EXTENDED REPORT

Expression of macrophage migration inhibitory factor in diffuse systemic sclerosis

E Selvi, S A Tripodi, M Catenaccio, S Lorenzini, D Chindamo, S Manganelli, R Romagnoli, F Ietta, L Paulesu, C Miracco, M Cintorino, R Marcolongo

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See end of article for authors' affiliations

Correspondence to:
Dr E Selvi, Institute of Rheumatology, University of Siena, Viale Bracci, I-53100 Siena, Italy; eselvi@katamail.com

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Objective: To evaluate whether, in patients with the diffuse form of systemic sclerosis (dSSc), macrophage migration inhibitory factor (MIF) production is dysregulated.
Methods: 10 patients with dSSc and 10 healthy controls, matched for age and sex, were studied. MIF expression was evaluated by immunohistochemistry on formalin fixed skin biopsies of patients with dSSc and controls. MIF levels were assayed in the sera and in the supernatants of skin cultured fibroblasts by a colorimetric sandwich enzyme linked immunosorbent assay (ELISA). MIF concentrations in culture medium samples and in serum samples were compared by Student's two tailed *t* test for unpaired data.
Results: Anti-MIF antibody immunostained the basal and mainly suprabasal keratinocytes. Small perivascular clusters of infiltrating mononuclear cells were positive; scattered spindle fibroblast-like cells were immunostained in superficial and deep dermal layers. The serum concentrations of MIF in patients with dSSc (mean (SD) 10705.6 (9311) pg/ml) were significantly higher than in controls (2157.5 (1288.6) pg/ml; p=0.011); MIF levels from dSSc fibroblast cultures (mean (SD) 1.74 (0.16) ng/2×10^5 cells) were also significantly higher than in controls (0.6 (0.2) ng/2×10^5 cells; p=0.008).
Conclusion: These results suggest that MIF may be involved in the amplifying proinflammatory loop leading to scleroderma tissue remodelling.

Macrophage migration inhibitory factor (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 tumoral cells.[1–3] Recent data indicate that other types of cell, such as macrophages, endothelial cells, and fibroblasts, can produce MIF,[4–6] and many other functions have been attributed to this molecule, such as the regulation of cell growth, including tumorigenesis, T cell activation, and angiogenesis.[7 8] Furthermore, recent reports suggest that MIF has a critical role in inflammatory and immune responses. In particular, MIF has been shown to induce the synthesis of proinflammatory cytokines, including tumour necrosis factor α (TNFα), interleukin (IL)1, IL6, and IL8 in immunocompetent cells, and to exert the unique ability of counteracting the inhibition of cytokine production by glucocorticoids.[9] Moreover, it has recently been verified that MIF acts as a powerful stimulator for nitric oxide production.[10–12] The dysregulation of MIF has recently been described in several inflammatory diseases[13–15]: Leech *et al* demonstrated the high expression of MIF in inflamed synovial tissue from rheumatoid arthritis, with a unique up and down regulation, respectively, induced by low and high glucocorticoid concentrations[14]; Sampey *et al* showed that MIF exerts an up regulation of fibroblast-like synoviocyte phospholipase A2 and cyclo-oxygenase 2.[16] As indicated in several previous reports, MIF also seems to have a role in several inflammatory skin diseases and in wound healing processes.[17–20]

Systemic sclerosis (SSc) is a connective tissue disease characterised by an abundant deposition of collagen in the skin and internal organs. Fibroblasts are considered to be the main effector cells of fibrogenesis occurring in scleroderma, but they also play an active part in inflammation, showing the ability to constitutively express proinflammatory factors.[21–23]

Furthermore, mononuclear cells and T cells known to produce MIF,[24 25] are present in the dermis infiltrate in the inflammatory stages of SSc, and they show excessive functional activity.[26] In view of the relationship between MIF and the cytokine network, we aimed at determining whether MIF production was up regulated in patients with SSc.

PATIENTS AND METHODS

Patients

We studied 10 patients consecutively enrolled at our outpatient clinic who fulfilled the preliminary American Rheumatism Association criteria for SSc[27] (mean (SD) age 52 (13.1) years) and 10 controls matched for age and sex. Disease duration from the first non-Raynaud manifestation was 6.3 (2.7) years (mean (SD)). According to the classification proposed by LeRoy *et al*,[28] all patients enrolled in our study were classified as having diffuse cutaneous SSc (dSSc). Exclusion criteria were current infections or neoplasms and treatment with glucocorticoids. Table 1 shows the major clinical characteristics of the patients.

Skin specimens were obtained by 6 mm^2 punch biopsy under local anaesthesia from the leading edge of the skin concerned, from the anterior surface of the upper arm. Control biopsy samples were taken from the anterior part of the forearm of healthy control subjects. At the time of biopsy, the blood samples were collected, centrifuged, and the sera were stored at –20°C. All subjects enrolled gave their informed consent to the study; the study was approved by the local ethical committee

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; dSSc, diffuse systemic sclerosis; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; MIF, migration inhibitory factor; PBS, phosphate buffered saline; SSc, systemic sclerosis; TBS, Tris buffered saline; TNFα, tumour necrosis factor α

Table 1 Clinical features of patients with dSSc

Patient	Age (years)	Sex	Duration of disease from the first non-Raynaud manifestation (years)	ANA	Raynaud	Oesophageal dysmotility	TLCO (%)	FVCL	Myocardiopathy	Pulmonary interstitial fibrosis	Skin score[29]	Drug treatment
1	50	F	4	Neg	+	+	32	1.52	–	+	32	Nifedipine, Carboprostacyclin
2	61	F	9	Scl-70	+	+	83	2.37	–	–	19	Nifedipine, NSAIDs
3	57	F	10	Neg	+	+	55	2.4	–	+	15	Captopril, Carboprostacyclin
4	31	F	6	Scl-70	+	+	95	2.91	–	+	16	Carboprostacyclin
5	52	F	4	Scl-70	+	+	67	2.55	–	–	13	Nifedipine
6	73	F	2	Neg	+	+	58	2.81	–	+	13	Enalapril
7	43	F	7	Neg	+	+	47	2.02	–	+	40	Carboprostacyclin, Nifedipine
8	52	F	5	Scl-70	+	–	87	3.57	–	+	14	Enalapril
9	35	F	6	Anticentromere	+	+	55	2.75	–	+	46	Carboprostacyclin, Nifedipine
10	66	F	10	Neg	+	+	67	1.81	–	+	22	Nifedipine
Mean (SD)	52 (13.1)	–	6.3 (2.7)		–	–	64.6(19.3)	2.47(0.59)	–	–	23(12.06)	

TLCO %, carbon monoxide transfer factor (ml/mm Hg/min); FVCL, forced vital capacity-L; ANA, antinuclear antibodies; Neg, negative; NSAIDs, non-steroidal anti-inflammatory drugs.

Immunohistochemistry

One slide from each skin specimen was stained by routine histological methods.[30] Immunohistochemistry was performed on cutaneous biopsy specimens of patients with dSSc 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 serum (Dako, Copenhagen, Denmark) to prevent non-specific binding, and incubated overnight at 4°C with the antihuman MIF goat polyclonal antibody diluted 1:300 in TBS. Slides were then washed three times with TBS for five minutes, and incubated with a rabbit antigoat antibody labelled with biotin (Dako), at a dilution of 1:500, for 30 minutes. The reaction was demonstrated using streptavidin-biotin complex (Dako). Sections were not counterstained. Slides were mounted and examined under a light microscope. For each case, a negative control was obtained by replacing the specific antibody with non-immune serum immunoglobulins at the same concentration as the primary antibody. Qualitative evaluations were carried out by the pathologist, who was unaware of the status of the samples.

Fibroblast cultures

Dermal fibroblasts were obtained from the first five patients with dSSc enrolled in our study and also from five controls matched for age and sex. Cells were grown by the standard explant technique.[31] Briefly, the skin was cut into 2–3 mm^2 pieces and placed in 25 cm^2 flasks. Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg /ml streptomycin, and 30% (vol/vol) fetal calf serum (FCS) was added to each flask. Cultures were incubated at 37°C with 95% air-5%CO_2; the culture medium was changed twice weekly. Fibroblasts were removed at subconfluence with 0.25% trypsin containing 0.02% EDTA and transferred to other 25 cm^2 flasks. As shown by the trypan blue viable stain, 90–95% of the cells recovered were alive. Cells were used for experiments at the second passage. Fibroblasts were then plated out in 200 μl of DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) FCS in 48 well tissue culture plate (2×10^5 cells/ well) and allowed to attach for 24 hours. Culture medium was then replaced with flash serum-free DMEM. After 48 hours, the cell count was obtained and the supernatant was collected and stored at –20°C. All experiments were performed in triplicate.

MIF enzyme linked immunosorbent assay (ELISA)

The concentrations of immunoreactive MIF 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 μg/ml) 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, appropriately diluted in Tris buffered saline-BSA (2 0 mM Tris-HCl, 150 mM NaCl (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 then washed three times and 100 μl of biotinylated goat antihuman MIF antibody (200 ng/ml) 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

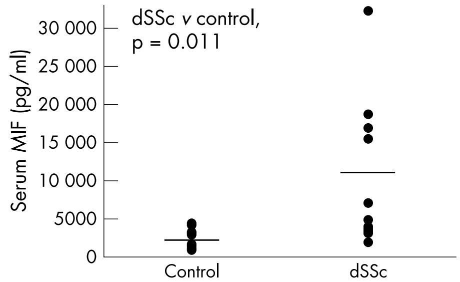

Figure 1 Serum MIF levels (pg/ml) from 10 patients with dSSc and 10 healthy controls matched for age and sex. The concentration of MIF in patients with dSSc was significantly higher than in controls ($p<0.05$; Mann-Whitney rank sum test).

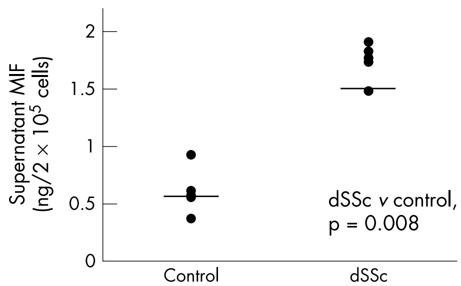

Figure 2 Supernatant MIF levels from fibroblast cell cultures (ng/2×10^5cell). The samples were obtained from five patients with dSSc and five healthy controls matched for age and sex. The concentration of MIF in patients with dSSc was significantly higher than in controls ($p<0.01$; Mann-Whitney rank sum test).

added to each well and incubated for 20 minutes at room temperature. The plates were then washed and 3,3′,5,5′-tetramethylbenzidine (Zymed) was added. After 20 minutes, the reaction was stopped by adding H_2SO_4. Absorbance was measured at 450 nm using an ELISA SR 400 microplate reader (Sclavo, Siena, Italy). MIF concentration was expressed as pg per ml or ng per cell number. The sensitivity limit was 18 pg/ml. Intra- and interassay coefficients of variation were 3.86 (0.95)% and 9.14 (0.47)%, respectively.

Data analysis

A Mann-Whitney rank sum test was used to compare the concentrations of MIF in cultured dermal fibroblast supernatant and in serum. Statistical significance was set at $p<0.05$.

RESULTS

Measurement of MIF in serum samples

Serum concentration of MIF in patients with dSSc, measured by colorimetric sandwich ELISA (mean (SD) 10705.6 (9311) pg/ml), was significantly higher than that of controls (2157.5 (1288.6) pg/ml; p=0.011) (fig 1).

Detection of MIF released by cultured dermal fibroblasts

Because serum MIF levels in patients with dSSc were significantly higher than those of controls, we evaluated MIF concentrations in the supernatant from cultured dermal fibroblasts of patients with dSSc and controls. MIF production of the five dSSc fibroblast cultures (mean (SD) 1.74 (0.16) ng/2×10^5 cells) was significantly greater than that of the five controls (0.6 (0.2) ng/2×10^5 cells; p=0.008) (fig 2).

Immunohistological detection of MIF in skin biopsy specimens

Tissue distribution of MIF immunoreactivity protein in the sections was then analysed by immunohistochemistry. Ten cases of scleroderma skin were examined and immunostained

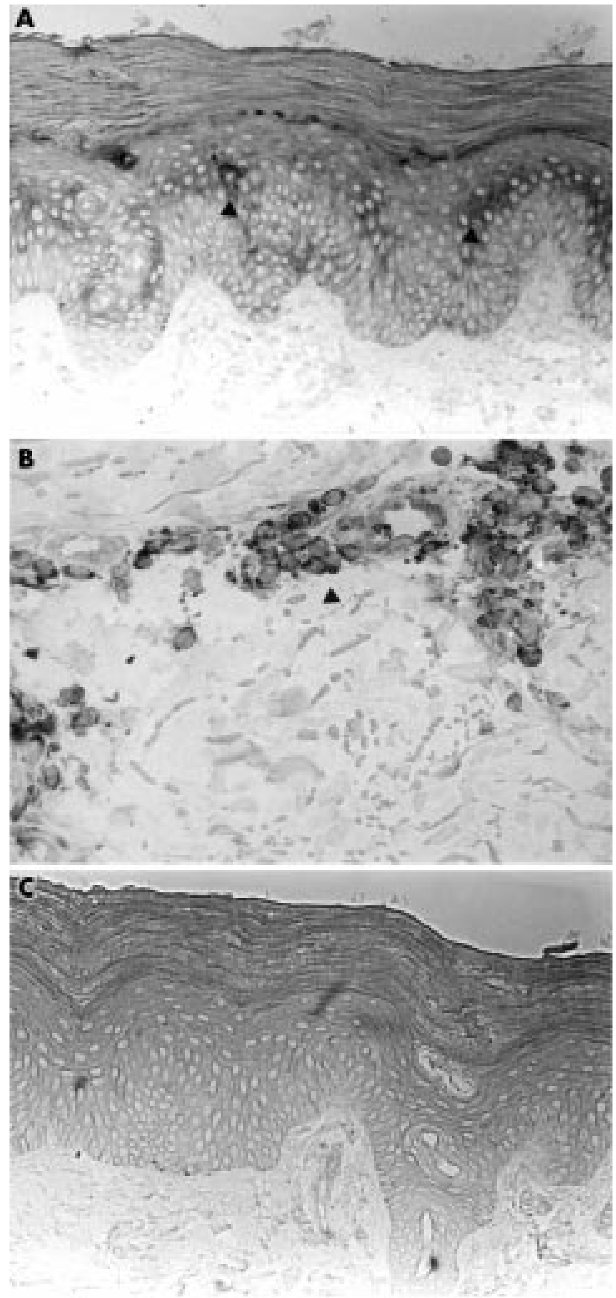

Figure 3 (A, B) Sclerodermal skin section immunostained by anti-MIF antibody. (A) Positive basal and suprabasal keratinocytes (arrow head). Original magnification ×20. (B) Several positive perivascular mononuclear cells (arrow head). Original magnification ×60. Antigen retrieval was carried out by incubating sections with the antihuman MIF goat polyclonal antibody diluted 1:300 in TBS. The reaction was shown using the streptavidin-biotin complex. (C) Negative control: SSc skin section immunostained by replacing the specific antibody with non-immune serum immunoglobulins at the same concentration as the primary antibody. Original magnification ×20.

with the MIF antibody. In routine histological staining, all tissues showed the histological hallmarks of scleroderma disease, such as the paucity of epidermal appendages and scant cellularity of the reticular dermis, which showed thickened collagen bundles. In the epidermis, MIF antibody immunostained the basal and mainly subbasal keratinocytes (fig 3A). In the dermal layer, acinar and ductal segments of the sweat glands, as well as the endothelial cells of small dermal vessels, were immunostained. Infiltrating mononuclear cells stained in the dermis were either scattered or gathered in small perivascular clusters (fig 3B). Spindle fibroblast-like cells were occasionally positive. By contrast in the control skin specimens, MIF immunoreactivity was mostly found in epidermal basal layers, as well as in endothelial cells, as previously observed.[4]

DISCUSSION

In this study we have demonstrated for the first time the increase of MIF in the sera and in the medium of skin cultured fibroblasts of patients with dSSc, compared with specimens from healthy control subjects. In particular, the constitutive increase of MIF production demonstrated in fibroblast culture is worth noting. Fibroblasts are considered to be the cells responsible for the progressive fibrosis occurring in scleroderma, which is the hallmark of the disease. Fibroblasts are also known to be not only mere effectors but also able to produce crucial molecules for the development of the disease. Furthermore, fibroblasts can modify their constitutive behaviour in physiological states, such as tissue repair, but they are also responsible for abnormal responses leading to several diseases, including SSc.[21–23]

The demonstration of a net increase of MIF production, particularly in culture supernatant, adds an additional interesting feature to the critical role of this cell line in scleroderma pathogenesis.

We found that fibroblast and mononuclear infiltrating cells produced MIF in skin tissue, probably acting through an autocrine/paracrine mechanism[20]; on the other hand, although the source of increased serum MIF is uncertain, it is likely that this cytokine may also act through a systemic action. This hypothesis is supported by evidence which showed that pituitary secreted MIF can also exert a systemic action—for example, by developing the lethality of endotoxin shock,[32] or counteracting the excessive anti-inflammatory activity of corticosteroids.[14]

To date, MIF is known to be a proinflammatory cytokine and its role in some diseases is under investigation because it has been shown to be up regulated in several inflammatory conditions, including rheumatoid arthritis.[14 15 33] Furthermore, it is likely that MIF has a pivotal role in several cutaneous diseases, such as psoriasis[20] and atopic dermatitis.[18]

MIF has also been shown to be increased in an experimental model of cultured fibroblasts from injured epidermis.[17] These findings suggest that MIF is a multipotent immunomodulatory molecule, which can both regulate the physiological reparative processes occurring in wound healing and cooperate in the development of several inflammatory and immunomediated skin diseases.

Although scleroderma is considered to be a multifactorial disease, activation of the immune system plays a central part in its development; interestingly, several cytokines, including IL2, IL6, and TNFα, which are known to be crucial in the development tissue remodelling of SSc, seem to be increased under the stimulatory effect of MIF.[22] Moreover, some inflammatory cytokines, including transforming growth factor β and platelet derived growth factor, which are representative fibrogenic proinflammatory cytokines,[34] have been shown to stimulate MIF mRNA expression in human tumour cells.[35]

In conclusion, our results showed that MIF synthesis is up regulated in SSc, suggesting that MIF participates in the amplifying proinflammatory loop leading to scleroderma tissue remodelling. This work, if confirmed by further studies such as, for example, detection of MIF mRNA by polymerase chain reaction in cell cultures, would amplify the number of pathogenic mechanisms in which MIF has been shown to play a part. This would confirm that this cytokine has an increasingly important role in immunomediated, as well as inflammatory, diseases. The identification of MIF as a putative proinflammatory factor, with a role in SSc pathogenesis, might lead to the future development of drugs which would be able to interact with such molecular processes,[8] thereby expanding the therapeutic strategy against SSc.

Authors' affiliations

E Selvi, M Catenaccio, S Lorenzini, D Chindamo, S Manganelli, R Marcolongo, Institute of Rheumatology, University of Siena, Italy
S A Tripodi, C Miracco, M Cintorino, Institute of Pathological Anatomy and Histology , University of Siena, Italy
R Romagnoli, F Ietta, L Paulesu, Department of Physiology, University of Siena, Italy

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