Role of Monocyte Chemoattractant Protein-1 and its Receptor, CCR-2, in the Pathogenesis of Bleomycin-Induced Scleroderma

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Systemic sclerosis is a connective tissue disease characterized by excessive deposition of extracellular matrix in the skin as well as various internal organs. Cellular infiltrates are found in the dermis in early systemic sclerosis, which are suggested to play an important part. Recent studies suggest the involvement of monocyte chemoattractant protein-1, a C-C chemokine, in the fibrotic process. This study examines the role of monocyte chemoattractant protein-1 in the induction of dermal sclerosis in a murine model of bleomycin-induced scleroderma. Immunohistochemical analysis showed that expression of monocyte chemoattractant protein-1 in the infiltrating mononuclear cells was enhanced at 2 to 3 wk following bleomycin treatment, whereas expression of monocyte chemoattractant protein-1 in fibroblasts was detected at later stages in the sclerotic skin. Reverse transcriptase–polymerase chain reaction analysis showed that monocyte chemoattractant protein-1 mRNA expression in the lesional skin peaked at 2 to 3 wk following bleomycin treatment. Expression of CCR-2, a major receptor for monocyte chemoattractant protein-1, was also upregulated in the lesional skin at both protein and mRNA levels following bleomycin treatment. Administration of anti-monocyte chemoattractant protein-1 neutralizing antibody together with local bleomycin treatment reduced dermal sclerosis, along with a decrease of collagen content in the skin as well as mRNA expression of type I collagen. *In vitro* analysis showed that stimulation with monocyte chemoattractant protein-1 (10 ng per mL) upregulated α1(I) collagen and decorin mRNA expression in normal dermal fibroblasts, whereas mRNA levels of fibronectin and biglycan were not altered. These data suggest that monocyte chemoattractant protein-1 and CCR-2 signaling plays an important part in the pathogenesis of bleomycin-induced scleroderma. Monocyte chemoattractant protein-1 may contribute to the induction of dermal sclerosis via its direct effect of upregulation of mRNA expression of extracellular matrix on fibroblasts, as well as indirect effect mediated by a number of cytokines released from immunocytes recruited into the lesional skin. ***Key words: animal model/bleomycin/CCR-2/monocyte chemoattractant protein-1/scleroderma. J Invest Dermatol 121:510–516, 2003***

Systemic sclerosis (SSc) is an autoimmune disorder with unknown etiology, characterized by excessive accumulation of extracellular matrix in the involved skin or various internal organs and vascular involvement (Krieg and Meurer, 1988; LeRoy *et al*, 1991). Activated fibroblasts in the affected areas produce high amounts of collagen (Bostein *et al*, 1982; Krieg *et al*, 1986; Mauch *et al*, 1993). One of the characteristic histologic features is the inflammatory infiltrates of mononuclear cells (Fagundus and LeRoy, 1994; Postlethwaite, 1995), which is associated with increased collagen synthesis in the surrounding fibroblasts (Fleischmajer *et al*, 1977; Scharffetter *et al*, 1988). Inflammatory cells are potent candidates for releasing cytokines, which play a crucial part in initiating and/or leading to the sequential events of fibrosis (LeRoy *et al*, 1989; Kovacs and DiPietro, 1994).

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Abbreviations: MCP-1, monocyte chemoattractant protein-1; SSc: systemic sclerosis.

Monocyte chemoattractant protein (MCP)-1 is a chemoattractant for monocytes and T cells, belonging to a C-C chemokine superfamily of small proteins that are important in recruiting and activating leukocytes during inflammation (Rollins *et al*, 1988; Leonard and Yoshimura, 1990). MCP-1 has also been characterized as the murine JE gene product. Previous studies have shown that numerous types of cells, including fibroblasts, endothelial cells, epithelial cells, mononuclear cells, smooth muscle cells, and mast cells are capable of expressing MCP-1 in the presence of serum or specific stimuli (Leonard and Yoshimura, 1990; Selvan *et al*, 1994). Recent studies have demonstrated that MCP-1 gene expression is upregulated in human idiopathic pulmonary fibrosis (Antoniades *et al*, 1992), as well as rodent models of bleomycin-induced pulmonary fibrosis (Zhang *et al*, 1994) or crescent nephritis and interstitial kidney fibrosis (Lyoid *et al*, 1997). A recent *in vitro* study shows that MCP-1 upregulates type I collagen mRNA expression in rat lung fibroblasts, which is indirectly mediated by endogenous upregulation of transforming growth factor (TGF)-β gene expression (Gharaee-Kermani *et al*, 1996). We previously reported that MCP-1 enhances expression of matrix metalloproteinase-1 and -2, as well as tissue inhibitor of metalloproteinase-1 in cultured skin fibroblasts (Yamamoto *et al*, 2000a). In addition, MCP-1 expression was increased in scleroderma fibroblasts both *in vitro* (Yamamoto *et al*, 2001a) and *in vivo*

(Hasegawa *et al*, 1999; Yamamoto *et al*, 2001b). These results suggest an important involvement of MCP-1 in the pathogenesis of scleroderma.

We have recently established a mouse model for scleroderma by repeated application of bleomycin (Yamamoto *et al*, 1999a, b, 2000b, c; Yamamoto and Nishioka, 2002). Local injections of bleomycin-induced dermal sclerosis which mimics human scleroderma both histologically and biochemically in several mice strains. In this study, we examined the role of MCP-1 and its receptor, CCR-2, in the pathogenesis of bleomycin-induced dermal sclerosis.

MATERIALS AND METHODS

Animals Specific pathogen-free, female C3H/HeJ mice (6 wk old; weighing about 20 g) were purchased from Japan Clea (Tokyo, Japan) and maintained with food and water ad libitum. All mice were treated humanly, and all the experiments were performed in accordance with proper institutional approval.

Development of dermal sclerosis by bleomycin Bleomycin (Nippon Kayaku Co. Ltd, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg per mL. One hundred microliters of bleomycin or control PBS were injected into the shaved back of mice every other day for 4 wk with a 26-gauge needle. In each group, six mice were examined.

Immunohistochemical expression Biopsy was performed from the shaved back skin on the next day of the final treatment. The skin pieces were cut into two. One was fixed in 10% formalin solution and embedded in paraffin, and the other was snap-frozen in OCT compound (Miles, Elkhart, Indiana) in liquid nitrogen and stored immediately at −80°C. Five micrometer thick cryostat sections were prepared on poly L-lysin coated slides. Immunohistochemistry was performed by the avidin–biotin peroxidase technique using anti-murine MCP-1 antibody (Genzyme Techne, Minneapolis, Minnesota) (diluted in PBS, 1:250) or anti-CKR-2B antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California) 1:250. The sections were developed with 3,3′-diaminobenzidine solution as chromogen. They were counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were prepared by omission of the primary antibody and by its substitution for corresponding IgG.

RNA isolation and reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR) Total RNA was isolated from biopsied skin tissues using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Complementary single-stranded DNA was synthesized from total RNA by reverse transcription. Initially, 100 ng of total RNA in DEPC-treated water was heated at 65°C for 5 min and cooled rapidly. After adding 1 μL of 10 × PCR buffer (500 mM KCl, 100 mM Tris–HCl buffer, pH 8.4, 15 mM $MgCl_2$ and 0.01% gelatin), 1 mL of 25 mM aNTP (Takara, Tokyo, Japan), 1 μL of 10á × hexanucleotide mixture (Roche Diagnostics GmbH, Mannheim, Germany), 20 U of ribonuclease inhibitor (Takara) and 3 U of RAV-2 reverse transcriptase (Takara), the mixture was incubated at 42°C for 60 min, heated at 94°C for 5 min and quick-chilled on ice. The cDNA was amplified by PCR using Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, New Jersey) with the specific primers for MCP-1 (sense; 5′CTCACCTGCTGCTACTCATTC 3′, anti-sense; 5′ GCATGAGGTGGTTGTGAAAAA 3′), CCR-1 (sense; 5′ GTGTTCATCATTGGAGTGGTGG 3′, anti-sense 5′ GGTTGAACAGGTAGATGCTGGTC 3′), CCR-2 (sense; 5′ TGTTACCTCAGTTCATCCACGG 3′, anti-sense; 5′ CAGAATGGTAATGTGAGCAGGAAG 3′), type I collagen (sense; 5′ TGGTGCCAAGGGTCTCACTGGC 3′, anti-sense; 5′ GGACCTTGTACACCACGTTCACC 3′), and GAPDH (sense; 5′ TGAAGGTCGGTGTGAACGGATTTGGC 3′, anti-sense; 5′ CATGTAGGCCATGAGGTCCACCAC 3′). Other primers for MIP-1α, MIP-1β, and RANTES were purchased from Biosource International (Camarillo, California). The PCR conditions for amplification were as follows; the mixture was first incubated for 1 min and 30 s at 94°C and then cycled 30 times at 94°C for 25 s, 55°C for 30 s, 72°C for 45 s, followed by extension at 72°C for 10 min. For GAPDH, amplification was performed 25 cycles. Cycle curve studies confirmed that amplification occurred in a linear range. After amplification, PCR products were subjected to electrophoresis on 1.7% agarose gels and detected by ethidium bromide under ultraviolet illuminator. For negative control, total cellular RNA without reverse transcription was used.

Cell culture Primary human normal dermal fibroblasts were established by outgrowth from skin biopsies of healthy donors (n = 4, aged 40–65 y old) after informed consent. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 50 μg sodium ascorbate per mL, 100 U per mL penicillin, 100 μg streptomycin per mL, and grown in the moist atmosphere of a CO_2 incubator at 37°C. Cells were used at passage numbers 3 to 6.

Stimulation by MCP-1 After the seeded fibroblasts were grown semiconfluent in the monolayer, the medium was changed to Dulbecco's modified Eagle's medium without fetal bovine serum. Twenty-four hours later, cells were incubated with 10 ng MCP-1 per mL (R&D Systems, Minneapolis, Minnesota) for 6 to 24 h.

Northern blot analysis Total RNA was extracted from cultured fibroblasts as described above. Aliquots of 5 μg per lane were electrophoresed in denaturing agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen membranes (NEN Life Science Products, Bostan, Massachusetts), fixed by ultraviolet cross-linking and hybridized to cDNA probes specific for α1(I) collagen (Hf 677) (Chu *et al*, 1982), fibronectin (FN 711) (Bernard *et al*, 1985), decorin (Krusius and Ruoslahti, 1986), biglycan (Fisher *et al*, 1989), and GAPDH (Fort *et al*, 1985), which were labeled by random priming using [α-^{32}P]deoxycytidine triphosphate. Filters were hybridized overnight at 42°C in 50% formamide, 5á × sodium citrate/chloride buffer, 100 μg denatured salmon sperm DNA per mL, 5á × Denhardt's, washed twice at room temperature in 2á × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate, followed by washing step at high stringency (62–65°C in 0.1á × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate). Autoradiography was performed from 6 h to 1 d at −80°C using intensifying screens (Kodak, Rochester, New York). Signal intensities were determined by densitometry, and normalized to GAPDH.

Administration of anti-MCP-1 antibody To determine the effect of the antibody on the induction of dermal sclerosis, we intravenously administered anti-murine MCP-1 neutralizing antibody (Genzyme Techne) 250 μg per mL 30 min before subcutaneous injections of bleomycin (1 mg per mL). Fifty microliters of anti-MCP-1 antibody was injected from tail vein (n = 6) every other day for 4 wk and the back skins were harvested. Normal goat serum (NGS) (n = 6) and anti-RANTES neutralizing antibody (Genzyme Techne) 250 μg per mL (n = 6) were used as a control under the same protocol.

Collagen content Collagen deposition was estimated by determining the total collagen content of the 8 mm punch biopsy specimens using the Sircol Collagen Assay kit (Biocolor, Northern Ireland) according to the manufacturer's instructions. The biopsies were homogenized in 0.5 M acetic acid, and 1 mL of Sircol dye reagent that binds to collagen was added to each sample and then mixed for 30 min. After centrifugation, the pellet was suspended in 1 mL of the alkali reagent included in the kit and assessed colorimetrically at 540 nm by a spectrophotometer. Collagen standard solutions were utilized to construct a standard curve. Results were expressed as a percentage compared with control group that received only PBS injections.

Statistics Results were expressed as mean ± SD. Significance testing was analyzed using Mann–Whitney U test. $p < 0.05$ was considered to be significant.

RESULTS

Expression of MCP-1 and CCR-2 in the lesional skin of bleomycin-induced scleroderma To begin with, immunohistochemical detection of MCP-1 was examined in the lesional skin. Fibroblasts and mononuclear cells were morphologically identified by a few dermatologists in our department. MCP-1-positive cells were weakly detected on scattered mononuclear cells in PBS-treated skin (**Fig 1*A***). Fibroblastic cells were negatively stained with MCP-1 in PBS-treated skin. By contrast, MCP-1 was detectable on the infiltrating mononuclear cells in the dermis at 1 wk, and the number of MCP-1-positive cells peaked at 2 to 3 wk in bleomycin-treated mice (**Fig 1*B***). In addition, the positively labeled fibroblastic cells were also localized in the sclerotic

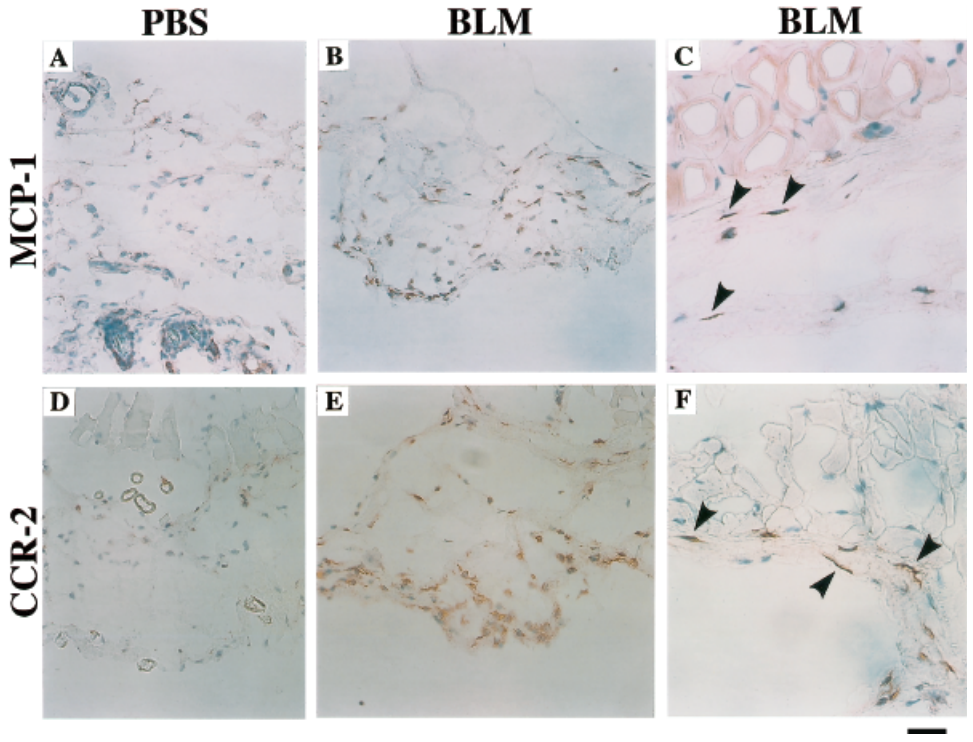

Figure 1. Immunohistologic detection of MCP-1 and CCR-2 in the lesional skin following treatment with either PBS (4 wk) or bleomycin (BLM; 1–4 wk). MCP-1 expression was faint to absent in mononuclear cells in the PBS-treated skin (*A*). By contrast, MCP-1 expression is upregulated on the infiltrating mononuclear cells in the lower dermis at 2 wk (*B*), and furthermore on fibroblastic cells *(arrowheads)* in the sclerotic skin at 4 wk (*C*). CCR-2 expression was weakly detected in the PBS-treated skin (*D*), whereas increased on the infiltrating mononuclear cells (*E*) as well as on fibroblastic cells (*arrowheads*) (*F*) in the bleomycin-treated skin. Magnification: (*A,B,D,E*) × á200; (*C,F*) × á400. *Scale bar:* (*A,B,D,E*) 50 μm; (*C,F*) 25 μm.

dermis at later stages (**Fig 1*C***). MCP-1 was also detected in keratinocytes, endothelial cells, and possibly mast cells.

CCR-2 is the major receptor for MCP-1. MCP-1 and CCR-2 have been implicated in several inflammatory diseases. In this study, we examined the involvement of CCR-2 in the induction of dermal sclerosis induced by bleomycin. Immunohistochemical examination revealed that CCR-2 expression was enhanced on the infiltrating mononuclear cells at 2 to 3 wk following bleomycin treatment, as compared with PBS treatment (**Fig 1*D*,*E***). Thereafter, CCR-2 expression on the infiltrating mononuclear cells was decreased. On the contrary, CCR-2 was also detected on the fibroblastic cells in the sclerotic dermis after 4 wk (**Fig 1*F***). This immunohistologic distribution demonstrated concurrent upregulation of MCP-1 and CCR-2 with enhanced expression on mononuclear cells at early inflammatory stages and on fibroblasts at later sclerotic stages.

Results of reverse transcriptase–PCR analysis showed that mRNA expression of MCP-1 was increased and peaked at 2 to 3 wk following bleomycin treatment (**Fig 2**). The highest mRNA expression of MCP-1 coincides with the kinetics followed by the infiltrating leukocytes. In addition to MCP-1, mRNA expression of MIP-1α and MIP-1β was also upregulated at 3 wk after bleomycin treatment. On the other hand, RANTES mRNA expression was not significantly altered during the course.

Results of reverse transcriptase–PCR analysis showed increased expression of CCR-2 mRNA, which reached a maximum at 2 to 3 wk following bleomycin treatment (**Fig 3**). CCR-1 mRNA level was mildly upregulated at 2 wk following bleomycin treatment.

Inhibition of dermal sclerosis by antibody against MCP-1 Next, effect of anti-MCP-1 neutralizing antibody was examined to determine whether blockade of MCP-1 activity suppresses the induction of dermal sclerosis. Mice treated with

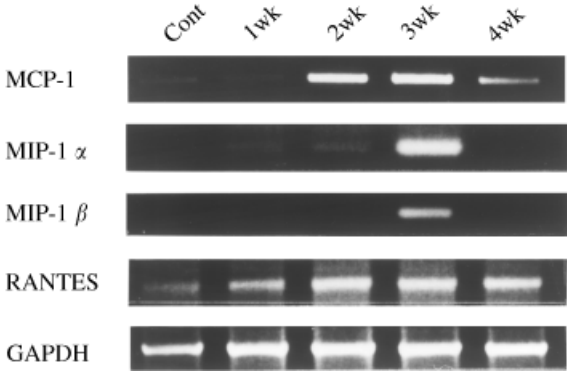

Figure 2. Reverse transcriptase–PCR analysis of MCP-1, MIP-1α, MIP-1β, and RANTES. Total RNA was isolated from skin samples treated with either PBS for 4 wk (Cont) or bleomycin (1–4 wk), and analyzed with specific primers. Results shown are representative of three independent experiments.

bleomycin (1 mg per mL) and intravenous NGS showed definite dermal sclerosis with thickened collagen bundles and deposition of homogeneous materials in the dermis (**Fig 4*A***). On the contrary, systemic administration of anti-MCP-1 antibody together with local bleomycin treatment every other day for 4 wk suppressed the induction of dermal sclerosis (**Fig 4*A***). The number of infiltrating mononuclear cells was reduced up to 50% after anti-MCP-1 treatment. Dermal thickness was also decreased after the treatment with anti-MCP-1 antibody. mRNA expression of type I collagen showed a partial reduction in mice treated with bleomycin and anti-MCP-1 antibody, as

compared with that treated with bleomycin and NGS (**Fig 4*B***). Collagen content in the skin treated with bleomycin and NGS (181 ± 8.1% of control) showed a significant decrease after the treatment with bleomycin and anti-MCP-1 antibody (150°C 9.9% of control) ($p < 0.05$) (**Fig 4*C***). On the contrary, treatment with anti-RANTES antibody together with local bleomycin injections did not reduce dermal sclerosis (not shown), and there was no significant difference in the collagen contents in the skin between mice treated with bleomycin and NGS and those treated with bleomycin and anti-RANTES antibody (177 ± 8.9% of control) (**Fig 4*C***).

MCP-1 enhances mRNA expression of α1(I) collagen in cultured fibroblasts Finally, we investigated the direct effect of MCP-1 on the induction of extracellular matrix in cultured fibroblasts. The time course of α1(I) collagen, fibronectin, decorin, and biglycan mRNA expression was examined by northern blot analysis in normal dermal fibroblasts exposed to 10 ng MCP-1 per mL for different time periods (6–24 h). As shown in **Fig 5**, α1(I) collagen mRNA was upregulated as early as 6 h and further increased at 24 h. Densitometric quantification revealed up to 4.3-fold increase of α1(I) collagen mRNA expression by MCP-1. Decorin mRNA expression showed its peak at 12 h, and waned to basal level at 24 h. MCP-1 upregulated α1(I) collagen and decorin mRNA expression in a concentration-dependent manner ranging from 1 to 50 ng per mL (data not shown). On the contrary, mRNA levels of fibronectin and biglycan were not significantly altered.

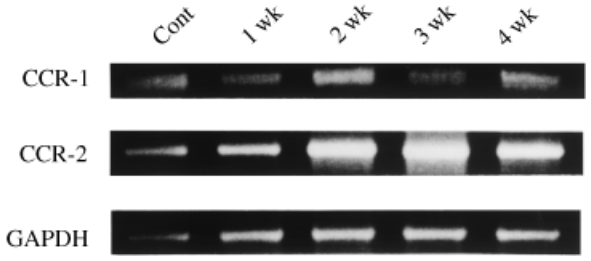

Figure 3. Reverse transcriptase–PCR analysis of CCR-1 and CCR-2. Total RNA was isolated from skin samples treated with either PBS for 4 wk (Cont) or bleomycin (1–4 wk), and analyzed with specific primers. Results shown are representative of three independent experiments.

DISCUSSION

Recent studies suggest that MCP-1 is involved in fibrotic process, including human idiopathic pulmonary fibrosis (Antoniades *et al*, 1992), acute hepatic fibrogenesis (Marra *et al*, 1998), as well as experimental animal models of wound healing (DiPietro *et al*, 1995), lung fibrosis induced by bleomycin (Zhang *et al*, 1994), and renal fibrosis (Lyoid *et al*, 1997). We have recently shown that MCP-1 expression is upregulated in scleroderma skin (Yamamoto *et al*, 2001b), as well as cultured scleroderma fibroblasts (Yamamoto *et al*, 2001a). Increased expression of MCP-1 in scleroderma has also been reported by other groups (Distler *et al*, 2001; Galindo *et al*, 2001a). Here we demonstrate that MCP-1 expression is enhanced in the lesional skin of an *in vivo* model for scleroderma. Immunohistologic localization revealed that both mononuclear cells and fibroblasts were the predominant cellular sources of MCP-1. MCP-1 was detected on the infiltrating mononuclear cells at relatively early phases, and thereafter also detected on fibroblasts in the sclerotic stages. MCP-1 expression in fibroblasts can play an important part in the development of fibrosis in scleroderma. It has been shown to modulate connective tissue deposition by both inducing collagen synthesis and collagenase production. MCP-1

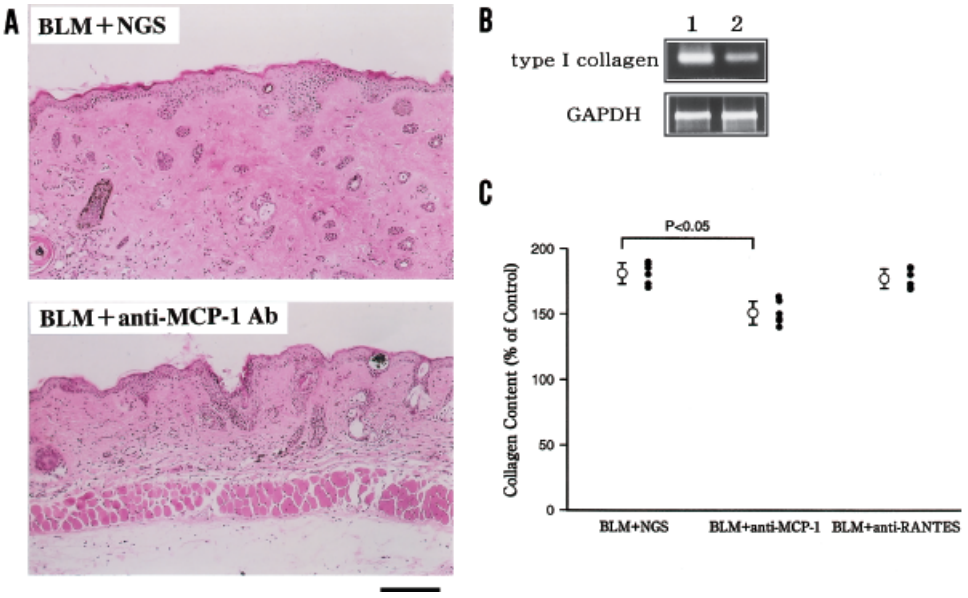

Figure 4. Effect of anti-MCP-1 antibody on bleomycin-induced dermal sclerosis. (*A*) Anti-MCP-1 antibody blocks the induction of scleroderma induced by bleomycin. Mice were treated with bleomycin (1 mg per mL) together with intravenous administration of either anti-MCP-1 antibody ($n = 6$) or NGS ($n = 6$). Mice treated with bleomycin and NGS for 4 wk demonstrate definite dermal sclerosis with thickened collagen bundles in the thickened dermis (upper). Mice treated with bleomycin and anti-MCP-1 neutralizing antibody for 4 wk show reduction of dermal sclerosis (lower). Hematoxylin and eosin stain, original magnification × á140. *Scale bar*: 100 μm. (*B*) Type I collagen mRNA expression. Total RNA was isolated from the lesional skin and analyzed by reverse transcriptase–PCR. *Lane 1*: mice treated with bleomycin and NGS for 4 wk. *Lane 2*: anti-MCP-1 treated mice together with bleomycin for 4 wk. Results shown are representative of three independent experiments. (*C*) Collagen content in the skin. Each value (% of control) was shown as closed circles. Mean ± SD are shown. Collagen content was significantly suppressed in mice treated with intravenous administration of anti-MCP-1 antibody together with local bleomycin, compared with those treated with NGS and bleomycin ($p < 0.05$).

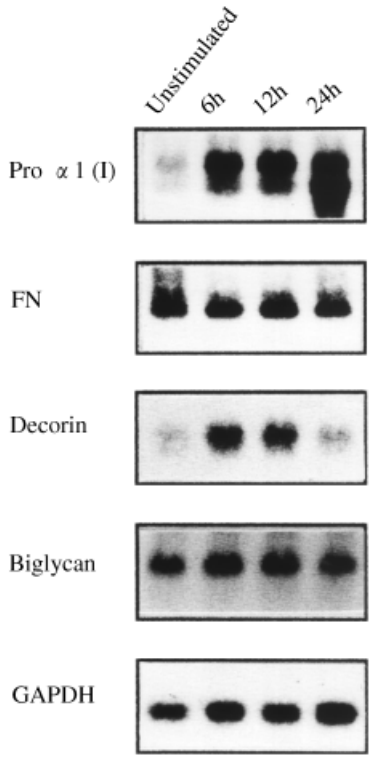

Figure 5. Effect of MCP-1 on α1(I) collagen, fibronectin, decorin, and biglycan mRNA expression in human skin fibroblasts. Semiconfluent cultures of normal dermal fibroblasts were incubated for various time periods (6–24 h) in the presence of 10 ng per mL MCP-1 in serum-free medium. Total RNA (5 μg per lane) was analyzed by northern blot hybridization with cDNA probes, as described in *Materials and Methods.* As a control, RNA from a parallel culture (unstimulated), which was maintained for 24 h without MCP-1, was loaded. Results shown are representative of three independent experiments.

produced by activated fibroblasts could act as a chemoattractant for mononuclear cells, which contribute to the initial inflammatory infiltrate localized around small blood vessels in the dermis in the early stages of scleroderma and could be involved in the perpetuation of the process. These infiltrating cells have been shown to play a disease-triggering role releasing a number of growth factors, cytokines, and chemokines, many of which are known to activate matrix production, fibroblast proliferation, and migration.

We previously reported that mast cells are increased in number in the lesional skin in parallel with the induction of dermal sclerosis by bleomycin (Yamamoto *et al*, 1999a). In particular, marked degranulation is prominent at an early phase. Mast cells are suggested as important initiators of SSc (Gruber, 1995), as mast cells produce a number of cytokines, growth factors, and mediators that are capable of activating fibroblasts or endothelial cells (Galli, 1989). MCP-1 is shown to be abundantly expressed on mast cells (Selvan *et al*, 1994; Yamamoto *et al*, 2001d). We and others previously showed that stem cell factor upregulates MCP-1 gene expression in human mast cells (Baghestanian *et al*, 1997; Yamamoto *et al*, 2001d). We speculated that mutual interaction of stem cell factor released from fibroblasts and MCP-1 from mast cells may play an important part in fibrosis (Yamamoto *et al*, 2001d). Also, previous studies demonstrated that MCP-1 can induce degranulation of mast cells (Conti *et al*, 1995). Taken together, MCP-1 released from mononuclear cells, mast cells, and fibroblasts are supposed to play an important part in the induction of cutaneous sclerosis in this model.

Recently, excessive oxidative stress has been implicated in the pathogenesis of scleroderma (Bruckdorfer *et al*, 1995; Stein *et al*, 1996; Casciola-Rosen *et al*, 1997; Sambo *et al*, 1999). Bleomycin is known to generate reactive oxygen species, such as superoxide and hydroxyl radicals. Reactive oxygen species can cause several abnormalities such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines, including platelet-derived growth factor and TGF-β. Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations (Murrell *et al*, 1990), and the production of increased amounts of collagen (Chojkier *et al*, 1989). A recent study has demonstrated that constitutive production of reactive oxygen species by scleroderma fibroblasts is essential to fibroblast proliferation and expression of type I collagen gene (Sambo *et al*, 2001). We previously reported the inhibitory effect of lecithinized superoxide dismutase on bleomycin-induced scleroderma (Yamamoto *et al*, 1999c). On the other hand, it is also reported that oxidative stress transiently induces MCP-1 mRNA and protein expression in cultured dermal fibroblasts (Galindo *et al*, 2001b). Therefore, elevated levels of MCP-1 in this model might be induced, in part, via reactive oxygen species by bleomycin.

Current studies suggest that an imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. IL-4, which is produced by activated memory T cells and mast cells, is known to promote fibroblast proliferation, collagen gene expression, and collagen synthesis (Gillery *et al*, 1992; Postlethwaite *et al*, 1992; Sempowski *et al*, 1994). A recent report suggests that most $CD4^+$ T cell clones generated from scleroderma skin biopsies exhibited type 2 cytokine profiles (Mavilia *et al*, 1997). In addition, serum in the majority of SSc patients showed elevated levels of CD30 (Mavilia *et al*, 1997). MCP-1 is a crucial factor for the development of type 2 responses. MCP-1 has been shown to induce the expression of IL-4 (Hogaboam *et al*, 1999; Gu *et al*, 2000; Trautman *et al*, 2000). Thus, increased levels of MCP-1 are supposed to shift the type 2 polarization, which may favor the imbalance in SSc. On the other hand, several studies have demonstrated that type 2 cytokines induce MCP-1 from multiple structural cell populations (Rollins and Pober, 1991; Goebeler *et al*, 1997). Therefore, enhanced expression of MCP-1 may be induced, in part, by type 2 cytokines, which are supposed to play a part in the induction of sclerotic skin. Such mutual interaction may play a significant part in the induction of scleroderma both at initial and later phases.

In this study, mRNA levels of MIP-1α and MIP-1β were also upregulated at 3 wk after bleomycin treatment, in addition to MCP-1. MIP-1 are secreted by activated T cells, B cells monocytes, and mast cells. MIP-1α is also reported to be highly expressed during many stages of interstitial fibrotic diseases (Hogaboam *et al*, 1998). MIP-1α was shown to play a part in the recruitment of mononuclear and polymorphonuclear cells to the lung in the bleomycin-induced pulmonary fibrosis model (Smith *et al*, 1994). These results suggest that MIP-1 may also be a key molecule in macrophage trafficking into inflammatory area.

Chemokine responses are mediated by the interaction of the soluble chemokine proteins with receptors. CCR-2 is the major MCP-1 receptor, and MCP-1 binds to CCR-2 with high affinity. CCR-2 belongs to the family of G protein-coupled receptors. Two isoforms with alternatively spliced intracellular carboxyl tails, CCR2A and CCR2B, were isolated; however, they differ only in their cytoplasmic carboxyl tails. CCR-2 is expressed on monocytes, activated T cells, B cells, natural killer cells, fibroblasts, and mast cells (Kunkel *et al*, 1993; Frade *et al*, 1997; Hogaboam *et al*, 1999). Our results also show that bleomycin-induced CCR-2 expression in the lesional skin in association with augmented levels of MCP-1. Upregulation of CCR-2 in fibroblasts in parallel with the induction of dermal sclerosis may enhance the susceptibility of the MCP-1 effect. Thus, activation of CCR-2 was suggested to exert profound effects on the fibrotic process.

The inhibition of MCP-1 partially attenuated dermal sclerosis induced by bleomycin, when anti-MCP-1 antibody was

administered throughout all stages. Blocking the activity of MCP-1 led to the reduction of collagen production, resulted in the suppression of dermal sclerosis in this model. On the contrary, administration of anti-RANTES antibody reduced neither histologic dermal sclerosis nor collagen content in the skin. There was a reduction in the number of skin monocytes/macrophages up to nearly 50%, although we examined only one time point at 4 wk. As mononuclear cells accumulation was not completely inhibited by MCP-1 neutralization, other chemokines may contribute to migration of mononuclear cells in this model. Mononuclear cells are considered to be a major TGF-β producing cells. MCP-1 can stimulate the production of TGF-β1 (Hogaboam *et al*, 1999), which is a key cytokine in fibrosis. It was reported that inhibition of MCP-1 reduced TGF-β and procollagen synthesis, thus improving renal fibrosis in an animal model (Lyoid *et al*, 1997). Therefore, amelioration of dermal sclerosis by neutralizing MCP-1 activity may also be mediated through reduction of TGF-β.

In vitro analysis showed that MCP-1 upregulates the mRNA levels of α1(I) collagen and decorin in normal skin fibroblasts, whereas those of fibronectin and biglycan were not significantly altered. Biglycan/PG-I and decorin/PG-II are two small proteoglycans with a core protein of similar size (42 kDa), containing most often two and one chondroitin/dermatan sulfate glycosaminoglycan side chains, respectively. Although these molecules display a high degree of structural similarities, our results showed differential regulation of these molecules by MCP-1. These data indicate the direct effect of MCP-1 to produce high amounts of extracellular matrix (collagen and decorin) on fibroblasts.

In conclusion, enhanced expression of MCP-1 and its corresponding receptor, CCR-2, on both infiltrating mononuclear cells and fibroblasts suggest an important involvement of these molecules in the pathogenesis of experimental scleroderma induced by bleomycin. MCP-1 may play an important part in the fibrotic process not only via indirect effect through inflammatory cytokines derived from mononuclear cells recruited into the lesional skin, and activation and production of matrix metalloproteinases, as well as via direct effect of upregulation of type I collagen gene expression. Modulation of MCP-1/CCR-2 signaling may implicate the therapeutic approach of experimental scleroderma.

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