Serum levels, tissue expression and cellular secretion of macrophage migration inhibitory factor in limited and diffuse systemic sclerosis

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ABSTRACT

Objective. *To investigate serum levels, tissue/cellular expression of macrophage migration inhibitory factor (MIF) in patients with limited (lSSc) and diffuse (dSSc) systemic sclerosis.*

Methods. *10 lSSc-patients, 10 dSSc-patients and 10 controls were enrolled. MIF serum levels were assayed by ELISA. MIF and its receptors CD74/CD44 were evaluated by immunohistochemistry on skin biopsies from patients with dSSc, lSSc (affected and not-affected skin) and controls. MIF levels were assessed (ELISA) in supernatants of healthy dermal microvascular endothelial cells (MVECs) and in control (CTR), non-affected SSc (NA) and affected (SSc) fibroblasts treated for 48h with 10% control serum and 10% SSc-serum. MIF supernatant (ELISA) and mRNA (quantitative real-time PCR) levels were determined in SSc dermal fibroblasts and in control dermal fibroblasts untreated or stimulated at 6h-24h-48h with bleomycin (50mU/ml).*

Results. *Serum MIF was significantly higher in dSSc (18.7±4.1 ng/ml, $p<0.001$) and in lSSc (10.4±4.4 ng/ml, $p<0.001$) patients respect to controls (2.6±1.4 ng/ml). Enhanced MIF immunoreactivity was found in keratinocytes, fibroblasts, endothelium, sebaceous/sweat glands from lSSc/dSSc affected skin. Faint MIF immunoreactivity was found in control skin and not-affected skin of lSSc patients. No differences were found in CD74/CD44 receptors' analysis among control and dSSc/lSSc affected and non-affected skin. MVECs and fibroblasts (CTR, NA and SSc) produced significantly more MIF, when stimulated with SSc serum respect to control-serum ($p<0.001$). Finally, MIF mRNA levels significantly increased at 6h ($p<0.001$) and decreased at 48h ($p<0.001$) in control fibroblasts treated with bleomycin compared to control untreated. Simultaneously, MIF supernatant protein levels increased after 48h ($p<0.01$) in bleomycin-treated fibroblasts respect to untreated ones.*

Conclusion. *These results suggest that MIF could be implicated in the pathogenesis of SSc, probably acting as protective factor against the SSc stressful conditions.*

Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterised by microvascular damage and progressive skin and internal organ fibrosis (1). Its pathogenesis remains incompletely understood (1). Immune activation, vascular impairment, and excessive synthesis of extracellular matrix are all known to be important in the development of this illness (2). Despite SSc is considered as a fibrotic disease with relatively poor inflammatory component (3), recent data demonstrated the contribution of some pro-inflammatory cytokines in the development of the disorder (4). Macrophage migration inhibitory factor (MIF) is a multi-functional protein that operates as a cytokine and an enzyme (5). As a cytokine, MIF acts as a major regulator of inflammation and a central upstream mediator of innate immune response (5); moreover, it functions as a key mediator to counter-regulate the immune-inhibitory effects of glucocorticoids (6). MIF was initially identified as the protein secreted by activated T lymphocytes, capable of inhibiting random migration of macrophages, concentrating macrophages at inflammation loci, and enhancing their ability to kill intracellular parasites and tumoural cells (7, 8). Nowadays, several types of cells (macrophages, endothelial cells,

and fibroblasts) are considered capable to produce MIF (9). The role of MIF has been studied in some rheumatic diseases, such as systemic lupus erythematosus (SLE) (10) and rheumatoid arthritis (RA) (11). In these conditions, MIF polymorphisms seem to be associated with both higher risk and severity of RA (12) and with SLE susceptibility (13). In contrast, its potential role in the pathogenesis of SSc remains poorly understood. Selvi *et al*. in 2003 demonstrated that MIF serum levels were higher in dSSc patients respect to controls and that MIF is expressed prevalently by suprabasal keratinocytes and perivascular mononuclear cells in the sclerodermic skin (14). Since then, literature lacks data concerning a more detailed analysis of MIF expression in serum and skin of sclerodermic patients, as regards its hypothetic role in the development of the disease, as regards its possible link to the clinical picture of the disorder.

We believe that this scarcity of studies depends also on the controversial experimental data related to the effect of MIF in the skin, during the wound healing process (15). Mechanisms underlying MIF target cell action were discovered when MHC class II chaperone CD74 was identified as MIF receptor (16, 17). Recently, transmembrane CD44 was considered as a potential accessory protein required for MIF-CD74 signal transduction (18). However, little is still known about the exact role of the MIF coupled CD74/CD44 receptors in the human skin, even if it has been supposed that CD74 and CD44 are necessary to MIF protection from apoptosis (19).

Based on these assumptions, the aim of the present study is to better investigate the role of MIF in SSc, by analysing serum MIF levels in lSSc, dSSc and controls, by evaluating both MIF and CD74/CD44 receptors' expression in sclerodermic and control skin, and by considering the MIF secretion from cultured endothelial cells and fibroblasts.

Lastly, to better understand when fibroblasts start to produce MIF during the differentiation process into myofibroblasts, MIF mRNA and protein levels were evaluated in healthy cultured fibroblasts activated by bleomycin, a pro-fibrotic molecule.

Materials and methods

Patients

Skin samples obtained from the dorsal mid-forearm of 20 SSc women were examined (20). Of the 20 patients, 10 presented lSSc and 10 presented dSSc, diagnosed in accordance with LeRoy and Colleagues (21). Unaffected areas of skin from the same lSSc patients and control skin from 10 gender and age/sex-matched voluntary healthy subjects were also evaluated. The lSSc patients' unaffected skin (taken from the unaffected areas of dorsal forearms) was graded as zero according to the modified Rodnan skin score (22). Table I shows the major clinical characteristics of the patients. Moreover, blood samples were collected, centrifuged, and the sera were used immediately or stored at -20ºC until use. All patients and control subjects gave fully informed voluntary written consent, according to the principles of the Declaration of Helsinki and in compliance with the Ethics Committee of the University of Siena (CEL AOUS 28/02/12).

Fibroblast cultures

Fibroblasts obtained from skin biopsies were cultured in DMEM (Sigma-Aldrich. St. Louis, MO, USA) supplemented with penicillin (100 U/mL) (Sigma-Aldrich), streptomycin (100 µg/mL) (Sigma-Aldrich), 0.25 µg/ml amphotericin B (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich) and 10% FBS (Sigma-Aldrich) and incubated at 37°C in atmosphere of 5% CO_2 95% air, until confluence (1 week) onto 75 cm^2 flasks (BD Costar Cambridge, MA, USA). Viability was estimated by Trypan blue (Sigma-Aldrich). Confluent cultures of control (CTR), not affected sclerodermic (NA) and affected sclerodermic (SSc) fibroblasts were washed twice with PBS and incubated overnight in DMEM supplemented with 2% FBS. Twelve hours before the experiments, fibroblasts (CTR, NA and SSc) were left in serum-free medium.

Fibroblast treatments with control and SSc sera

Fibroblasts were challenged with DMEM supplemented with pooled (n=10) 10% lSSc serum, pooled (n=10) 10% dSSc serum and pooled (n=10) 10% control serum, respectively. After 48 hours, culture supernatants were centrifuged at 1500 revolutions per minute for 10 minutes and either tested immediately or stored at -80ºC until use. It is important to underline that before treatments, all the conditioned media containing 10% human serum were tested (ELISA) for MIF concentration. Therefore, to assess the real endogenous MIF production by fibroblasts, MIF concentration levels, calculated in the conditioned media before the experiments, were subtracted to MIF levels recorded after treatments.

MVECs cultures

MVECs (originated from the digital microvasculature of an healthy 40-year-old female donor) were purchased from Medicyte (Heidelberg, Germany), and cultured in upcyte® MVEC Growth Medium (Medicyte, Heidelberg, Germany), supplemented with 10% fetal calf serum (FCS) (Medicyte) onto 75 cm^2 flasks (BD Costar), and incubated at 37ºC in atmosphere of 5% CO_2 95% air, till confluence (1 week). Confluent cultures of MVECs were washed twice with PBS and incubated overnight in upcyte® MVEC Growth Medium supplemented with 2% FCS. Twelve hours before the experiments, MVECs were left in serum-free medium.

MVECs treatments with control and SSc sera

MVECs were challenged with upcyte® MVEC Growth Medium supplemented with pooled (n=10) 10% lSSc serum, pooled (n=10) 10% dSSc serum and pooled (n=10) 10% control serum, respectively. After 48 hours, culture supernatants were centrifuged at 1500 revolutions per minute for 10 minutes and either tested immediately or stored at -80ºC until use. All the conditioned media containing 10% human serum were tested (ELISA) for MIF concentration. Therefore, to assess the real endogenous MIF production by MVECs, MIF concentration levels, calculated in the conditioned media before the experiments were subtracted to MIF levels recorded after treatments.

Table I. Clinical parameters of patients with lSSc and dSSc.

Patient	Age (years)	Sex	Disease duration (years)	Digital ulcers	PAH	*Drug treatment	ANA-ENA
1 lSSc	64	F	8	no	no	Nifedipine	Anticentromere
2 lSSc	35	F	9	no	no	Nifedipine, NSAIDs	Anticentromere
3 lSSc	72	F	11	yes	yes	Nifedipine, Bosentan	Anticentromere
4 lSSc	49	F	7	no	no	Nifedipine	Anticentromere-CenpB
5 lSSc	37	F	5	no	no	Nifedipine	Anticentromere
6 lSSc	52	F	10	no	no	Nifedipine, NSAIDs	Anticentromere-SSA/ro
7 lSSc	59	F	8	no	yes	Nifedipine, Bosentan	Anticentromere-CenpB
8 lSSc	66	F	9	no	no	Nifedipine, NSAIDs	Antinucleolar-CenpB
9 lSSc	58	F	6	no	no	Nifedipine	Anticentromere
10 lSSc	65	F	10	no	no	Nifedipine, NSAIDs	Anticentromere-CenpB
Mean (SD)	55.7 (12.3)		8.3 (1.8)				
11 dSSc	46	F	7	no	no	Nifedipine	Antinucleolar-Scl-70
12 dSSc	48	F	8	no	no	Nifedipine	Anticentromere-Scl-70
13 dSSc	55	F	8	no	no	Nifedipine	Antinucleolar-Scl-70
14 dSSc	71	F	12	no	no	Nifedipine, NSAIDs	Antinucleolar-Scl-70
15 dSSc	61	F	8	no	no	Nifedipine, NSAIDs	Anticentromere-Scl-70
16 dSSc	63	F	9	no	yes	Nifedipine, Bosentan	Antinucleolar-Scl-70
17 dSSc	50	F	6	yes	yes	Nifedipine, Bosentan	Anticentromere-Scl-70
18 dSSc	42	F	9	yes	yes	Nifedipine, Bosentan	Anticentromere-Scl-70
19 dSSc	58	F	11	yes	yes	Nifedipine, Bosentan	Antinucleolar-Scl-70
20 dSSc	56	F	10	yes	yes	Nifedipine, Bosentan	Antinucleolar-Scl-70
Mean (SD)	55 (8.7)		8.8 (1.8)				

ANA: Anti-nuclear antibodies; ENA: Extractable nuclear antigens; NSAIDs: non-steroidal anti-inflammatory drugs; PAH: pulmonary arterial hypertension. *Drug treatment: It is important to underline that patients with gastroesophageal involvment (16 dSSc, 20 dSSc) were receiving also prokinetic and proton pump inhibitor agents. Moreover, patients with digital ulcers (3 lSSc, 17 dSSc, 18 dSSc, 19 dSSc and 20 dSSc) interrupted therapy with Iloprost one year before the initiation of the study; three of them (17 dSSc, 19 dSSc and 20 dSSc) because of the ineffectiveness of the treatment, two of them (3 lSSc, 18 dSSc) because of drug intolerance.

MIF enzyme linked immunosorbent assay (ELISA)

MIF concentrations in culture supernatants and in serum samples were measured by a colorimetric sandwich ELISA. Ninety six well ELISA plates were coated with 100 µl/well of antihuman MIF monoclonal antibody (2.0 mg/ml; R&D System, Abingdon, UK) and incubated overnight at room temperature. The plates were washed three times with washing solution (10 mM phosphate buffered saline (PBS; pH 7.4), 0.05% (vol/vol) Tween 20), blocked by adding 300 µl of blocking solution (10 mM PBS (pH 7.4), 1% (wt/vol) bovine serum albumin (BSA), and 5% (wt/vol) sucrose), and incubated at room temperature for 1.5 hours. After washing three times, the samples and the standard (bacterially expressed recombinant human MIF; R&D Systems), appropriately diluted in Tris buffered saline-BSA (20mMTris-HCl, 150mMNaCl (pH 7.3), 0.1% (wt/vol) BSA, 0.05% (vol/vol) Tween 20) were added in duplicate (100 µl/well) and incubated for two hours at room temperature. The plates were washed three times and 100 µl of biotinylated goat anti-human MIF antibody (200 ng/ml; R&D Systems) was added to each well and incubated for two hours at room temperature. The plates were washed again and streptavidin horseradish peroxidase (Zymed, San Francisco, CA) was added to each well and incubated for 20 minutes at room temperature. After washing, 3,3',5,5'- tetramethylbenzidine (Zymed, San Francisco, CA) was added. After 20 minutes, the reaction was stopped by adding H_2SO_4. Absorbance was measured at 450 nm. MIF concentration was expressed as pg per ml in serum samples or ng per cell number in cell culture supernatants. The sensitivity limit was 18 pg/ml. Intra- and inter-assay coefficients of variation were 3.86 (0.95)% and 9.14 (0.47)%, respectively.

MIF and CD74/CD44 Immunohistochemistry

Immunohistochemistry was performed on skin biopsy specimens of patients with dSSc, lSSc and controls. Briefly, 4 µm sections were obtained from skin specimens fixed in 10% buffered formalin and embedded in paraffin. Sections were dewaxed, rehydrated, and washed in Tris buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl (pH 7.6)). Antigen retrieval was carried out by incubating sections in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven at 750 W for five minutes. Slides were preincubated with normal rabbit or goat serum (Dako, Copenhagen, Denmark) to prevent non-specific binding, and incubated overnight at 4°C with the anti-human MIF goat polyclonal antibody (Abcam, Cambridge, UK) diluted 1:300 in TBS, anti-human CD74 rabbit polyclonal (Abcam) diluted 1:100 in TBS and anti-human CD44 rabbit polyclonal (Abcam) diluted 1:50 in TBS. Slides were then washed with TBS, and incubated with a rabbit anti-goat (for MIF) or goat anti-rabbit (for CD74 and CD44) antibodies labelled with biotin (Dako), both at a dilution of 1:500, for 30 minutes. The reaction was developed using streptavidin-biotin complex (Dako). Sections were

counterstained. Slides were mounted and examined under a light microscope. Negative control was obtained by replacing the specific primary antibody with TBS. The positivity of reaction was assessed by using AxioVision 4.7 Microscope Software (Zeiss, Germany) for Windows. This system creates a binary image through color intensity thresholds and calculates the ratio between the blank area and the measured area of interest. The analysis was performed by two independent operators in a blinded fashion, and repeated measurements ensured constant and reproducible results.

Fibroblast treatments with bleomycin

Fibroblasts isolated from skin samples of control subjects (n=10) were passaged twice and then were left untreated or treated with bleomycin (50mU/ml) (Sigma-Aldrich) for 48 hours to induce the fibrogenic phenotype (myofibroblast) (23). Simultaneously, SSc fibroblasts isolated from skin samples of lSSc (n=10) and dSSc (n=10) patients were passaged twice and left untreated in culture for 48 hours.

RNA isolation and real time PCR

After 6, 24 and 48 hours over culture, control fibroblasts (n=10 healthy donors) (untreated or treated with bleomycin) and SSc fibroblasts (n=10 patients, 5 with lSSc and 5 with dSSc) were collected in TRIZOL reagent (Sigma-Aldrich). Total RNA was extracted following the manufacturer's instructions. The total RNA content of the samples was quantified by measuring the absorbance at 260 nm, by Ultrospec2000 spectrophotometer (Amersham Pharmacia Biotech, NJ, USA). The RNA was then reverse transcribed using random hexamer MultiScribe enzyme (Applied Biosystems Group, Foster City, CA). Quantitative PCR (qRT-PCR) reactions were run in the StepOne Real-Time PCR System instrument (Applied Biosystems Group) using TaqMan chemistry. Two microliters of cDNA in a final volume of 20 µl was amplified using the 20× Assays-on-Demand gene expression assay mix (Applied Biosystems Group). TaqMan probes and specific primers for MIF and ribosomal 18S, selected as housekeeping gene, were purchased from Applied Biosystems Group. The MIF mRNA levels were normalised to those of 18S, and the relative mRNA levels after the treatment were calculated using the $\Delta\Delta C_T$ method (24).

Statistical analysis

A Mann-Whitney rank sum test was used to compare serum MIF in control subjects *versus* lSSc patients, control subjects *versus* dSSc patients and finally lSSc *versus* dSSc patients. Data are reported as ng/ml ± standard deviation (SD). Statistical significance was set at $p<0.05$. To assess MIF differences in MVECs and fibroblast supernatants (both sera and bleomycin experiments) ANOVA and Tukey's post test were used. Data are reported as ng/cell number ± SD. The software used to perform Mann-Whitney rank sum test, ANOVA and Tukey's GraphPad Prism 5® for Windows. Real time PCR statistics was performed using REST© (Relative Expression Software Tool). Data are reported as mean fold change ± SD of healthy fibroblasts stimulated with bleomycin and SSc fibroblasts *versus* healthy control fibroblasts untreated at each time point.

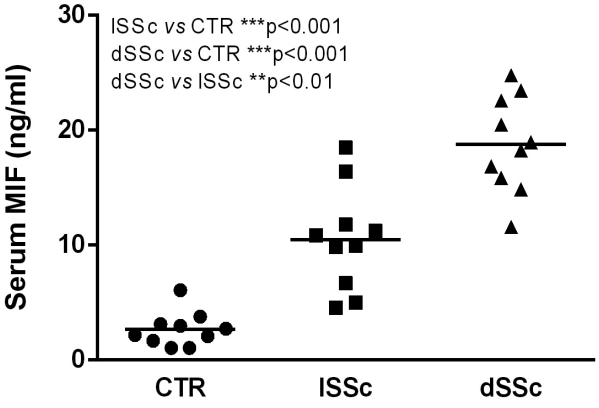

Fig. 1. Serum MIF levels (ng/ml) from 10 healthy controls (CTR), 10 patients with limited systemic sclerosis (lSSc) and 10 patients with diffuse systemic sclerosis (dSSc) matched for age and sex. The concentration of MIF in patients with lSSc and dSSc was significantly higher than in controls ($p<0.001$). MIF concentration was significantly higher in the dSSc patients than in the lSSc ($p<0.01$).

Results

MIF serum levels in SSc patients and healthy controls

Serum MIF levels were significantly higher in lSSc (10.4±4.4 ng/ml, $p<0.001$) and in dSSc (18.7±4.1 ng/ml, $p<0.001$) patients respect to healthy controls (2.6±1.4 ng/ml) (Fig. 1). Moreover, MIF serum levels were significantly higher in dSSc respect to lSSc patients ($p<0.01$).

MIF in MVECs supernatants treated with control and SSc sera

Figure 2 shows MIF supernatant levels in MVECs treated with control serum, lSSc and dSSc serum respectively. After 48 hours, MIF levels were significantly higher in MVECs treated with lSSc (6.90±0.60 ng/2*10^5 cells, $p<0.001$) and dSSc (8.61±0.59 ng/2*10^5 cells, $p<0.001$) serum, respect to control serum (3.46±0.47 ng/2*10^5 cells). Interestingly, differences were found also between lSSc and dSSc serum ($p=0.025$)

MIF in fibroblast supernatants treated with control and SSc sera

Figure 3 shows MIF levels in supernatant of CTR, NA and SSc fibroblasts treated with medium alone, control, lSSc and dSSc sera, respectively. In all the above mentioned fibroblast groups, MIF levels were significantly higher in fibroblasts stimulated with lSSc ($p<0.001$) and dSSc ($p<0.001$) sera, in comparison to those stimulated with control sera. Moreover, significant differences in CTR fibroblasts, were found between lSSc (2.12±0.31 ng/2*10^5 cells) and dSSc (2.79±0.37 ng/2*10^5 cells) sera ($p<0.05$). The same relevant MIF differences between lSSc (2.24±0.36 ng/2*10^5 cells) and dSSc (3.37±0.51 ng/2*10^5 cells) were found in NA fibroblasts ($p<0.05$).

MIF, CD74/CD44 immunohistochemistry on skin biopies

Figure 4 shows MIF and its receptors CD74/CD44 immunostaining in control skin, in both not-affected and affected skin of lSSc patients and in affected skin of dSSc patients. In control skin (a) and in unaffected skin from lSSc patients

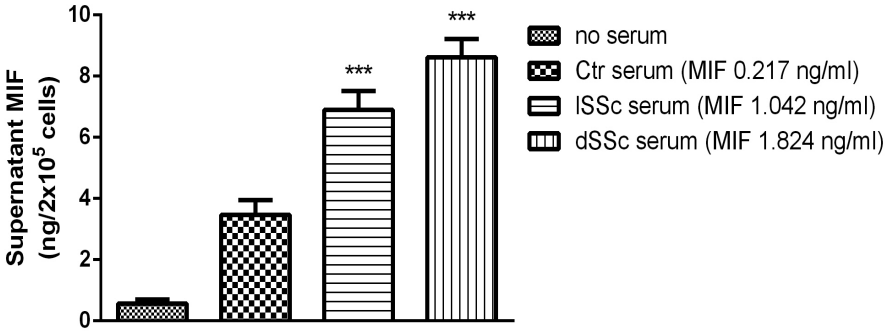

Fig. 2. Supernatant MIF from healthy dermal microvascular endothelial cells (MVECs) untreated (no serum) or treated for 48 hours with 10% pooled (n=10) sera from healthy donors (Ctr serum containing 0.217 ng/ml of MIF), 10% pooled (n=10) sera from lSSc patients (lSSc serum containing 1.042 ng/ml of MIF) and 10% pooled (n=10) sera from dSSc patients (dSSc serum containing 1.824 ng/ml of MIF). Data are reported as ng/2*10^5 cells. Tukey's Multiple Comparison Test respect to Ctr serum (*** p<0.001). MIF supernatant levels were significantly higher in MVECs treated with dSSc serum respect to lSSc serum (p=0.025; patients than in lSSc ones (p<0.01; Tukey's Multiple Comparison Test).

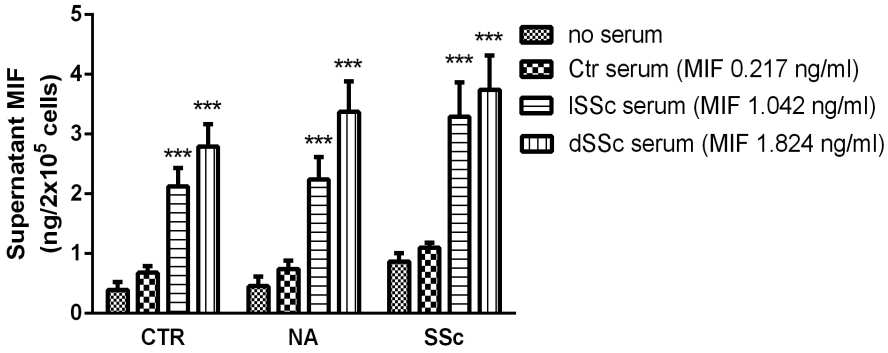

Fig. 3. Supernatant MIF levels from control (CTR), not-affected lSSc (NA) and affected lSSc/dSSc (SSc) fibroblasts untreated (no serum) or treated for 48 hours with 10% pooled (n=10) sera from healthy donors (Ctr serum containing 0.217 ng/ml of MIF), 10 % pooled (n=10) sera from lSSc patients (lSSc serum containing 1.042 ng/ml of MIF) and 10 % pooled (n=10) sera from dSSc patients (dSSc serum containing 1.824 ng/ml of MIF). Data are reported as ng/2*10^5 cells. Tukey's Multiple Comparison Test in each group respect to Ctr serum (*** p<0.001).

(b), MIF immunoreactivity was faint and mostly found in epidermal basal layers, as previously reported (9, 14). By contrast, both lSSc (c) and dSSc (d) affected skin showed fibroblasts positive for MIF in the dermis and keratinocytes in the epidermal layer. Endothelium (e), sweat glands (f) and sebaceous glands (g) resulted positive for MIF in both lSSc and dSSc affected skin. No alterations in CD74 immunoreactivity were found in control (h), not-affected lSSc (i), affected lSSc (l) and affected dSSc (m) skin samples. Finally, CD44 immunostaining appeared to be the same in control (n), not affected lSSc (o), affected lSSc (p) and affected dSSc (q) skin samples.

MIF mRNA and protein levels in fibroblasts treated with bleomycin

Figure 5 shows MIF mRNA levels (a) and protein levels of secreted MIF (b) in control fibroblasts (untreated or treated with bleomycin) and in SSc fibroblasts. Control fibroblasts, treated with bleomycin, showed increased MIF mRNA levels at 6 hours (fold change 1.9±0.3, p<0.001) and decreased levels at 48 hours (fold change 0.47±0.1, p<0.001) respect to control untreated fibroblasts. MIF supernatant protein levels have proved to have an opposite behavior respect to mRNA levels: in fact, they were low (0.26±0.08 ng/2*10^5 cells) when mRNA levels were the highest (6 hours) and were high (0.70±0.15 ng/2*10^5 cells, p<0.01) when mRNA levels were the lowest (48 hours). SSc fibroblasts MIF mRNA levels increased not significantly at 24 hours and decreased significantly at 48 hours (p<0.05). Whereas, SSc fibroblasts MIF supernatant protein levels gradually increased from 6 to 48 hours, with statistical significance recorded at 48 hours (0.59±0.10 ng/2*10^5 cells, p<0.05).

Discussion

In the present study, we showed that serum levels of MIF are significantly increased in patients with SSc respect to healthy controls, which is consistent with previous studies (14, 25). In particular, we demonstrated that MIF serum levels are significantly higher in dSSc patients respect to lSSc ones, probably related to the extent of tissue damage. For the first time, we showed that MIF immunostaining is similar between control skin and not affected skin from lSSc patients, confirming that MIF immunoreactivity was faint and mostly found in epidermal basal layers (14).

On the contrary, affected skin of both lSSc and dSSc patients showed MIF positive fibroblasts in the dermis, keratinocytes in the epidermal basal layers, endothelial cells and sweat and sebaceous gland cells.

These last findings seem to indicate the topic presence and activity of MIF at the lesional sites of the disease. The question arises: how and why MIF is mostly expressed in SSc?

Before all, we underline that the activation of the immune system plays a central part in SSc development: in particular, several cytokines, including interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α), which are involved in the development of SSc tissue remodeling (26-28), seem to be increased under the stimulatory effect of MIF (28). We found that fibroblasts, keratinocytes, vascular endothelial cells and gland epithelial cells from the affected skin of both lSSc and dSSc patients are able to produce MIF, probably acting through an autocrine/paracrine mechanism, as demonstrated prevalently in many dermatological and some rheumatic diseases (9, 15, 29).

Another question arises: has MIF a local or a systemic activity? At present, it is reasonable to hypothesise that the cytokine acts also at systemic levels: in fact, pituitary-derived MIF exerts a systemic action by developing the lethality of endotoxin shock (30), and that it

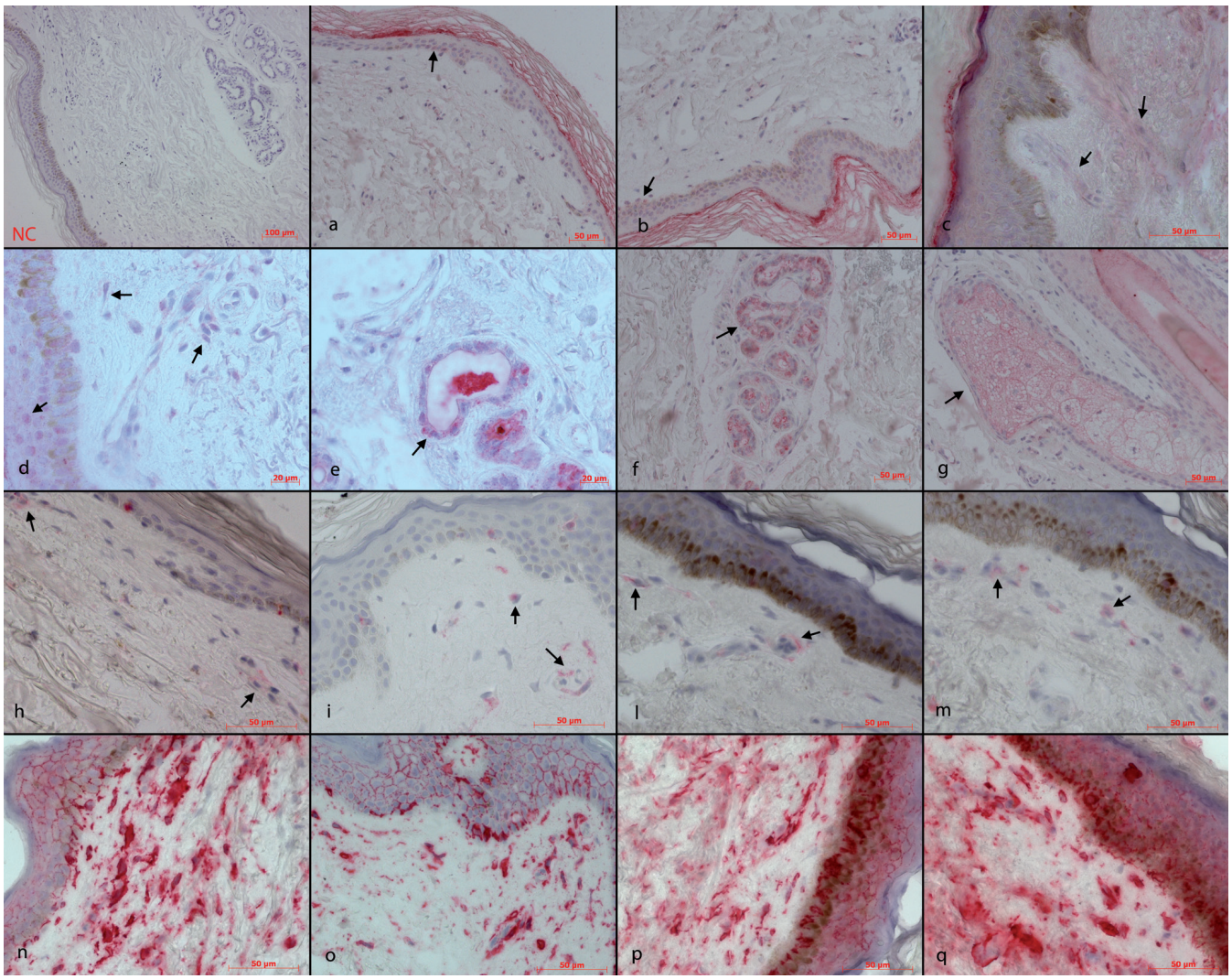

Fig. 4. MIF (a-g) and its receptors CD74 (h-m) and CD44 (n-q) immunostaining in control skin, in both not-affected and affected skin of lSSc patients and in affected skin of dSSc patients. Negative control (NC). Control skin (a) and unaffected skin from lSSc patients (b) showed faint immunoreactivity to MIF in epidermal basal layers (black arrows). lSSc (c) and dSSc (d) affected skin showed MIF positivity in fibroblasts (black arrows) and keratinocytes (black arrows). Endothelium (e) (black arrows), sweat glands (f) (black arrows) and sebaceous glands (g) (black arrows) resulted positive for MIF in both lSSc and dSSc affected skin. No differences in CD74 immunoreactivity were found in control (h), not affected lSSc (i), affected lSSc (l) and affected dSSc (m) skin samples. Finally, CD44 immunostaining appeared to be the same in control (n), not affected lSSc (o), affected lSSc (p) and affected dSSc (q) skin samples.

counteracts the anti-inflammatory activity of corticosteroids (31).

The sharp increase of MIF production in MVECs and fibroblasts culture supernatants, after stimulation with SSc sera, adds further interesting feature to the critical role of these two cell phenotypes (MVECs and fibroblasts) in SSc pathogenesis. In particular, we demonstrated that healthy MVECs are able to produce two times more MIF when stimulated with SSc serum, compared to what happens if they are stimulated with control serum; moreover, we evidenced that healthy MVECs produce more MIF when stimulated with dSSc serum respect to lSSc serum.

At present, we cannot explain these findings: we can hypothesise that MVECs and fibroblasts, when stimulated with soluble factors of SSc serum, increase the secretion of MIF, as a defense response against the inflammatory process. In fact, literature suggests that MIF plays a protective role on MVECs by increasing the expression of intercellular adhesion molecule 1 (ICAM-1) and trombomodulin (TM) *in vitro* (32).

ICAM-1 is involved in the trafficking of inflammatory cells and in mediating the adhesion between leukocytes and endothelium (33). In addition, TM plays a central role in regulating not only haemostasis, but also inflammation, thus providing a close link between these processes (34).

Another study confirms the protective role of MIF on vascular cells, showing that MIF is able to recruit endothelial progenitor cells (EPC), to promote angiogenesis, revascularisation and wound healing (35). Our study demonstrated that not only MVECs but also fibroblasts, when stimulated with SSc sera, increased the secretion of MIF. Therefore, fibroblasts are not only mere effectors of tissue damage, but can interfere directly with the development of the disease (27, 36).

We demonstrated that dermal fibroblasts of healthy subjects in culture are

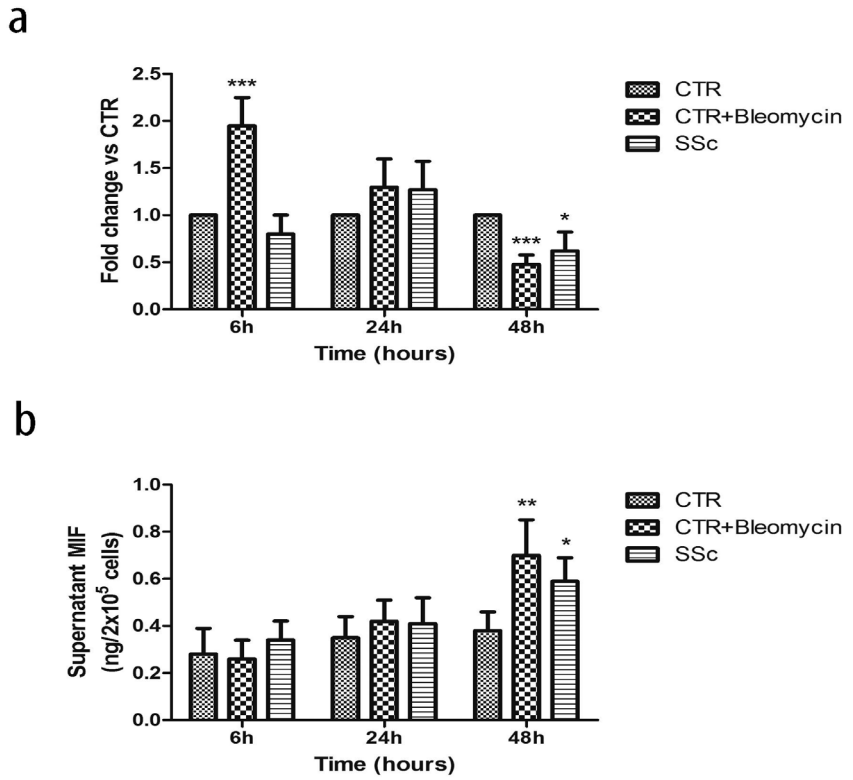

Fig. 5. a. MIF mRNA levels at 6, 24 and 48 hours in control healthy fibroblasts, untreated (CTR) or treated with bleomycin (50mU/ml) (CTR+bleomycin) and in lSSc/dSSc affected fibroblasts (SSc). Data are reported as fold change vs control healthy fibroblasts untreated (CTR). (*p<0.05; ***p<0.001). **b.** MIF supernatant levels at 6, 24 and 48 hours in control healthy fibroblasts, untreated (CTR) or treated with bleomycin (50mU/ml) (CTR+bleomycin) and in lSSc/dSSc affected fibroblasts (SSc). Data are reported as ng/2*10^5 cells. (*p<0.05; **p<0.01; Tukey's Multiple Comparison Test).

able to produce MIF, even if in lower concentrations respect to SSc fibroblasts. In addition, we evidenced that control fibroblasts, fibroblasts derived from the not affected skin of lSSc patients and fibroblasts derived from the affected skin of both lSSc and dSSc patients, significantly increased MIF secretion only when stimulated with SSc sera.

So we suggest that pro-infammatory soluble factors, present in SSc serum and different by nature and quantity from those in control serum, could induce fibroblasts to secrete MIF, as a protective factor. In fact, some researchers reported that MIF is able to inhibit apoptosis of SSc dermal fibroblasts *in vitro*, suggesting that endogenous MIF confers apoptotic resistance to SSc fibroblasts (25).

Our study showed that MIF production is higher in cultured SSc fibroblasts than in control ones, and that MIF secretion increases when fibroblasts are stimulated with SSc sera: so, it can be postulated that apoptotic resistance of SSc fibroblasts (37) may be caused, at least in part, by local overproduction of MIF. Based on our findings, elevated serum MIF levels in SSc patients may come from dermal fibroblasts, keratinocytes and MVECs, as well as activated T cells in the affected skin or peripheral blood as reported in literature (38).

Therefore, for future studies, it could be intriguing to speculate triggering between fibroblast-derived MIF and secondarily T cell activation of blood derived leucocytes infiltrated in the dermis, or activated T cell-derived MIF that might augment the further activation of fibroblasts in the dermis, suggesting an autocrine/paracrine contribution of MIF in the tissue.

Moreover, we observed how healthy dermal fibroblasts, activated by profibrotic molecules (bleomycin) *in vitro* (23, 39), start to produce MIF by analysing both MIF mRNA and supernatant protein levels; it follows that pro-fibrotic induction represents alone a stimulus to MIF secretion for fibroblasts. Consequently, we tried to evaluate when fibroblasts start to produce MIF during their differentiation process into myofibroblasts, under bleomycin stimulation.

We evidenced that MIF mRNA levels increased significantly after 6 hours of stimulation and then decreased significantly in 48 hours. Simultaneously, MIF supernatant levels were highest at 48 hours of incubation with bleomycin. Therefore, it could be postulated that fibroblasts start to produce MIF during their differentiation process into myofibroblasts, and that MIF probably will prevent their apoptosis in SSc.

In conclusion, we showed that MIF is abundant both in serum and in the skin of SSc patients, and that several types of cells (including fibroblasts, keratinocytes and endothelial cells) contribute to the local production of MIF. We showed also that there are no differences in MIF receptors CD74/CD44 expression between control and SSc skin, thus excluding the hypothesis that MIF overexpression in lSSc/dSSc affected tissue could be a consequence of the overexpression of its receptors CD74/CD44 at lesional site.

In our opinion, all these data support the hypothesis that MIF plays an important role in the development of inflammatory/fibrotic lesions of the skin in course of SSc.

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