The −2518 Promotor Polymorphism in the MCP-1 Gene Is Associated with Systemic Sclerosis

Sigrid Karrer,* Anja Kathrin Bosserhoff,† Petra Weiderer,* Oliver Distler,‡ Michael Landthaler,* Rolf-Markus Szeimies,* Ulf Müller-Ladner,§ Jürgen Schölmerich,§ and Claus Hellerbrand§

*Department of Dermatology, University of Regensburg, Regensburg, Germany; †Institute of Pathology, University of Regensburg, Regensburg, Germany; ‡Department of Rheumatology, University Hospital Zurich, Zurich, Switzerland; §Department of Internal Medicine I, University of Regensburg, Regensburg, Germany

Factors influencing the initiation or progression of sclerosis in patients with systemic sclerosis (SSc) are poorly understood. Monocyte chemotactic protein-1 (MCP-1) is a potent chemokine, which is upregulated in fibroblasts during development of sclerosis. In this study, we investigated the frequency of the functional −2518G MCP-1 promoter polymorphism in 18 patients with SSc and 139 healthy controls. In the lesional skin of the same SSc patients, expression of MCP-1 protein was examined by immunohistochemistry. To investigate a genotype/phenotype correlation, basal as well as tumor necrosis factor (TNF)-induced MCP-1 expression was analyzed in fibroblasts isolated from the skin of SSc patients with different MCP-1 genotypes by quantitative RT-PCR and ELISA. Genotyping for the −2518 (A/G) MCP-1 promotor polymorphism showed that GG homozygotes were significantly more frequent in patients with SSc than in controls (28% *vs* 6%). Results of immunohistochemistry revealed that MCP-1 was expressed in keratinocytes, infiltrating inflammatory cells, fibroblasts, and endothelial cells in scleroderma skin, whereas normal control skin showed no MCP-1 expression. MCP-1 expression in fibroblasts from GG-homozygote individuals tended to be stronger as compared to AG or AA genotypes. Furthermore, basal as well as TNF-induced MCP-1 expression of fibroblasts isolated from a GG-homozygote SSc patient was significantly higher than MCP-1 expression of fibroblasts isolated from heterozygote or AA-homozygote donors. The A −2518G polymorphism of the MCP-1 gene appears to affect MCP-1 expression of skin fibroblasts of patients with SSc. In accordance, the G/G genotype may predispose patients to SSc.

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Systemic sclerosis (SSc) is a rare autoimmune disease of the connective tissue characterized by sclerosis of the skin, subcutaneous tissue, and internal organs. One of the characteristic histological features in SSc is the presence of infiltrating mononuclear cells in the dermis in early stages in association with an increase in collagen synthesis. It is generally accepted that dermal fibroblasts play a crucial role in the pathogenesis of skin sclerosis by synthesizing increased amounts of collagen types I, III, VI, and VII, glycosaminoglycans, decorin, and fibronectin, whereas collagen degrading enzymes (matrix-metalloproteinases: MMP-1, -2, -3) are decreased (Hawk and English, 2001). The cause of the increased collagen synthesis is still not elucidated, however, inflammatory cells are regarded to be candidates for releasing cytokines that initiate and/or result in the sequential events of fibrosis and in perpetuation of the pathophysiologic process (Kovacs and DiPietro, 1994).

Chemokines are a large family of small cytokines with potent chemoattractive effects on specific leukocyte subsets, which can also induce a variety of cellular responses such as angiogenesis or matrix protein synthesis. Chemotactic cytokines are known to be critical mediators of cell trafficking into sites of injury and therewith modulators of tissue injury, inflammation and repair. Of these, monocyte chemotactic protein-1 (MCP-1) is one of the most potent chemokines especially for monocytes and macrophages (Boring *et al*, 1999). It belongs to the group of CC chemokines with two conserved adjacent cysteine residues. Recent studies have shown that MCP-1 expession is upregulated in fibrotic processes, including acute hepatic fibrogenesis (Marra *et al*, 1998), interstitial kidney fibrosis (Lloyd *et al*, 1997) as well as human idiopathic pulmonary fibrosis (Antoniades *et al*, 1992). *In vitro* studies have demonstrated that MCP-1 stimulates type I collagen gene expression in rat lung fibroblasts and in normal skin fibroblasts (Gharaee-Kermani *et al*, 1996; Yamamoto *et al*, 2001b). These results provide evidence that enhanced MCP-1 expression contributes to the development of tissue fibrosis by various mechanisms, although little is known about the factors, which regulate MCP-1 gene expression.

SSc fibroblasts have been shown to display an array of chemokines including an increased and abnormally regulated expression of MCP-1 *in vitro* (Galindo *et al*, 2001; Yamamoto *et al*, 2001a, b). In addition to the highly increased expression of MCP-1, *in vitro* SSc fibroblasts are more responsive to MCP-1, which could result in an

Abbreviations: MCP-1, monocyte chemotactic protein-1; SSc, systemic sclerosis; TNF, tumor necrosis factor

autocrine stimulation, perpetuation, and maintenance of hyperactivity of the SSc fibroblasts and the fibrotic process (Yamamoto *et al*, 2001b). Also *in vivo*, increased MCP-1 expression was found in fibroblasts derived from lesional skin of patients with SSc (Galindo *et al*, 2001; Yamamoto *et al*, 2001a, b). In these previous studies, MCP-1 expression correlated with the infiltration of mononuclear cells suggesting that MCP-1 plays a role in recruiting monocyte/macrophages to the lesional skin of SSc and that activated fibroblasts are involved in this process. Furthermore, elevated serum levels of MCP-1 were found in patients with SSc, which correlated with the presence of pulmonary fibrosis (Hasegawa *et al*, 1999).

Previous *in vitro* studies with different human cell types including peripheral blood mononuclear cells or activated hepatic stellate cells/myofibroblasts revealed considerable interindividual variability in basal as well as in cytokine-induced MCP-1 expression (Sylvester *et al*, 1993; Rovin *et al*, 1999; Kruger *et al*, 2002; Muhlbauer *et al*, 2003), suggesting that inherited factors influence the expression of this chemokine. Interestingly, also *in vitro* MCP-1 production of systemic fibroblast cell lines revealed considerable variation (Galindo *et al*, 2001).

Recently, a polymorphism in the distal regulatory region of the MCP-1 gene at position −2518 relatively to the transcription start site (based on the published sequence Gene Bank accession number D26087) was identified (Rovin *et al*, 1999). The functional relevance of this polymorphism has been shown by several *in vitro* studies analyzing cytokine-induced MCP-1 expression of peripheral blood mononuclear cells or activated hepatic stellate cells/myofibroblasts (Rovin *et al*, 1999; Kruger *et al*, 2002; Muhlbauer *et al*, 2003). Cells from individuals carrying the G-allele at −2518 expressed more MCP-1 than cells from A/A homozygous subjects. Moreover, the prevalence of these high MCP-1-producing genotypes has been shown to be associated with the presence and severity of bronchial asthma (Szalai *et al*, 2001), the risk for premature kidney graft failure (Kruger *et al*, 2002), the onset and disease behavior of Crohn's disease (Herfarth *et al*, 2003), the frequency of cutaneus vasculitis (Aguilar *et al*, 2001) and fibrosis progression in patients with chronic hepatitis C infection (Muhlbauer *et al*, 2003). Furthermore, it has been postulated to be a genetic risk factor for severe coronary artery disease (Szalai *et al*, 2001). The role of this genetic polymorphism, however, in SSc has not yet been investigated.

In this study, we investigated the hypothesis that the presence of the high MCP-1 producing G-allele may predispose to or support the development of SSc. The aims of this study were (1) to examine the prevalence of this polymorphism in patients with SSc as compared to normal controls, (2) to evaluate whether the expression of MCP-1 in the patients tissue correlates with the patients genotype, and (3) to investigate a genotype/phenotype correlation by analyzing MCP-1 expression in fibroblasts from donors with different MCP-1 genotypes.

Results

Frequency of the −2518 MCP-1 polymorphism in patients with SSc and healthy controls The frequencies of the individual genotypes of the control group were similar to those previously reported in other Caucasian control populations (Aguilar *et al*, 2001; Szalai *et al*, 2001a, b; Kruger *et al*, 2002), and in patients, as well as controls, individual genotypes were found to be in the Hardy–Weinberg equilibrium.

G/G-homozygote SSc-patients, A/G heterozygotes and non-G-allele carriers (A/A-homozygotes) did not differ significantly with regard to the distribution of demographic features, e.g., age (G/G: 48.8 ± 18.4 y, A/G: 58.6 ± 6.8 y, A/A: 56.7 ± 13.5 y) or gender (G/G: 1m/4w, A/G: 1m/4w, A/A: 2m/6w).

Carriers of the G-allele were more frequent in patients with SSc as compared to controls (ten of 18 (56%) *vs* 68 of 139 (48%), respectively) although these differences did not reach statistical significance.

Despite the relatively small sample size, however, the frequency of G/G-homozygotes was significantly higher in SSc patients than in controls (five of 18 (28%) *vs* nine of 139 (6%), respectively; $p = 0.023$ based on Fischer's Exact Test) (Table I).

Characterization of MCP-1 expression by immunohistochemistry Next, we investigated MCP-1 expression in lesional skin of SSc patients with different MCP-1 genotypes by immunohistochemistry. Specimens from healthy subjects and from patients with inflammatory skin disorders (contact dermatitis, vasculitis, urticaria, eczema) served as controls. Although no immunosignal was detectable in normal skin (Fig 1*A*), immunostaining of scleroderma skin revealed MCP-1 expression in keratinocytes, inflammatory cells, fibroblasts, and endothelial cells (Fig 1*B–D*).

As cellular source of MCP-1 expression, we found infiltrating inflammatory cells in patients with an inflammatory stage of SSc (Fig 1*B*) as well as in positive controls (contact dermatitis, vasculitis, urticaria, eczema) (Fig 1*E*). Weak, moderate or strong MCP-1 expression was detected in fibroblasts of SSc specimens. Interestingly, in contrast this pronounced MCP-1 expression in fibroblasts could not be observed in skin specimens of positive controls.

A semiquantitative score was applied to characterize MCP-1 expression in different cell types. Results are summarized in Table II. Comparing MCP-1 expression in fibroblasts from SSc patients with different MCP-1 genotypes revealed a strong expression of MCP-1 in 60% of the specimens from GG-homozygote individuals, in 40% of AG-heterozygote patients and in 37.5% of AA-homozygote individuals. In the only two patients with an inflammatory

Table I. Frequency of the −2518 MCP-1 polymorphism in patients with SSc and healthy controls

−2518 MCP-1 genotypes	Systemic sclerosis, n = 18 (%)	Controls, n = 139 (%)
A/A	8 (44)	71 (51)
A/G	5 (28)	59 (42)
G/G	5 (28)[a]	9 (6)[a]

[a]Indicates a significant difference ($p = 0.023$ based on Fischer's Exact Test).
MCP-1, monocyte chemotactic protein-1; SSc, systemic scelorosis.

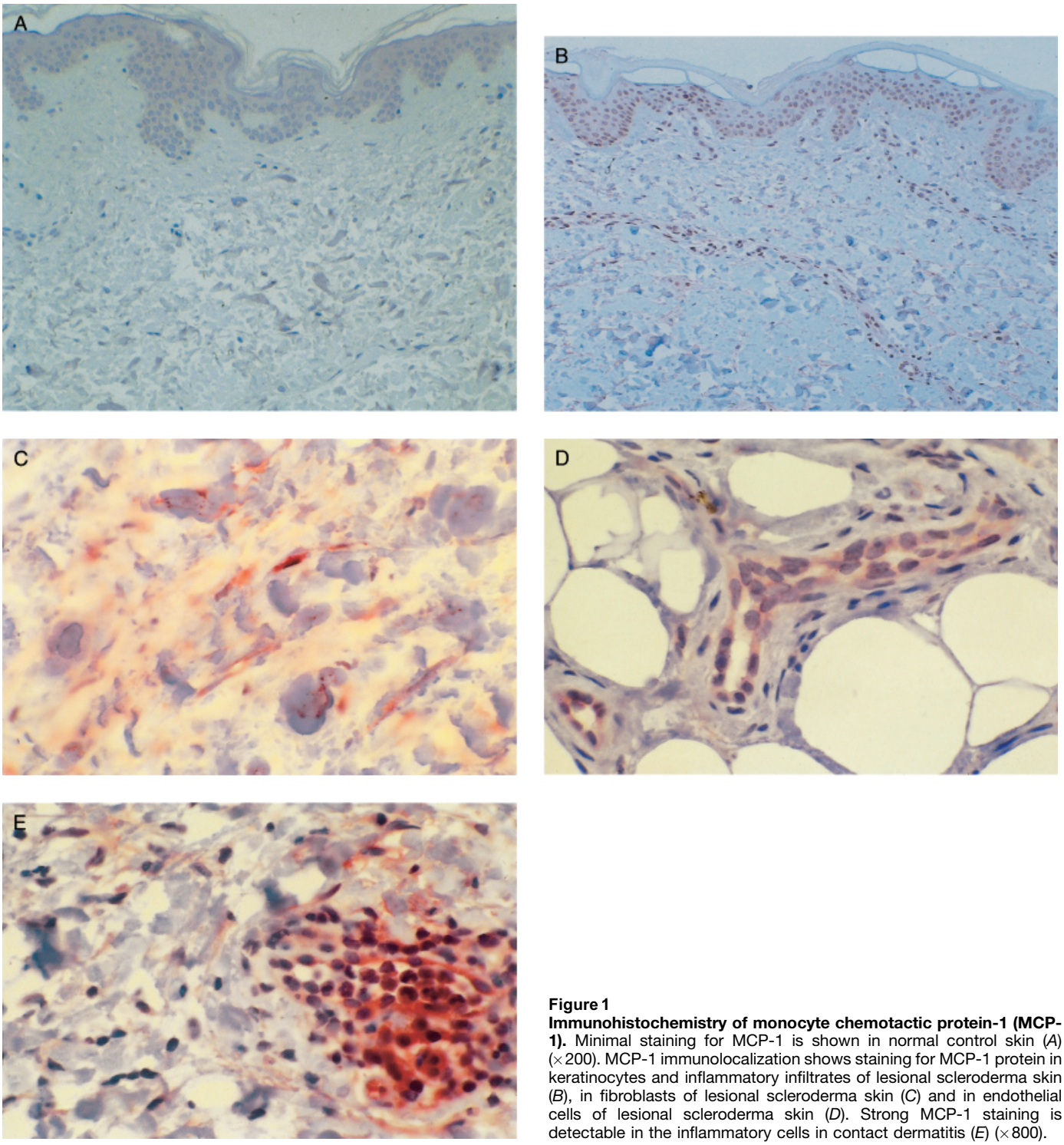

Figure 1
Immunohistochemistry of monocyte chemotactic protein-1 (MCP-1). Minimal staining for MCP-1 is shown in normal control skin (*A*) (×200). MCP-1 immunolocalization shows staining for MCP-1 protein in keratinocytes and inflammatory infiltrates of lesional scleroderma skin (*B*), in fibroblasts of lesional scleroderma skin (*C*) and in endothelial cells of lesional scleroderma skin (*D*). Strong MCP-1 staining is detectable in the inflammatory cells in contact dermatitis (*E*) (×800).

stage of SSc (both GG-homozygote), MCP-expression was most pronounced within the inflammatory infiltrates. All of the AA and AG groups were in the sclerotic stage of disease and showed mostly negative MCP-1 expression within the absent or sparse inflammatory infiltrates. Due to the limited amount of data, it is difficult to make quantitative statements regarding results of histochemistry.

MCP-1 expression in dermal fibroblasts from individuals with different MCP-1 genotypes To further analyze the functional relevance of the MCP-1 promoter polymorphism in SSc, we investigated the *in vitro* MCP-1 expression of three SSc fibroblast cell strains isolated from donors with different MCP-1 genotypes (A/A, A/G and G/G).

The G/G-homozygote fibroblasts revealed a 4-fold higher basal MCP-1 mRNA expression as compared to A/G heterozygote fibroblasts and a 8.3-fold higher MCP-1 mRNA expression as compared to A/A homozygote fibroblasts. In all three cell lines, as tumor necrosis factor (TNF) induced a significant increase of MCP-1 mRNA compared to unstimulated controls. Also after TNF stimulation GG-homozygote fibroblasts revealed significantly higher MCP-1 expression

Table II. Immunohistochemical detection of MCP-1 in the skin tissues of SSc patients and correlation with MCP-1 genotype and stage of the disease

Patient no.	Genotype	Stage of disease	MCP-1 expression[a]			
			Keratino-cytes	Inflam-matory cells	Fibro-blasts	Endo-thelial cells
1	AA	Sclerotic	+	−	+	+
2	AA	Sclerotic	+	−	±	−
3	AA	Sclerotic	+	−	+	−
4	AA	Sclerotic	±	−	±	−
5	AA	Sclerotic	+	−	+	−
6	AA	Sclerotic	+ +	−	+ +	+
7	AA	Sclerotic	+	−	+ +	+
8	AA	Sclerotic	+ +	+	+ +	+ +
9	AG	Sclerotic	±	−	+	±
10	AG	Sclerotic	+	−	+	±
11	AG	Sclerotic	+	−	+	±
12	AG	Sclerotic	+	−	+ +	−
13	AG	Sclerotic	+ +	±	+ +	±
14	GG	Inflammatory	+	+ +	+ +	+
15	GG	Inflammatory	+	+	+ +	+
16	GG	Sclerotic	+	−	+	−
17	GG	Sclerotic	+	−	+	−
18	GG	Sclerotic	+ +	±	+ +	±
Control sections: diagnosis						
Vasculitis			+	+ +	+	+ +
Contact dermatitis			+ +	+ +	±	+
Contact dermatitis			+ +	+ +	±	+
Urticaria			+	+ +	±	+ +
Eczema			±	±	±	±

[a] + +, strong; +, moderate; +, weak; −, negative (according to Yamamoto *et al*, 2001a, b).
MCP-1, monocyte chemotactic protein-1; SSc, systemic sclerosis.

than AA-homozygote (3.1-fold) or heterozygote fibroblasts (3.4-fold), respectively (Fig 2).

Basal expression of MCP-1 protein as measured by ELISA was significantly higher in fibroblasts from G/G-homozygote donors (9452 ± 250 pg per mL) as compared to A/G heterozygote (4739 ± 1028 pg per mL, $p = 0.024$) and A/A homozygote fibroblasts (2355 ± 50 pg per mL, $p < 0.001$). In all three cell lines, TNF induced a significant increase of MCP-1 expression compared with unstimulated controls. Also after TNF stimulation GG-homozygote fibroblasts (18,921 ± 66.5 pg per mL) revealed significantly higher MCP-1 secretion than AA-homozygote (3980 ± 500 pg per mL, $p = 0.001$) or heterozygote fibroblasts (14,106 ± 379, $p = 0.01$). TNF induced MCP-1 secretion was also significantly higher in heterozygote fibroblasts as compared to AA-homozygote fibroblasts ($p < 0.001$) (Fig 3).

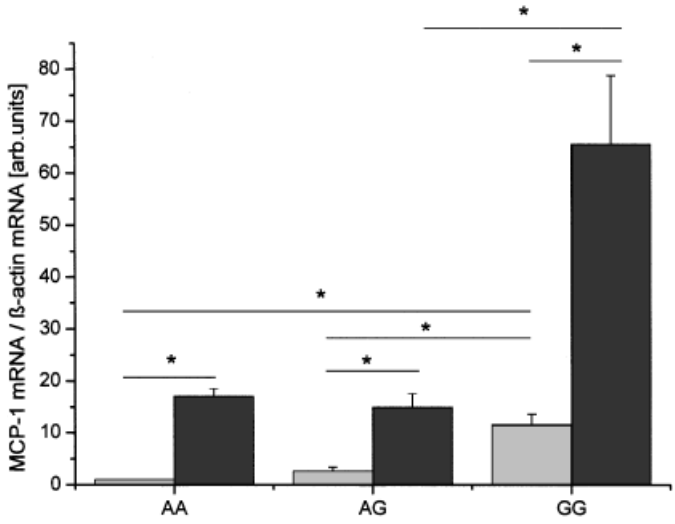

Figure 2
Monocyte chemotactic protein-1 (MCP-1) mRNA expression of dermal fibroblasts from systemic sclerosis (SSc) donors with different MCP-1 genotypes (A/A, A/G, G/G). MCP-1 mRNA expression was analysed by quantitative PCR in unstimulated cells (basal expression, light gray bars) and after stimulation with tumor necrosis factor (TNF) (10 ng per mL) (dark gray bars) for 6 h. Results shown are representative of three independent experiments. *Indicates a significant difference ($p < 0.05$).

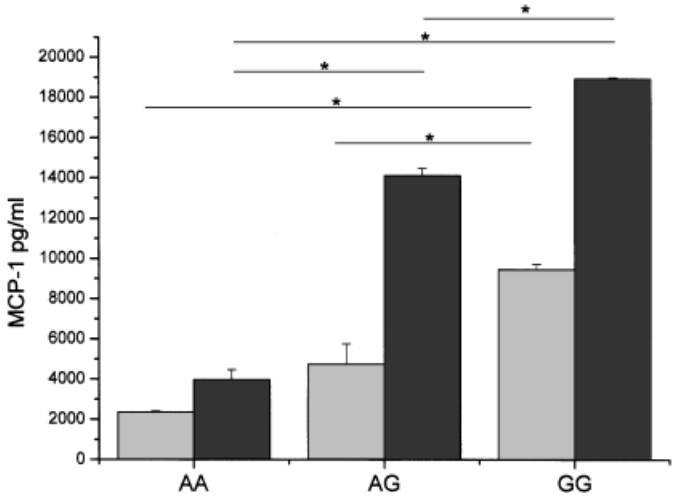

Figure 3
Monocyte chemotactic protein-1 (MCP-1) secretion of dermal fibroblasts isolated from systemic sclerosis (SSc) donors with different MCP-1 genotypes (A/A, A/G, and G/G). MCP-1 protein secretion was analysed by ELISA in the supernatants of unstimulated fibroblasts (basal expression, light gray bars) and after stimulation with tumor necrosis factor (TNF) (10 ng per mL) (dark gray bars) for 16 h. Results represent mean ± SD. Results shown are representative of three independent experiments. *Indicates a significant difference ($p < 0.05$).

Discussion

In this study, we could support our hypothesis that inheritance of the G-allele of the functional promotor MCP-1 polymorphism A −2518G may contribute to the development of SSc. We found homozygote carriers of the G-polymorphism to be significantly more frequent in SSc patients than in healthy controls.

Recently, several important prerequisites for the design of association trials were recommended (Ioannidis *et al*, 2001; Day, 2003). It is important to define (I) a sound rationale for the candidate genes; (II) a plausible *a priori* hypothesis based on the functional significance of the studied polymorphisms; and (III) a detailed characterization of cases ensuring comparable exposure to the pathogenic trigger. Furthermore, the study sample (IV) should have a homogeneous genetic background to avoid ethnic heterogeneity and (V) exclude confounding factors. All these requirements were accounted for in this study. As in most genetic association studies analyzing rare diseases, however, it has to be taken into account that the number of patients and the corresponding power of calculation are limited and results have to be confirmed in other cohorts of patients.

Here, unlike almost all previous genetic association studies performed in SSc patients so far, we provide additional functional data indicating the relevance of the MCP-1 polymorphism in SSc.

Chemokines, such as MCP-1, play a pathogenetic role in SSc, potentially by augmenting leucocyte migration into the affected tissues (Yamamoto *et al*, 2001a). Patients with SSc seem to be predisposed to high MCP-1 production. MCP-1 serum levels as well as spontaneous MCP-1 expression levels of peripheral blood mononuclear cells have been shown to be significantly elevated in patients with SSc compared to normal controls (Hasegawa *et al*, 1999). Furthermore, elevated serum levels of MCP-1 correlated with the presence of pulmonary fibrosis in SSc patients (Hasegawa *et al*, 1999). Since MCP-1 expression in inflammatory mononuclear cells and endothelial cells was more frequently detected immunohistochemically in the skin of SSc patients with short disease duration than in those with long disease duration, it was suggested that MCP-1 expression in the skin might contribute to the initiation of skin sclerosis (Hasegawa *et al*, 1999). Furthermore, it has been shown that MCP-1 stimulates collagen synthesis in fibroblasts indirectly via endogenous upregulation of transforming growth factor-β expression (Gharaee-Kermani *et al*, 1996). Also in dermal sclerosis, MCP-1 has been suggested to contribute to the increased fibrosis via its direct effect on the upregulation of mRNA expression of extracellular matrix proteins in fibroblasts, as well as its indirect effect mediated by a number of cytokines released from proinflammatory cells recruited into the lesional skin (Yamamoto and Nishioka, 2003).

The polymorphism at position −2518 of the MCP-1 gene has been implicated before in the progression of coronary artery disease, asthma, cutaneous vasculitis, kidney graft failure, hepatitis C virus-related liver damage and the onset of Crohn's disease (Aguilar *et al*, 2001; Szalai *et al*, 2001; Kruger *et al*, 2002; Herfarth *et al*, 2003; Muhlbauer *et al*, 2003). Considering the known pathophysiological role of the chemokine MCP-1 in these diseases, these examples further indicate that the G allele may be the allele associated with a higher MCP-1 expression in certain inflammatory conditions. There are few studies, however, investigating the molecular mechanisms responsible for the effect of the −2518G MCP-1 promoter polymorphism on MCP-1 transcription. Rovin *et al* (1999) performed *in vitro* studies using appropriate reporter plasmids and provided evidence that the region of the polymorphism is influencing the transcriptional activity of the MCP-1 gene. Moreover, we could recently demonstrate a genotype–phenotype correlation in activated hepatic stellate cells from donors with different MCP-1 genotypes (Muhlbauer *et al*, 2003). Activated hepatic stellate cells are a myofibroblast-like cell type and are responsible for the excessive hepatic extracellular matrix deposition during liver fibrosis. During chronic liver disease hepatic MCP-1 is mainly secreted by these cells.

Similarly, previous studies identified dermal fibroblasts as a main source of MCP-1 in scleroderma lesions (Distler *et al*, 2001; Galindo *et al*, 2001, Yamamoto *et al*, 2001a, b). In accordance, also in this study we could confirm strong MCP-1 expression in fibroblasts of affected skin biopsies of SSc patients. Interestingly, this MCP-1 expression in fibroblasts was not found in control patients with inflammatory skin disorders. These findings are in line with a previous study (Yamamoto *et al*, 2001a), indicating a specific role of fibroblasts in the pathogenesis of SSc.

This prompted us to investigate *in vitro* cultured fibroblasts from donors with different −2518 MCP-1 genotypes to compare their basal and TNF-induced MCP-1 expression. Proinflammatory cytokines such as TNF and IL-1 are known to be potent inducers of MCP-1 production (Gu *et al*, 1999). In line with the above-mentioned findings, we found a significantly higher expression of MCP-1 mRNA and protein in fibroblasts from donors carrying at least one G-allele compared to A/A-homozygotes. Furthermore, cells from the G/G homozygote donor revealed significantly higher MCP-1 secretion than heterozygotic fibroblasts. Stimulation with TNF resulted in a significant increase of MCP-1 mRNA and protein expression. These findings provide further evidence for the functional relevance of the MCP-1 polymorphism. Interestingly, *in vitro* differences between SSc fibroblasts from donors with different MCP-1 genotypes were evident also after TNF stimulation. This may indicate the relevance of this polymorphism in SSc also under inflammatory conditions.

In SSc, the activation of the immune system is an early event in the disease process. Fibroblasts play an important role in the regulation of the immune system, e.g., by the secretion of chemokines and participate already in the initiation of the disease process (Hogaboam *et al*, 1998). Infiltration of the skin with mononuclear cells precedes the development of fibrosis and vasculopathy (Hawk and English, 2001). Investigation of the mechanisms that initiate and perpetuate the inflammatory infiltration in SSc is important for understanding the early steps in the pathogenesis of the disease. Several lines of evidence indicate that MCP-1 expression in fibroblasts plays an important role in the development of fibrosis in SSc. MCP-1 has been shown to modulate connective tissue deposition by inducing collagen synthesis (Gharaee-Kermani *et al*, 1996). Furthermore,

MCP-1 expression has been shown to induce the proinflammatory cytokine IL-1 and to lead to the induction of signal transduction cascades controlling inflammatory gene expression as the NFκB system (Hisada *et al*, 2000; Yamamoto *et al*, 2000).

MCP-1 produced by activated fibroblasts in scleroderma patients could therefore act as a chemoattractant for mononuclear cells, which contribute to the initial inflammatory infiltrate localized around small blood vessels in the dermis in early stages of scleroderma and could be involved in the perpetuation of inflammation. Thus, this chemokine plays an important role in the complex networks that ultimately result in tissue fibrosis (Distler *et al*, 2001).

In summary, the association between −2518 MCP-1 polymorphism and SSc, together with the results that −2581 MCP-1 polymorphism affects mRNA and protein expression in fibroblasts, suggests that MCP-1 genotyping might provide a reliable indicator of disease susceptibility and eventually also of prognosis of the disease. These findings, however, have to be confirmed in other cohorts and further studies have to be performed to fully elucidate the pathophysiological role of MCP-1 and its functional genetic polymorphism in SSc.

Materials and Methods

Patients and patients material We investigated 18 patients (14 female, four male; mean age: 55.0 ± 15.0 y), who were admitted consecutively to the hospital of the University of Regensburg with the clinically suspected diagnosis "SSc". The diagnosis of SSc was confirmed by histological examination and according to the criteria of the American College of Rheumatology for SSc (Subcommittee for scleroderma criteria of the American Rheumatism Association diagnostic and therapeutic criteria committee, 1980).

Routine skin biopsies of affected areas were obtained in all SSc patients. Parts of the biopsies were fixed in formalin, paraffin embedded and subsequently histological sections were stained with hematoxylin and eosin. Furthermore, parts of the biopsies were used for DNA isolation and isolation of skin fibroblasts, respectively.

Histological examination of the samples showed fibrosis of the dermis with hyalinization and homogenization of the collagen bundles, as confirmed also by a collagen-specific stain (Masson–Goldner stain). Two of the patients presented an inflammatory stage with marked, mostly perivascular inflammatory cell infiltrates (lymphocytes, plasma cells, and macrophages) in the dermis. The other 16 patients presented a sclerotic stage with no or only sparse inflammatory infiltrates.

In addition, skin biopsies of four healthy individuals and five patients with inflammatory skin disorders (contact dermatitis, vasculitis, urticaria, eczema) were obtained and served as controls for immunohistochemical examinations.

Informed consent was obtained from all patients and the study was approved by the local ethics committee. The study was conducted according to the Declaration of Helsinki Guidelines.

Frequency of MCP-1 genotypes was compared to 139 healthy controls from the same geographic region (Muhlbauer *et al*, 2003). Patients and controls were of Caucasian origin.

DNA isolation and MCP-1 genotyping Genomic DNA from SSc patients was extracted from skin tissue using a commercially available DNA isolation kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The G to A polymorphisms at position −2518 of the MCP-1 gene was analyzed by performing PCR and subsequent restriction fragment length polymorphism (RFLP) analysis. PCR was performed under standard conditions (35 cycles, annealing temperature: 55°C) in a total reaction volume of 50 μL containing 2 μL of diluted genomic DNA, using the following pair of primers: *forw:* 5′-CCG AGA TGT TCC CAG CAC AG-3′ and *rev:* 5′-CTG CTT TGC TTG TGC CTC TT-3′. PCR products were digested by *Pvu*II, and the resulting fragments were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. With the −2518 A polymorphic base, the recognition sequence 5′-CAG/CTG-3′ is modified to 5′-CAG/CTA-3′, which is not cut by *Pvu*II.

Controls consisted of healthy individuals that had already been genotyped for a recent study of our group (Muhlbauer *et al*, 2003).

MCP-1 immunohistochemical studies Immunohistochemistry for MCP-1 was performed on formalin-fixed and paraffin-embedded 2-μm-thick sections obtained from all 18 patients with SSc, from four healthy controls and from five patients with inflammatory skin disorders different from SSc (urticaria, contact dermatitis, vasculitis, eczema). The slides were deparaffinized by xylol and decreasing concentrations of ethanol. The sections were then treated with citric acid (2.1 g per liter, pH 6.0) and steamed for 30 min followed by cooling for further 30 min. Serial sections were immunostained for MCP-1 (anti-MCP-1 human monoclonal antibody, 1:100 dilution, 5D3-F7, Alexis Biochemicals, Grünberg, Germany) according to the manufacturer's recommendations and counterstained with hemalaun. Negative controls were performed by omitting the primary antibody.

MCP-1 expression was evaluated semiquantitatively using a score (++, strong; +, moderate; ±, weak; −, negative) representing the product of the intensity of immunosignals and the number of positive cells as previously described (Yamamoto *et al*, 2001a).

All diagnoses were confirmed by a second dermatopathologist blind of clinical information and without knowledge of the patients' genotypes.

Isolation and culture of human dermal fibroblasts Primary human dermal fibroblast strains were established by outgrowth from biopsies of involved skin from patients with SSc. Fibroblasts were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 μg per mL sodium ascorbate, 100 U per mL penicillin, and 100 μg per mL streptomycin and grown as monolayers on plastic Petri dishes in a humidified atmosphere of a CO_2 incubator at 37°C. Fibroblast cultures were subcultured by trypsinisation and used between the third and the tenth passages.

MCP-1 genotyping of the fibroblast strains was performed according to the methods described above. Three SSc fibroblast cell lines with different genotypes were chosen for further experiments (one cell line: A/A, one cell line: A/G, and one cell line: G/G, respectively.)

MCP-1 expression of dermal fibroblasts Fibroblasts were stimulated with recombinant human TNF (10 ng per mL; R&D Systems, Wiesbaden-Nordenstadt, Germany) for 6 and 16 h, respectively.

After 6 h of stimulation, RNA was isolated using the RNeasy Mini Kit including a RNase-free DNase step following the instructor's manual (Qiagen). RNA amounts were analyzed by using a fluorescence microplate reader and following the instructor's manual of the RiboGreen RNA Quantitation Reagent and Kit (MoBiTec, Göttingen, Germany). Integrity of the RNA was verified by agarose gel electrophoresis and by visualization of ribosomal bands with ethidium bromide staining. First strand cDNA was synthesized using 1 μg of total RNA and the AMV-reverse transcription reaction (Promega, Madison, Wisconsin). MCP-1 mRNA expression was quantified using real-time PCR technology (Lightcycler, Roche Diagnostics, Mannheim, Germany) with specific sets of primers for human MCP-1 and β-actin based on published sequences: β-actin forward 5′-CTA CGT CGC CCT GGA CTT CGA GC-3′ and β-actin reverse 5′-GAT GGA GCC GCC GAT CCA CAC G-3′; MCP-1 forward 5′-GCG GAG CTA TAG AAG AAT CAC-3′ and MCP-1 reverse 5′-TTG GGT TGT GGA GTG AGT GT-3′.

Furthermore, after 16 h stimulation with TNF supernatants were collected, centrifuged to remove cellular debris and MCP-1 protein concentrations were analyzed by a sandwich ELISA following the instructor's manuals (Biosource, Camarillo, California).

Unstimulated SSc fibroblasts were analyzed to determine basal MCP-1 mRNA expression and MCP-1 secretion, respectively.

Statistical analysis Results are expressed as mean $\pm$ SD. Genotype frequencies are reported with their group percentages, a two-sided Fisher's Exact Test was used for comparison between these groups. Independent *in vitro* experiments were performed in triplicate. Statistical significance was evaluated in paired analyses using the Student's paired *t* test or the U-test (non-parametric), depending on the data distribution. A p value <0.05 was considered statistically significant.

Statistical evaluations were performed with the SPSS-10 for Windows statistical computer package (SSPS, Chicago, Illinois).

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Address correspondence to: Sigrid Karrer, MD, Department of Dermatology, University of Regensburg, 93042 Regensburg, Germany. Email: sigrid.karrer@klinik.uni-regensburg.de

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