc	Yes	+	PRED, AZA	−
18	48	F	36	lSSc	Yes	+	PRED	−
19	58	F	115	lSSc	Yes	+	PRED	−
20	41	F	108	dSSc	No	+	PRED	−

Disease duration: since first SSc symptom whether Raynaud or other. Type of SSc: limited cutaneous form (lSSc), diffuse cutaneous form (dSSc). Therapy: AZA, azathioprine; CYC, cyclophosphamide; PE, plasma exchange; PRED, prednisone; MMF, mycophenolate mofetil; MTX, methotrexate.

AFA-positive IgG increase steady-state mRNA levels of MMP-1, MMP-9, but not those of COL1A1, CTGF, α-SMA and TIMP-1

To determine whether the changes observed at the protein level were paralleled by changes in gene transcription, we tested by quantitative RT-PCR whether IgG would induce changes in COL1A1, MMP-1 and TIMP-1 mRNA steady-state levels. While MMP-1 mRNA was significantly increased by AFA-positive IgG, with a peak at 24 h (Fig. 2B), COL1A1 and TIMP-1 were not (Fig. 2A). Similar results were obtained by northern blot and ribonuclease protection assay (data not shown). The gelatinase MMP-9 mRNA was also increased in fibroblasts by AFA-positive IgG (Fig. 2C), whereas mRNA levels of CTGF and α-SMA, which are present in pro-fibrotic condition and over expressed in SSc fibroblasts, were not modified (Fig. 2D and E). AFA-negative and NHS IgG did not affect transcription in any of the genes studied (Fig. 2A–E). In conclusion, AFA-positive IgG increases the mRNA steady-state levels of MMP-1 and MMP-9, but does not affect those of genes associated with fibrosis such as type-I collagen, TIMP-1, CTGF and α-SMA.

AFA-positive IgG does not induce Smad 2/3 phosphorylation

Since there is increasing evidence that alterations in the TGF-β signalling pathway and in particular in the levels of Smad 2/3 may contribute to abnormal ECM deposition and it has been reported that scleroderma fibroblasts display an enhanced phosphorylation of Smad 2/3, also in the absence of TGF-β [9], we tested the expression of phospho-Smad 2/3 by western blotting. AFA-positive IgG did not induce any detectable Smad 2/3 phosphorylation, in contrast to TGF-β, used as positive control (Fig. 3). This finding is consistent with the lack of a pro-fibrotic activity of AFA-positive IgG on fibroblasts.

MMP-1 induction by AFA-positive IgG is not mediated by TNF or IL-1

TNF and IL-1 are key regulators of inflammatory responses and are potent inducers of MMPs [24, 25]. To test the hypothesis that

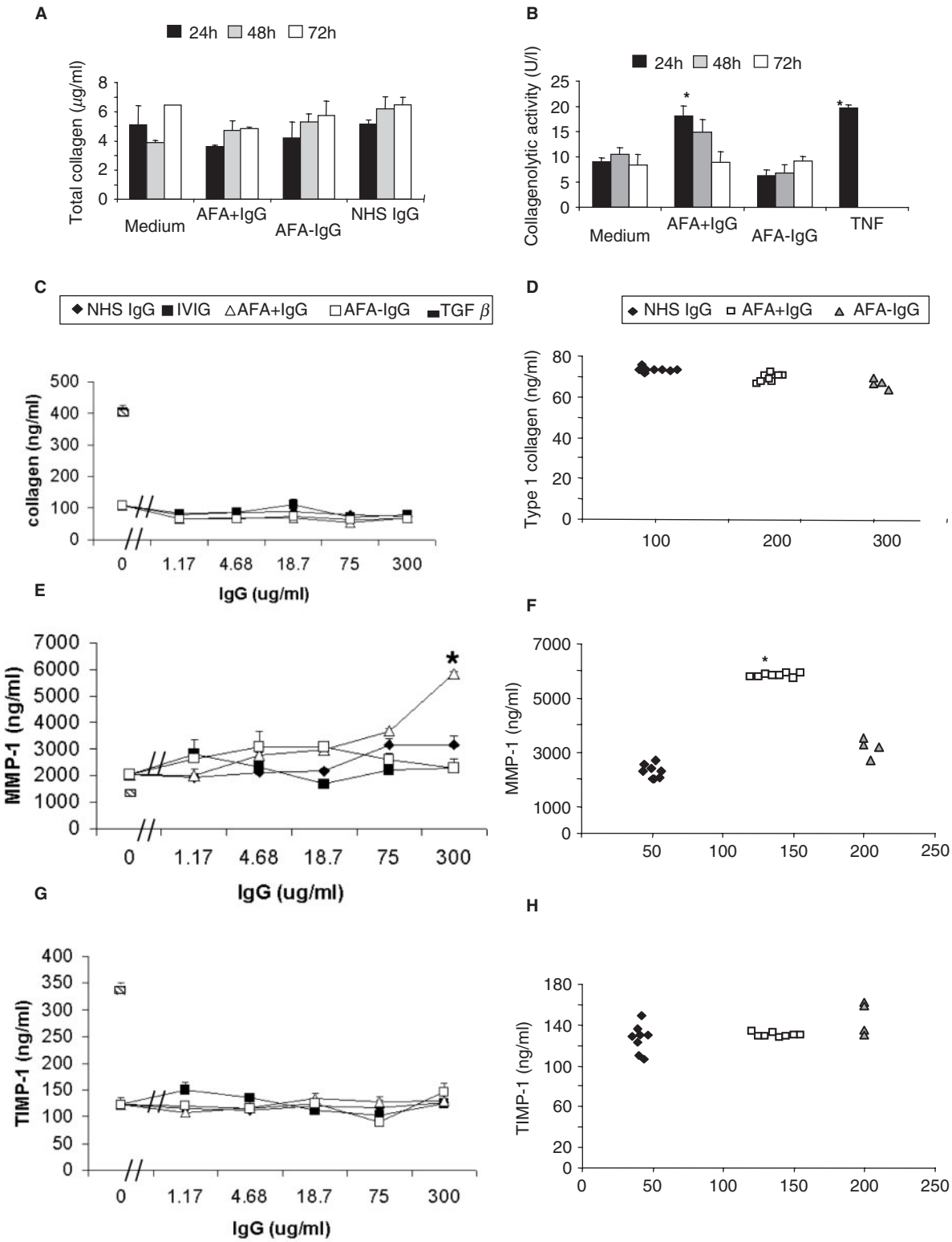

FIG. 1. Effect of AFA+IgG, AFA−IgG, NHS IgG and IVIg on type I collagen, MMP-1 and TIMP-1 proteins. (**A**) Total collagen detected by Sircol assay. (**B**) Collagenase activity. In **A** and **B** bars represent the mean ± S.E.M. of 4 AFA+IgG, 2 AFA−IgG and 4 NHS IgG tested in two distinct experiments (IgG = 300 μg/ml, TNF = 10ng/ml). (**C, E, G**) IgG mean ± S.E.M. of 8 AFA+IgG, 4 AFA−IgG, 8 NHS IgG and 1 IVIg. TGF-β (10 ng/ml) was used as positive control. (**D, F, H**) values at 300 μg/ml are represented. $^{*}P < 0.05$ ANOVA test.

AFA-positive IgG could induce MMP-1 indirectly by eliciting the autocrine production of IL-1 or TNF, the activity of AFA-positive IgG was assessed in the presence of IL-1Ra and of anti-TNF. While IL-1Ra abrogated MMP-1 production induced by IL-1β and anti-TNF that is induced by TNF (Fig. 4B), neither of these two inhibitors affected MMP-1 induced by AFA-positive IgG (Fig. 4A). This strongly indicates that AFA-positive IgG does not act by eliciting an autocrine production of IL-1 or TNF.

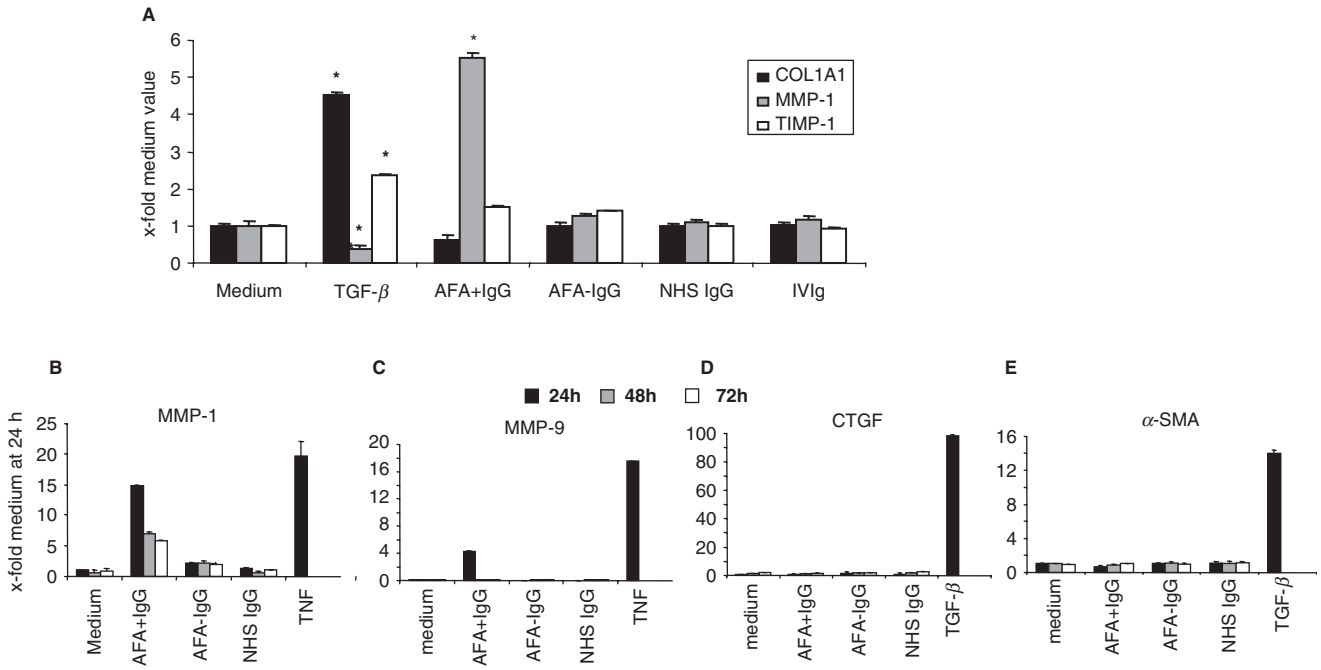

FIG. 2. Effect of AFA on COL1A1, MMP-1, TIMP-1, CTGF and α-SMA mRNA levels assessed by quantitative RT-PCR. (**A**) Bars represent the mean ± S.E.M. of 8 AFA+IgG, 4 AFA−IgG, 8 NHS IgG and an IVIg of mRNA at 24 h of culture. *$P < 0.05$ compared with the medium. (**B, C, D, E**) mean ± S.D. of a representative experiment of three in which 2 AFA+IgG, 2 AFA−IgG and 2 NHS IgG were tested.

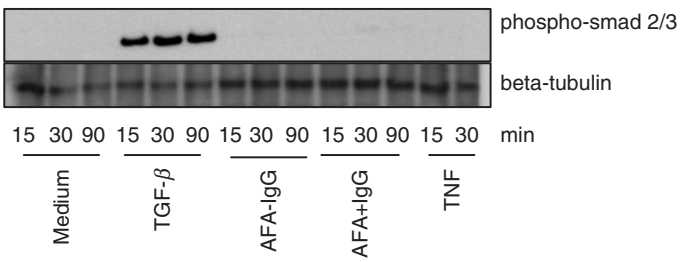

FIG. 3. AFA+IgG do not induce Smad 2/3 phosphorylation. (TGF-β 10 ng/ml, TNF 10 ng/ml, IgG 300 μg/ml). One representative western blot of two.

Anti-topoisomerase-I antibodies do not bind to fibroblast surface and do not induce MMP-1 production

Direct binding of anti-topo-I auto-Ab to the cell surface of fibroblasts that induces monocyte adhesion and activation has been reported in patients with SSc [12, 13]. To test the possibility that topo-I may be recognized by AFA, we screened AFA-positive and AFA-negative SSc sera for their anti-topo-I reactivity. Eight of eight AFA-positive sera and 11 of 12 AFA-negative sera were positive for anti-topo-I auto-Ab (Table 1), thus indicating a dissociation for the presence of AFA-positive and anti-topo-I IgG within individual sera. We further assessed whether a mouse anti-topo-I mAb could bind to fibroblast cell surface and induce MMP-1 production. The murine anti-topo-I mAb displayed a nuclear fluorescence in fibroblasts made permeable, but failed to bind the plasma membrane in intact cells (Fig. 5A, panels 3 and 4). This was distinctly different from AFA-positive IgG that showed a nuclear fluorescence in cells made permeable and a surface fluorescence in intact fibroblasts (Fig. 5A, panels 2 and 3). The lack of binding of anti-topo-I mAb on fibroblast surface up to a concentration of 100 μg/ml was confirmed in a cell-based ELISA (data not shown). Moreover no ICAM-1 (Fig. 5B) and MMP-1 (Fig. 5C) regulation was observed in the presence of anti-topo-I mAb, in contrast to AFA-positive IgG. Overall, these results indicate that antibodies directed against topo-I differ from AFA-positive IgG in terms of binding to fibroblasts and biological functions.

Discussion

The pathogenesis of SSc remains poorly understood and no unitary theory has been developed to clarify the interactions between autoimmunity and fibrosis. In this study, we addressed the question whether autoantibodies binding to fibroblast surface (AFA) may induce changes in fibroblast metabolism affecting ECM deposition. Our data strongly indicate that AFA enhance the collagenolytic activity of fibroblasts by favouring MMP-1 and MMP-9 gene transcription and MMP-1 production without affecting their production of TIMP-1 and of collagens including type I collagen. In addition, AFA failed to induce CTGF and α-SMA gene transcription, and Smad 2/3 signalling, currently considered markers of fibroblasts actively involved in ECM deposition. In the search for molecular mechanisms triggered by AFA in inducing MMP-1, we explored the possible contribution of IL-1 and TNF. Indeed, IL-1 and TNF are known to be potent inducers of MMPs [24, 25] and autocrine production of these cytokines by AFA-activated fibroblasts could explain our findings. In addition, while direct testing of our IgG preparations failed to detect IL-1 and TNF, we could not exclude the contaminating presence of minute, but biologically active, amounts of these cytokines. The results obtained with biological agents capable of neutralizing IL-1 and TNF, however, proved that these cytokines were not involved in MMP-1 production in our experiments. Thus, these findings suggest a direct effect of AFA on fibroblasts.

We have not yet identified the autoantigen(s) recognized by AFA. Several, different, non-ubiquitous autoantigens have recently been reported to be recognized by IgG from SSc patients. Of interest, antibodies recognizing fibrillin-1 have been shown to activate fibroblasts and MMP-1 was among the up-regulated genes [15]. At variance with our findings, however, fibrillin-1-specific IgG also induced collagen gene up-regulation

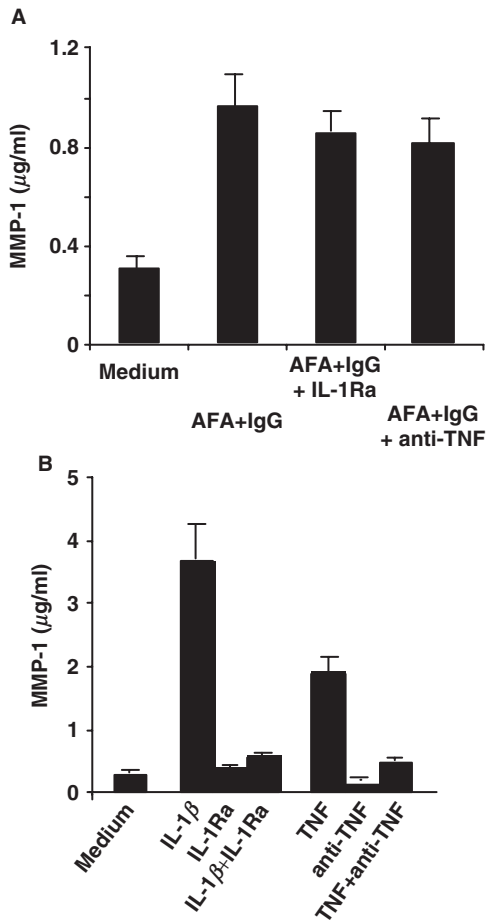

FIG. 4. AFA+ IgG induce MMP-1 in an IL-1 and TNF-independent manner. The bars represent the mean ± S.E.M. of 3 AFA+ IgG, and 3 NHS IgG. (IL1-β 10 ng/ml, IL-1Ra 2 μg/ml, TNF 10 ng/ml, anti-TNF-α 10^{-8} M and IgG 300 μg/ml). The inhibitors were added 1 h before the agonist. Proteins were determined by ELISA after 48 h of culture.

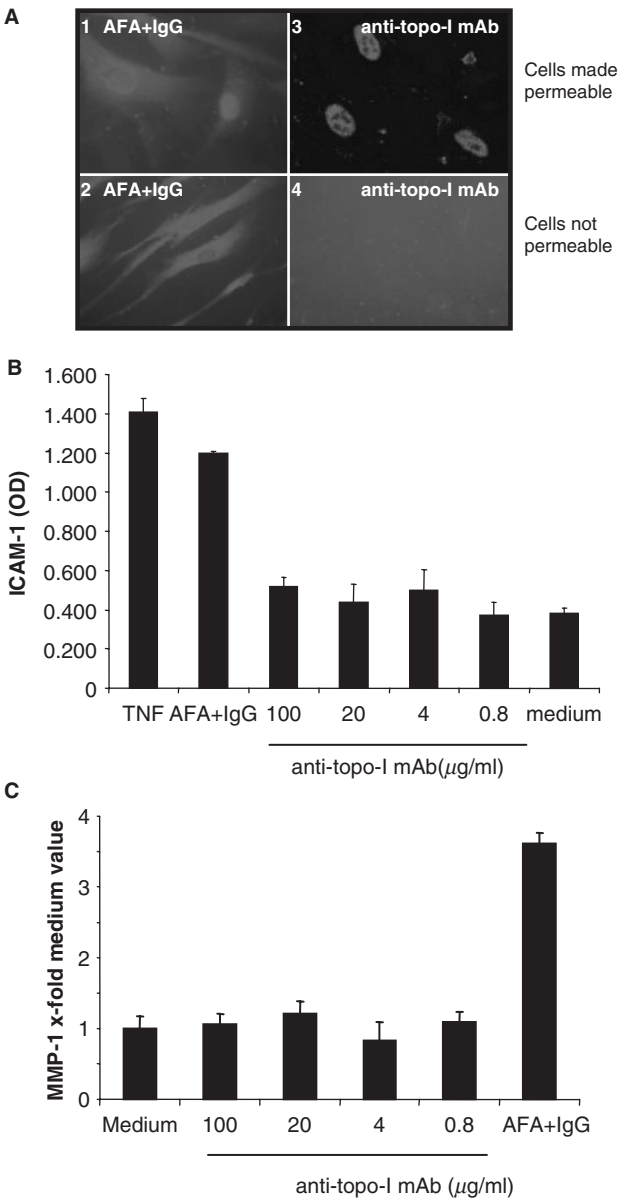

FIG. 5. Anti-topo-1 antibody do not bind to the surface of fibroblasts and do not induce ICAM-1 and MMP-1 up-regulation. (**A**) Indirect immunofluorescence microphotographs (original magnification 630×). In the upper panels, fibroblasts were made permeable and then incubated an hour with AFA+IgG (300 μg/ml) and anti-topo-I (20 μg/ml). In the lower panels, fibroblasts were not made permeable. Anti-topo-I mAb gives only a nuclear staining. AFA+ IgG, which is also anti-topo-I-positive, gives both nuclear and plasma-membrane staining depending on whether cells were permeable or not. One of the three independent experiments. (**B**) ICAM-1 expression tested in cell-based ELISA on fibroblasts cultured for 24 h. Mean ± S.D. of triplicates of a representative experiment of two. (**C**) MMP-1 ELISA on supernatants of fibroblasts cultured 48 h with AFA+ IgG (300 μg/ml) and anti-topo-I mAb. One representative experiment of three.

in a TGF-β-dependent manner [15]. Furthermore, autoantibodies, binding to the PDGF-R, have been shown to activate fibroblasts, favouring collagen deposition [16]. Antibodies cross-recognizing UL94, a CMV peptide, and NAG-2, an adhesion molecule expressed on fibroblast surface, also favoured collagen synthesis [18, 19]. Thus, a large panel of autoantibodies with presumed different specificities, present in SSc sera, is being characterized and appear to induce a variety of non-overlapping fibroblast responses, which may participate to events relevant to SSc pathogenesis. Within this variety, the anti-fibroblasts antibodies we detect by cell-based ELISA are enriched for IgG with predominant collagenolytic activity. In this respect, it is interesting to note that beside their involvement in matrix digestion, MMP induced by AFA could modulate inflammation by enhancing or dampening the biological activity of several mediators, including cytokines and chemokines [26]. Modulation of inflammation may then impact on the development of fibrosis [27].

Consistently with a lack of enhanced collagen deposition, we could not detect SMAD phosphorylation in fibroblasts stimulated by AFA. In preliminary experiments, we observed that AFA were activating other intracellular signalling pathways including the MAP kinase ERK 1/2, and the transcription factors NF-κB and AP-1.

The samples we tested were from patients with heterogeneous clinical presentations and different disease duration. It is also possible that previous treatment had an impact on AFA levels. However, the biological activities of IgG were robust and consistent across the various assays preformed (present article and Fineschi *et al.*, manuscript in preparation). In our hands, the most important characteristic for predicting biological activity was the capacity of IgG to bind to fibroblast surface as detected by cell-based ELISA. Indeed, binding was always accompanied by substantial biological activity, while no binding correlated with no or very low biological activity. In this respect, it is interesting to note that most of the IgG we tested were from individuals who were anti-topo-I-positive, but binding to fibroblasts did not correlate with anti-topo-I positivity. In addition, a mouse anti-topo-I mAb failed to induce MMP-1 or ICAM-1 in exposed fibroblasts, thus strongly suggesting that anti-topo-I Ab are not directly involved in fibroblast activation, while they may, as

suggested by others, participate to monocyte recruitment leading to a cascade of events relevant to SSc pathogenesis [12, 13].

In conclusion, our data concur with those reported by others in indicating that anti-fibroblast antibodies may not only be an epiphenomenon resulting from the loss of tolerance, but may have pathogenic relevance. Thus, AFA may participate, at least in a subset of SSc individuals, to events that indirectly, by favoring inflammation, or directly, by reprogramming fibroblast gene expression, result in pathological changes characteristic of SSc.

Rheumatology key messages

- Antibody binding to fibroblast surface in SSc sera activate fibroblast inflammatory responses.
- Humoral immunity may participate in pathogenetic events relevant to SSc development.

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