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Animal Model of Sclerotic Skin. I: Local Injections of Bleomycin Induce Sclerotic Skin Mimicking Scleroderma

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We have established a mouse model for scleroderma induced by repeated local injections of bleomycin (BLM). Daily injection of BLM at a dose of >10 μg per ml for 4 wk induced histologic changes of dermal sclerosis, but not fibrosis, with thickened and homogenous collagen bundles and cellular infiltrates in BALB/C mice, whereas clinical signs of scleroderma were not apparent. In addition, lung fibrosis was also induced preceding the cutaneous changes. Sclerotic changes were not found in other sites of the skin distant from the injection site. Dermal sclerosis could also be induced by injecting BLM only every other day. The sclerotic changes of the dermis were sustained after ceasing BLM applications for at least 6 wk. Mast cells gradually increased in number as the sclerotic changes developed. Marked degranulation of mast cells was observed with elevated histamine release. The amount of hydroxyproline in skin was significantly increased at 4 wk of BLM treatment as compared with that in untreated or phosphate-buffered saline-treated mice. Anti-nuclear antibody was detected in serum of BLM-treated mice. Transforming growth factor-β1 mRNA was detected at an early phase, while transforming growth factor-β2 mRNA was strongly expressed at 4 wk when the sclerotic features were prominent. These results suggest that dermal sclerosis induced by BLM closely resembles systemic sclerosis both histologically and biochemically. Our mouse model can provide a powerful tool of inducing dermal sclerosis to examine the pathogenesis and the therapeutic approach of scleroderma. ***Key words: fibrosis/mast cell/RT-PCR/sclerosis/TGF-β. J Invest Dermatol 112:456–462, 1999***

Systemic sclerosis (SSc) is an autoimmune disorder characterized by extensive fibrosis associated with increased collagen synthesis and accumulation, and affects the skin as well as various internal organs such as lung, heart, kidney, and gastrointestinal tract (Haynes and Gershwin, 1982; Krieg and Meurer, 1988). Scleroderma, sclerosis of the skin, is the typical symptom of SSc, affects the quality of life of the patients, and is resistant to all kinds of treatments. Although numerous efforts have been undertaken to elucidate the pathogenesis of SSc, the etiology and the initial events of SSc remain unclear. By now, there is no animal model that exhibits all aspects of SSc except the limited available models of the tight skin mouse and chronic graft-*versus*-host disease mouse. Tight skin mouse is a spontaneous mutant with a single gene defect on chromosome 2, characterized by extensive connective tissue deposition in skin and multiple internal organs (Katsuri *et al*, 1994). It develops cutaneous hyperplasia with histologic and biochemical changes in the skin similar to those of human scleroderma skin. Graft-*versus*-host disease mouse develops cutaneous fibrosis, loss of dermal fat, atrophy of dermal appendages, mast cell depletion, and a mononuclear cell infiltration (Claman *et al*, 1985). UCD line 200 chickens develop fibrotic changes and produce anti-nuclear antibody (Gershwin *et al*, 1981; Van de Water *et al*, 1984; Van de Water and Gershwin, 1985). This chicken model resembles SSc regarding particularly mononuclear cell infiltration in various organs and fibrosis of skin and esophagus.

Bleomycin (BLM), whose side-effects include pulmonary fibrosis or scleroderma-like conditions, is an antibiotic widely used for cancer treatment. BLM is also well known to induce lung injury/fibrosis in experimental animal models (Aso *et al*, 1976; Adamson, 1984; Chandler, 1990). In this study, we have attempted to establish an animal model of SSc skin by subcutaneous injections of BLM. Repeated injections of BLM induced not only skin sclerosis but also lung fibrosis in mice. Our mouse model could provide an aid to analyse the etiology and therapeutic strategy of scleroderma of SSc.

MATERIALS AND METHODS

Animals Specific pathogen-free, female BALB/C mice and C3H mice of 6 wk old (weighing about 20 g) were purchased from Japan Clea (Tokyo, Japan) and maintained with food and water *ad libitum*.

Bleomycin treatment BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) at different concentrations of 1 mg per ml, 100 μg per ml, 10 μg per ml, and 1 μg per ml, and sterilised with filtration. One hundred microliters of each concentration of BLM or PBS were injected subcutaneously into the shaved back of the mice daily for 1–4 wk with a 27 gauge needle. In each group, more than 10 mice were histologically examined. In several experiments, mice were injected only every other day.

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Abbreviations: BLM, bleomycin; SSc, systemic sclerosis.

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Histopathology and immunohistochemistry The back skin was removed on the next day after the final injection. The skin pieces were cut into two. One was fixed in 10% formalin solution and embedded in paraffin, and the other half was snap-frozen in OCT compound (Miles, Elkhart, IN) in liquid nitrogen and stored immediately at –80°C. Five micrometer thick cryostat sections were prepared on poly L-lysin-coated slides, and fixed with cold acetone for 10 min. To block the endogenous peroxidase activity, the sections were treated with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature and then washed in PBS. The sections were stained using a standard avidine-biotin peroxidase technique with anti-L3T4 monoclonal antibody (CD4, diluted in PBS, 1:200) (Seikagaku Kogyo, Tokyo, Japan), anti-Lyt 2 monoclonal antibody (CD8, 1:200) (Pharmacia, Uppsala, Sweden), anti-mouse pan-tissue-fixed macrophage antibody (1:100) (BM-8, Biomedicals, Switzerland), anti-stem cell factor monoclonal antibody (1:200) (Genzyme, Cambridge, MA), anti-transforming growth factor-β (1:250) (TGF-β) polyclonal antibody (R&D Systems, Minneapolis, MN), and anti-decorin antibody (1:100) (generous gift by Prof. Shinkai, Chiba, Japan). Cytokine expression of cellular infiltrates was also examined by several anti-cytokine antibodies against interleukin-6 (IL-6) (1:250) (Genzyme), IL-10 (1:250) (Upstate Biotech, NY), and intercellular adhesion molecule-1 (1:250) (R&D). 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 omitting the primary antibodies, and by their substitution with a nonspecific IgG antibody at the dilution used for the specific antibodies in this study. Dermal thickness was measured after taking photographs under a light microscope of hematoxylin and eosin sections.

Measurement of hydroxyproline Full-thickness punch-biopsied specimens of 6 mm diameter were obtained weekly from the shaved dorsal skin of each animal during the 4 wk of the injection and stored at –80°C. Collagen deposition was estimated by determining the total content of hydroxyproline in the skin. The stored skin pieces were hydrolyzed with 6 N hydrochloric acid under 110°C for 18 h according to the method previously described (Woessener, 1961). After neutralization with sodium hydroxide, the hydrolysates were diluted with distilled water. Hydroxyprofine in the hydrolysates was assessed colorimetrically at 560 nm with p-dimethylaminobenzaldehyde. Results were expressed as micrograms of hydroxyproline per 6 mm diameter skin pieces.

Collagen analysis of the sclerotic skin Six millimeter punch biopsied tissues were homogenized in acetic acid at 4°C to extract collagen. One milligram of pepsin was added to each homogenized sample and incubated at 4°C for 24 h with shaking. The pepsin-solubilized material was collected after removal of the insoluble residues by centrifugation at 35,000 × *g* for 60 min at 4°C. Thus extracted collagen was analyzed by polyacrylamide stacking gel electrophoresis, utilizing a 10% polyacrylamide-running gel overlaid with a 3.5% polyacrylamide stacking gel. Following electrophoresis, the gels were stained with Coomassie brilliant blue for identification of pepsin-resistant collagen.

Mast cell number and plasma histamine level Mast cells were identified by toluidine blue at a pH of 2.5, 4.1, and 7.0. Cells containing metachromatic granules were counted in 10 random grids under high magnification of ×400 power fields of a light microscope.

Blood samples were obtained weekly by cardiac puncture at each week, and stored at –80°C. Plasma histamine levels were measured by radioimmunoassay.

Enzyme-linked immunosorbent assay of serum cytokines Sera was obtained by a cardiac puncture at 1, 2, 3, and 4 wk treatment of BLM (n = 5) and stored at –80°C. Serum levels of IL-4, IL-6, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were assessed using enzyme-linked immunosorbent assay kit (Genzyme).

Reverse transcriptase-polymerase chain reaction (PCR) of cytokines Total RNA was extracted weekly from biopsied frozen tissues during the 4 wk of BLM injection with RNA zol (Chinna/Biotex, Houston, TX). RNA yield and purity was determined with spectrophotometry. Total RNA was then diluted with sterile diethylpyrocarbonate-treated water and stored at –80°C until use. Reverse transcriptase-PCR was performed using the DNA Thermocycler (Program Temp Control System, PC-700, ASTEC, Tokyo). Complementary single-stranded DNA was synthesized from total RNA by reverse transcription. Initially, 100 ng of total RNA (with or without prior treatment with RNAse-free DNAse) in diethylpyrocarbonate-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 αNTP (Takara, Tokyo, Japan), 1 μl of 10 × hexanucleotide mixture (Boehringer, 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 with the use of the specific primers for mouse TGF-β1, TGF-β2, platelet-derived growth factor (PDGF)-AA, TNF-α, and β-actin as described (Brown *et al*, 1995). The upstream and downstream primers are: TGF-β1, 5′ GCTAATGGTGGACCGCAACAACG, 3′ CTTGCTGTACTGTGTGTCCAGGC; TGF-β2, 5′ CACCTCCCCTCCGAAAATGCCAT, 3′ ACCCCAGGTTCCTGTCTTTGTGGT; PDGF-AA, 5′ CACATCGGCCAACTTCCT, 3′ TCACACGCCACGCACATC; TNF-α, 5′ AGCCCACGTCGTAGCAAACCACCAA, 3′ ACACCCATTCCCTTCACAGAGCAAT; β-actin, 5′ GTGGGCGCTCTAGGCACCAA, 3′ CTCTTTGATGTCACGCACGATTTC. The PCR conditions were optimized for each set of primers, and PCR was performed using different numbers of cycles to ensure that amplification occurred in a linear range. The PCR reaction mixture contained 10 μl cDNA, 5 μl of 10 × PCR buffer, 8 μl of 1.25 mM αNTP, 5 μl of 20 pM 5′ and 3′ primers, and 1.5 U of Taq polymerase (Perkin Elmer, Cetus, Norwalk, CT). Cycle number for amplification was 35, except for PDGF-AA (30 cycles). After amplification, PCR products were subjected to electrophoresis on 1.7% agarose gels and detected by ethidium bromide under UV illuminator. The intensity of each band was measured by a densitometer (EPA-3000, Chemiway, Tokyo) and compared with that of β-actin to quantitate the PCR products. As a negative control, total cellular RNA without reverse transcription was used.

Detection of autoantibodies in the serum 3T3 fibroblasts were used as the substrate to screen for the appearance of autoantibodies. After fixing with cold acetone/methanol for 15 min, 3T3 fibroblasts were incubated with various concentrations of mouse serum (n = 5) for 30 min at room temperature. They were washed with PBS and incubated with 1:100 dilution of fluorescein-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratory, Baltimore, MD) and examined with fluorescent epi-illumination.

Statistical analysis Results were expressed as means ± SD. Significance testing was assessed by Mann–Whitney U test. A p value < 0.05 was considered as significant.

RESULTS

Subcutaneous injections of BLM induces dermal sclerosis Subcutaneous injections of 1 mg BLM per ml for 4 wk induced marked dermal sclerosis in BALB/C mice, which was histologically characterized by thickened and homogenous collagen bundles, thickening of vascular walls, and inflammatory infiltrates, whereas PBS-treated mice did not develop either sclerosis or fibrosis (**Fig 1*A–D***). Preliminary results showed no histologic differences between males and females. Dermal sclerosis was also induced after injection of 10 μg per ml and 100 μg BLM per ml, whereas 1 μg BLM per ml did not cause definite sclerosis even at 4 wk. This dermal sclerosis was sustained for at least 6 wk after cessation of treatment (data not shown). The dermal sclerosis was also induced by injecting BLM only every other day. Sclerosis, but not fibrosis, was induced even in "BLM-resistant" BALB/C mice. In comparison with BALB/C mice, C3H "BLM-sensitive" mice developed dermal sclerosis already after 3 wk (data not shown). There was diffuse positive staining for colloidal iron stain in the sclerotic lesional skin, whereas only faint staining was noted in PBS-treated mice (**Fig 1*E*, *F***). Examination of the lung revealed thickened alveolar septa with infiltration of mononuclear cells preceding the cutaneous changes (at 1 wk) (**Fig 1*G***); however, sclerosis was not induced in uninjected sites of the skin, such as in toes, abdomen, and kidney even after 4 wk of treatment (data not shown). Toluidine blue stain (pH 2.5, 4.1, and 7.0) demonstrated increased numbers of mast cells around sclerotic lesions. Marked degranulation was observed especially in the early phase, prior to the increase of mast cell numbers (**Fig 1*H***). Positive staining for CD8, CD4, and BM-8 was also found in the cellular infiltrates (**Fig 1*I*, *J***).

Dermal thickness showed a time-dependent increase in BLM-treated skin, and exhibited a significant difference as compared

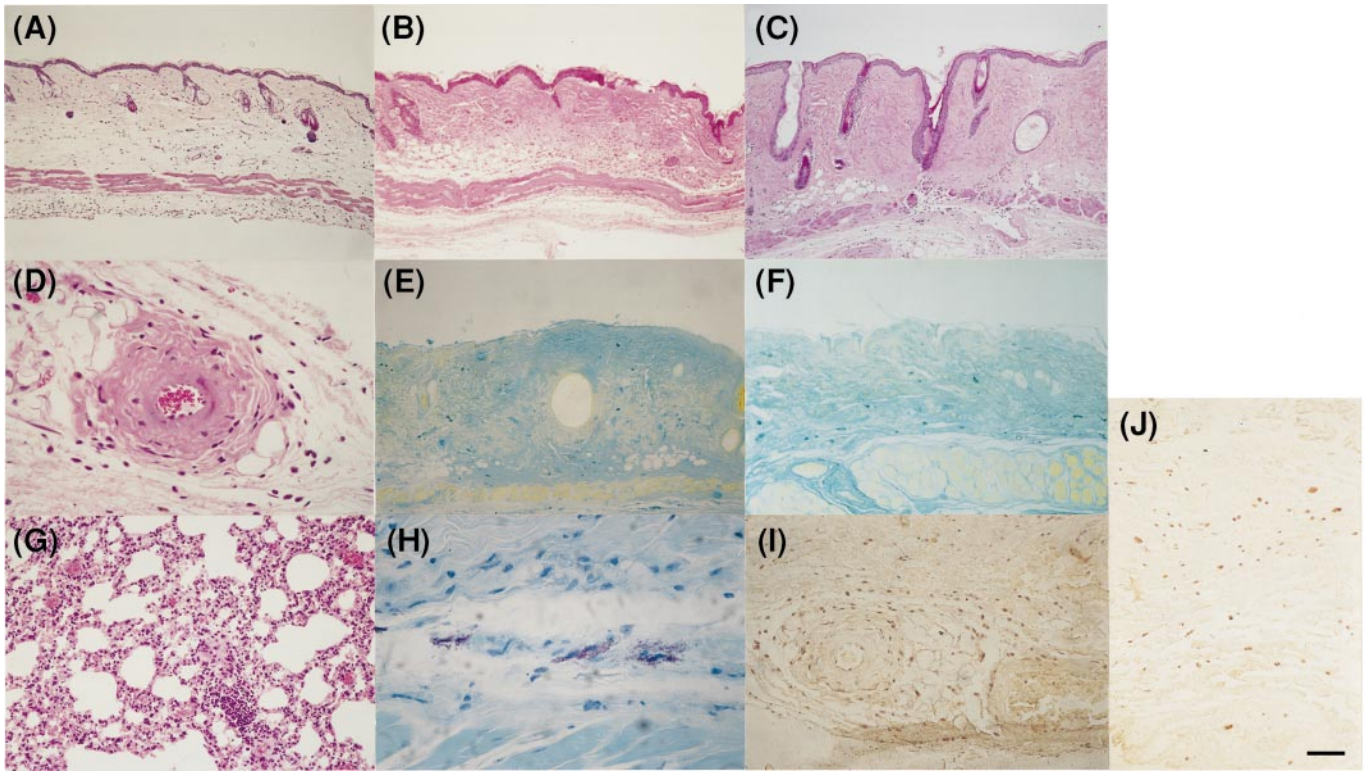

Figure 1. Histopathologic evaluation of dermal sclerosis in BALB/C mice. (*A*) Mice were treated with PBS for 4 wk (hematoxylin and eosin stain, ×50). Neither fibrosis nor sclerosis was noted. (*B*) Mice were treated with bleomycin (1 mg per ml) for 2 wk (hematoxylin and eosin stain, ×50). (*C*) Mice were treated with bleomycin (1 mg per ml) for 4 wk (hematoxylin and eosin stain, ×82). Marked dermal sclerosis with thickened and homogenous collagen bundles were induced with cellular infiltrates in the subcutaneous tissue. (*D*) Vascular walls were also thickened at 4 wk (hematoxylin and eosin stain, ×400). (*E*) Diffuse positive staining was noted in the sclerotic lesional skin by colloidal iron (×50). (*F*) Faint staining was noted in the dermis in the PBS-treated mice for 4 wk by colloidal iron (×50). (*G*) Mononuclear cell infiltrates were also prominent in the lung at 1 wk of bleomycin treatment (1 mg per ml) (hematoxylin and eosin stain, ×260). (*H*) Marked degranulation of mast cells was shown by toluidine blue stain (pH 7.0) at 2 wk (×520). (*I*) Positive staining for BM-8 in the infiltrating mononuclear cells was seen in the subcutaneous tissue of the sclerotic skin (×260). (*J*) Fewer numbers of immunoreactive cells for BM-8 were also seen in PBS-treated mice for 4 wk (×360). *Scale bar*: (*A*, *B*, *E*, *F*) 100 μm, (*C*) 60 μm, (*D*) 12.5 μm, (*G*, *I*) 19 μm, (*H*) 10 μm, (*J*) 14 μm.

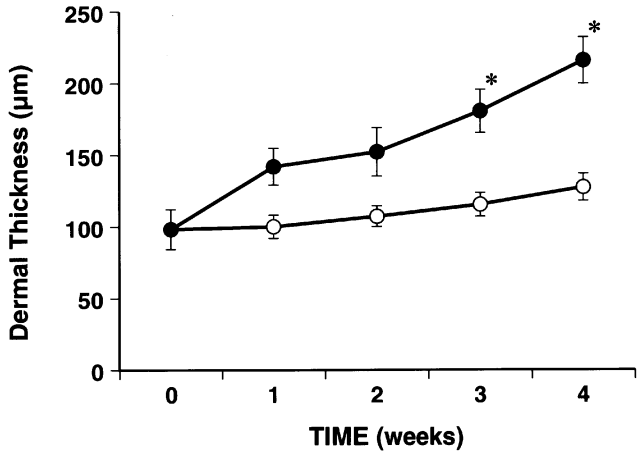

Figure 2. Dermal thickness in BALB/C mice treated with bleomycin. Mice were injected with 1 mg BLM per ml (dose per day, n = 6) (●) or PBS alone (n = 5) (○). Dermal thickness gradually increased as the sclerosis developed, whereas PBS-treated mice did not exhibit an increase of dermal thickness. Significant differences were noted after 3 wk. * $p < 0.05$.

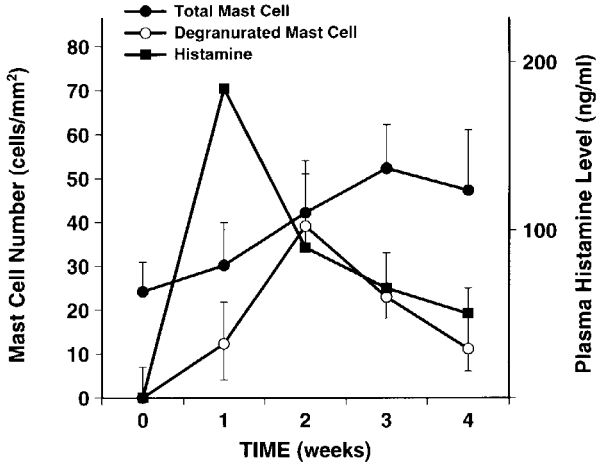

Figure 3. Changes of mast cell numbers and histamine levels during BLM treatment. The number of total and degranulated mast cells was quantitated by identification of toluidine blue stain (pH 7.0) and represents mean of six mice ± SD. Total mast cell number (●) was gradually increased as the sclerotic lesions were induced, whereas degranulated mast cell number (○) showed its peak at 2 wk of bleomycin treatment. Plasma histamine level (■), measured by radioimmunoassay, was elevated when the mast cell begins degranulation.

with that in PBS-treated skin after 3 wk (217 ± 17 *vs* 126 ± 8.7 μm at 4 wk, $p < 0.05$) (**Fig 2**).

Results of mast cell counts are shown in **Fig 3**. The numbers of mast cells reached a peak at 3 or 4 wk after BLM administration, and increased up to 2-fold as compared with untreated mice (48.5 ± 12.8 *vs* 23.0 ± 8.6 per mm^2, $p < 0.05$) or more than 1.5 times as compared with PBS-treated mice (29.3 ± 9.4, $p < 0.05$) at 4 wk. The numbers of degranulated mast cells increased more rapidly, and peaked at 2 wk. Elevation of plasma histamine level

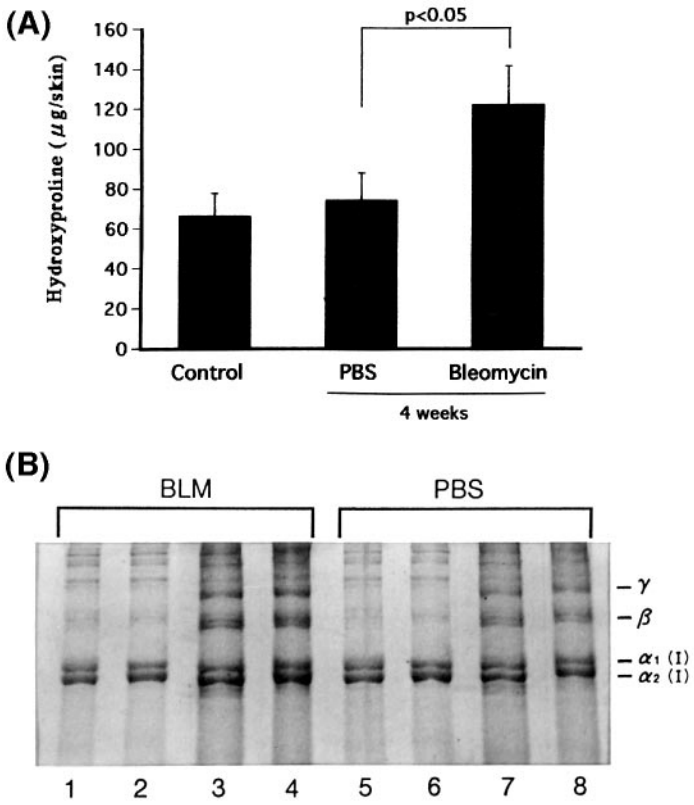

Figure 4. Biochemical analysis of bleomycin-induced sclerotic dermis in BALB/C mice. (*A*) Hydroxyproline assay by 6 mm punch biopsy skin tissue. BALB/C mice were given 1 mg bleomycin per ml or PBS subcutaneously for 4 wk and killed the next day after the final treatment. Skin hydroxyproline content was measured as outlined in *Materials and Methods*. At 4 wk, bleomycin-treated mice (n = 5) showed a significant increased hydroxyproline content as compared with PBS-treated (n = 5) or control untreated mice (n = 5) ($p < 0.05$). (*B*) Type 1 collagen content of skin tissue. Total protein extracted from 6 mm punch biopsied skin samples obtained from BALB/C mice with 4 wk treatment of bleomycin or PBS were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Lanes 1*, *5*, pepsin soluble, reductant (+); *lanes 2*, *6*, pepsin soluble, reductant (–); *lanes 3*, *7*, pepsin insoluble, reductant (+); *lanes 4*, *8*, pepsin insoluble, reductant (–). Representative data are shown.

preceded the increment of numbers of either degranulated or nondegranulated mast cells.

The amount of collagen is increased in the sclerotic skin In the sclerotic skin induced by 4 wk injections of BLM, the content of hydroxyproline was significantly increased as compared with that in normal untreated mice and PBS-treated mice (**Fig 4*A***). The hydroxyproline content in the BLM-treated skin (120 ± 17 μg per 6 mm punch biopsied skin) was almost double that in the skin of untreated mice (64 ± 8 μg per 6 mm punch biopsied skin) and PBS-treated mice (73 ± 10 μg per 6 mm punch biopsied skin) ($p < 0.05$ in both comparisons). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of pepsin-resistant collagen chains revealed the presence and an increment of both α1 and α2 chains of collagen, which comigrated with type I standard collagen, in the sclerotic skin (**Fig 4*B***).

Mononuclear cells and cytokine profile in the sclerotic skin Mononuclear cell infiltration was observed in the sclerotic dermis perivascularly and around the sclerotic collagen bundles. The infiltrating mononuclear cells mainly consisted of CD4 positive T cells and macrophages. Immunoreactive stem cell factor was abundantly detected in the fibroblasts and mast cells in the sclerotic skin. TGF-β was expressed diffusely in the extracellular matrix and infiltrating mononuclear cells in the sclerotic dermis. In contrast, decorin was hardly expressed in the sclerotic dermis. The results of the immunohistochemical studies are summarized in **Table I**.

Table I. Immunohistochemical characteristics of infiltrating mononuclear cells in the lesional skin of BLM or PBS-treated BALB/C mice.[a]

Cytokine/growth factor	BLM-treated mice (wk)				PBS-treated
	1	2	3	4	4
IL-2	–	–	–	+	–
IL-6	–	–	+	++	–
IL-10	–	–	+	+++	–
TGF-β	–	–	++	+++	–
ICAM-1	–	–	+	+++	–

[a] Staining intensity: –, no staining; +, weak; ++, moderate; +++, strong.

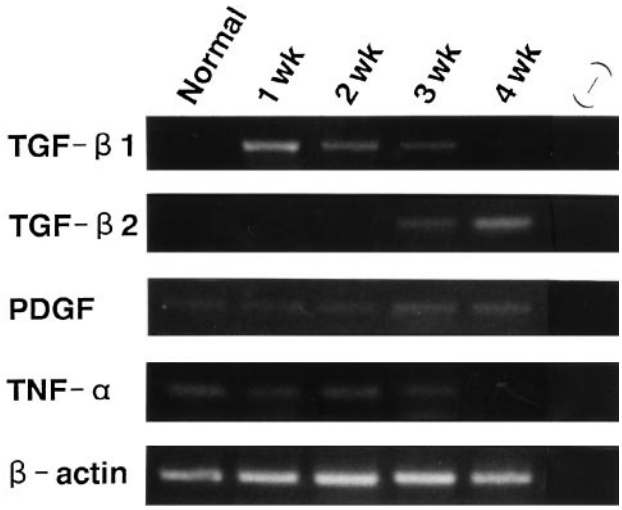

Figure 5. PCR analysis of cytokine gene expression in bleomycin-treated BALB/C mice. Mice received a subcutaneous injection of bleomycin (1 mg per ml), and the next day of the final application the back skin was removed. Total RNA was isolated from the lesional skin at 1, 2, 3, and 4 wk, and cDNA was prepared for the detection of TGF-β1, TGF-β2, PDGF-AA, and TNF-α mRNA. PCR was performed as described in *Materials and Methods*. –, negative control. Representative data are shown.

Table II. Kinetics analysis of serum cytokine in the BLM and PBS-treated BALB/C mice

Cytokine	Treatment	Weeks			
		1	2	3	4
IL-4 (pg per ml)	BLM (1mg per ml)	<5	16 ± 5	20 ± 6	54 ± 8
	PBS	<5	<5	<5	<5
IL-6 (pg per ml)	BLM	45 ± 10	43 ± 9	38 ± 11	62 ± 10
	PBS	<5	<5	<5	<5
TNF-α (pg per ml)	BLM	20 ± 4	30 ± 6	34 ± 7	40 ± 8
	PBS	<15	<15	<15	<15
IFN-γ (pg per ml)	BLM	<5	<5	<5	<5
	PBS	<5	<5	<5	<5

Reverse transcriptase-PCR was performed to analyze cytokine profiles during the sclerotic process. mRNA expression of TGF-β1 was detected at the early phase of dermal sclerosis, and that of TGF-β2 was expressed mostly at 4 wk when the dermal sclerosis became prominent (**Fig 5**). mRNA expression of TNF-α was detected at the early phase, and that of PDGF-AA was expressed all through the course.

Kinetics of serum cytokines during the sclerotic process are listed in **Table II**. IL-6 was elevated from an early phase. Th2-type

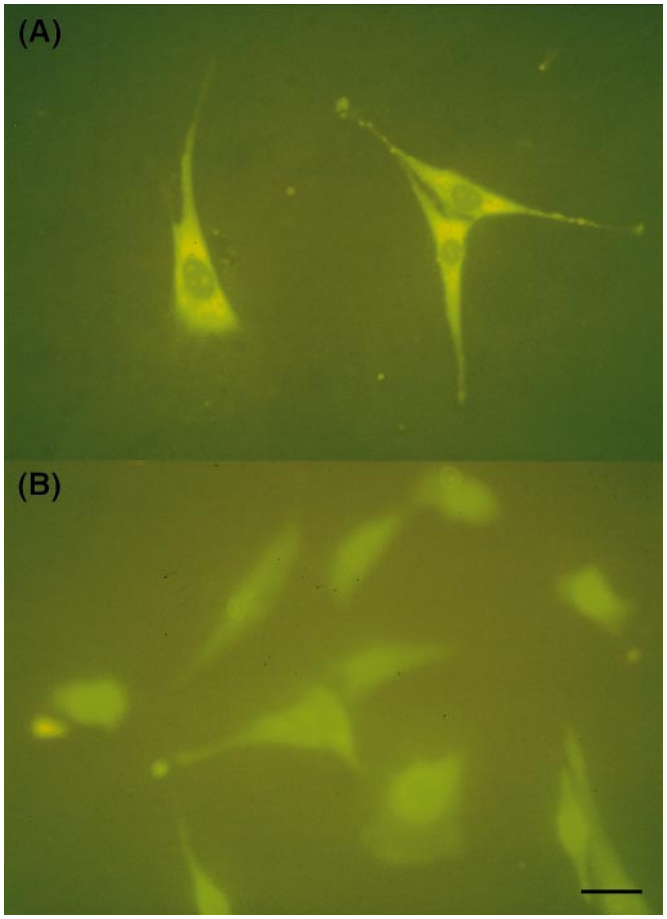

Figure 6. Detection of anti-nuclear antibody. 3T3 fibroblasts were stained with a monospecific BALB/C mouse serum recognizing FITC-goat anti-mouse IgG and examined by fluorescent microscopy. Diffuse cytoplasmic staining, as well as punctate intranucleolar structures, was seen using serum obtained at 4 wk with bleomycin treatment (*A*), and were not seen in cases using serum of PBS-treated mice (*B*). *Scale bar*: 32 μm.

cytokines such as IL-4 and IL-6 were elevated in the circulation during the sclerotic process.

BLM-treated mice produced autoantibodies In order to investigate whether autoantibodles are present in BLM-treated mice, we obtained serum from mice after 4 wk of BLM injection. Exclusive staining of punctate intranucleolar structures as well as diffuse cytoplasmic staining was observed on 3T3 fibroblasts using sera of BLM-treated mice (**Fig 6**), whereas there was no positive staining when similar experiments were performed using sera from either nontreated mice or PBS-treated mice. These antibodies were detected up to a dilution of 1:320.

DISCUSSION

In this study, we established a mouse model of sclerotic skin by repeated subcutaneous injections of BLM. Daily injection of BLM gradually induced dermal sclerosis with dermal thickening after 4 wk in BALB/C mice. Sclerosis was apparent only histologically, and was hardly recognized clinically. Histologically, BLM-treated dermis showed sclerosis with thickened, homogenous collagen bundles, perivascular infiltrates, and thickening of the vascular wall, which are all characteristic of histologic features of human scleroderma skin. Immunohistochemical studies revealed that this cellular infiltration mainly consisted of $CD4^+$ T cells, mast cells, and macrophages. Cutaneous sclerosis persisted for up to 6 wk after the final administration of BLM. Lung fibrosis was also induced much earlier than the formation of dermal sclerosis; however, histologic examination of the other sites of the skin distant from the sites of injection, such as toes, stomach, or kidney, failed to show any sclerosis. These results suggest that effects of BLM injection on the formation of sclerotic skin lesions are local and not generalized.

BLM-induced pulmonary fibrosis is a well-established histologic and biochemical animal model, which is characterized by the accumulation of collagen within the lung following enhancement of collagen synthesis (Aso *et al*, 1976; Adamson, 1984; Chandler, 1990). Previous studies examined chronic effects of BLM on mice when BLM was administered only once every 2 wk (Mountz *et al*, 1983). In their study, increased collagen fibers were demonstrated, whereas serum antibodies were not detected. To our knowledge, however, skin fibrosis/sclerosis using continuous injections of BLM has not been examined in detail. One of the reasons may be that dermal sclerosis is only histologically induced by BLM, as has been demonstrated in this study. Our results may reflect on acute dermal changes and it is of note that local administration of BLM caused sclerosis, but not fibrosis. Pulmonary fibrosis has been induced in mice in about 2 wk by a single intravenous injection of BLM (Adamson and Bowden, 1974), in 4–8 wk by intraperitoneal injections (Adamson and Bowden, 1979), and after a short interval by administration through the trachea (Anider *et al*, 1978). The extent of experimental lung fibrosis depends on the dose and the route of administration of BLM as well as on the mouse strain. C57/BL6 is a susceptible strain, whereas BALB/C is a resistant strain (Schrier *et al*, 1983). In this study, we could induce sclerosis even in the BLM-resistant BALB/C mice by injecting BLM for 4 wk at a dose of more than 10 μg per ml. Dermal sclerosis was also induced even by alternate day's injection of BLM for 4 wk. In other experiments using BLM-sensitive C3H mice, sclerosis was induced earlier, and interestingly, also with epidermal proliferation (Yamamoto *et al*, manuscript in preparation).

It is of note that mast cells are increased in number as the sclerotic lesion was induced, showing a peak at 3 or 4 wk. Furthermore, degranulation of mast cells was observed earlier, with a peak at 2 wk. Mast cells are abundant in the skin of tight skin mice and exhibit prominent degranulation (Walker *et al*, 1985). Inhibition of mast cell degranulation by cromolyn (Walker *et al*, 1987) or ketotifen (Walker *et al*, 1990)was associated with decreased fibrosis in this model. As mast cells form a heterogenous population with different developmental stages, mediator contents, ultrastructure, as well as ability to interact with their local environment (Dayton *et al*, 1989), and contain many cytokines or chemical mediators, including histamine, heparin, tryptase, proteinase, leukotriens, or prostaglandin D2, it is likely that secretion, activation, or degranulation of mast cells have the potential to cause microenvironmental changes. Mast cells influence fibroblast behavior by membrane contact (Dvorak, 1991). Recent findings demonstrated that mast cell precursors circulate as agranular mononuclear cells and undergo final maturation and granule synthesis after migration into tissues in a stem cell factor-dependent manner (Church *et al*, 1994). In this study, stem cell factor was expressed on fibroblasts and mast cells especially in the sclerotic phase (data not shown). Plasma histamine levels were also elevated during the early phase. Histamine has a mitogenic activity for fibroblasts (Russel *et al*, 1977) and is a potent modulator of collagen metabolism of fibroblasts (Hatamochi *et al*, 1985). Tryptase has also been shown to be mitogenic for fibroblasts (Ruoss *et al*, 1991) and stimulates the synthesis of type I collagen in fibroblasts (Cairns and Walls, 1997; Gruber *et al*, 1997). Thus, interaction of mast cell granule constituents with connective tissue cells, components of extracellular matrix, and inflammatory cells also suggests the participation of mast cells in active fibrosis. In scleroderma, mast cell numbers are increased, especially in the edematous phase (Nishioka *et al*, 1987); however, mast cell products alone are unlikely to be sufficient to induce sclerosis. In our recent observation using mast cell-deficient mice, sclerosis was also induced by injections of BLM in WBB6F1-W/W^v mice, which may indicate that infiltrating mononuclear cells are more important for the induction of sclerosis (Yamamoto *et al*, manuscript submitted).

It has been shown that mouse $CD4^+$ T cell clones could be classified into distinct subsets according to their cytokine production pattern, and it is widely accepted that the same dichotomy is applied to human T cells. The contribution of IL-4 to scleroderma leads to the classification of this disorder as a Th2 condition. IL-4 is produced by activated memory T cells and mast cells, both types of cells having a significant role in the pathogenesis of scleroderma. IL-4 stimulates fibroblast proliferation and fibroblast extracellular matrix production, and promotes T cell adhesion to endothelial cells, differentiation of lymphocytes, regulation of inflammatory responses, and growth of mast cells and lymphocytes (Monroe *et al*, 1988; Thornhill *et al*, 1990; Fertin *et al*, 1991; Gillery *et al*, 1992). Increased IL-4 production has been detected in the sera or by activated peripheral blood mononuclear cells of patients with SSc (Famularo *et al*, 1990; Needleman *et al*, 1992). IL-4 levels significantly correlate with skin scores and are inversely associated with the duration of the disease (Lindner and Frieri, 1991). IL-6 has also been shown to stimulate the synthesis of collagen and glycosaminoglycans (Duncan and Berman, 1991). *In vitro* study has shown that IL-4 induces the production of IL-6 by human skin fibroblasts (Feghali *et al*, 1992), which suggests that these cytokines are able to amplify the stimulating effects of collagen synthesis. Elevated IL-6 levels have also been frequently found in sera of SSc patients (Needleman *et al*, 1992). Our results regarding cytokine production in the sera of BLM-treated mice indicate a Th2 pattern.

Accumulation of several connective tissue components has also been studied in BLM-induced lung fibrosis, such as collagen, hyarulonan, fibronectin, or proteoglycan. Decorin is a small proteoglycan and its core protein binds to TGF-β, leading to a neutralization of the effect of TGF-β. Our immunohistochemical studies showed a reduced expression of decorin (data not shown) and an enhanced expression of TGF-β1 in the lesional skin of sclerosis at 4 wk. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed an increase of type I collagen, and biochemical assessment revealed a significant increase of hydroxyproline contents in the skin during the sclerotic stage. These results indicate that our mouse model is characterized by sclerotic skin changes that are very similar to scleroderma.

Recent studies suggest that cytokines released from macrophages may play an important role in the development of BLM-induced pulmonary fibrosis in animals. IL-1, IL-6, TGF-β, TNF-α, PDGF, or insulin-like growth factor I (IGF-I) have been suggested to be involved as effectors in the induction of experimental pulmonary fibrosis (Piguet *et al*, 1989; Kovacs, 1991; Phan and Kunkel, 1992). Focal release of cytokines is supposed to play a key role in fibroblast proliferation and collagen synthesis. In particular, TGF-β is supposed to play a crucial role in tissue fibrosis, which induces a rapid fibrotic response when injected subcutaneously into newborn mice (Roberts *et al*, 1986). TGF-β, which is able to upregulate its own synthesis (Wahl *et al*, 1990), has been shown to stimulate human fibroblasts to produce type I collagen *in vitro* (Varga *et al*, 1987). Multiple actions of TGF-β include the strong induction of extracellular matrix deposition by stimulating the production of matrix proteins, inhibiting proteases that degrade matrix, and modulating the expression of matrix receptors on the cell surface (Grande, 1997). In BLM-induced pulmonary injury, TGF-β was maximally elevated at 7 d after BLM administration (Khalit *et al*, 1989). Enhanced expression of TGF-β mRNA occurs within the first 2 wk after induction of lung injury (Hoyt and Lazo, 1988; Raghow *et al*, 1989). Alveolar macrophages obtained 7 d after BLM administration secreted TGF-β1 in large quantities, whereas TGF-β2 and TGF-β3 remained unchanged (Khalit *et al*, 1989). Recent studies showed that TGF-β1 is ubiquitously produced by all cells, whereas production of TGF-β2 is more selective (Phan *et al*, 1991). Our study revealed that TGF-β1 mRNA was detected during the early phase, whereas TGF-β2 mRNA was induced when sclerosis was most prominent. TGF-β protein was expressed in the extracellular matrix of the dermis most clearly in the sclerotic lesional skin at 4 wk. On the other hand, TNF-α gene expression was detected during the early phase before sclerosis started. PDGF-AA was constantly expressed during all stages. This discrepancy of cytokine expression between lung and skin lesions may be due to the timing examined, because lung fibrosis occurred earlier than cutaneous sclerosis. In our model, TGF-β1 may have a primary role in the pathogenesis of the sclerotic response and production of TGF-β2 starts as the sclerotic phase is completed. TGF-β2 mRNA has been found in close proximity to fibroblasts expressing pro α I(I) collagen near blood vessels in active SSc (Kulozik *et al*, 1990). Recent findings showed that TGF-β isoforms may have different functions and gene expressions during fibrotic processes (Shah *et al*, 1995; Coker *et al*, 1997), and the triggering mechanism of the production of TGF-β isoforms may be upregulated by other cytokines or by autocrine regulation. Our results support the notion that TGF-β1 is a key regulatory molecule to initiate the activity of fibroblasts to form cutaneous sclerosis.

Repeated subcutaneous injections of BLM induced autoantibodies against nuclear components in our model. Autoimmune mechanisms may be triggered by local administration of BLM. Our mouse model develops cutaneous sclerosis with lung fibrosis and autoantibodies, and may therefore provide a powerful tool for examining the pathogenesis and therapeutic approach of scleroderma.

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