Possible Role of Apoptosis in the Pathogenesis of Bleomycin-Induced Scleroderma

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To elucidate the role of apoptosis in cutaneous sclerosis, we examined the induction of apoptosis and expression of Fas, Fas ligand, as well as caspase-3 in a murine model of bleomycin-induced scleroderma. Dermal sclerosis was induced by local injections of bleomycin (1 mg per mL) in C3H/HeJ mice. Induction of apoptosis was examined by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) assay and DNA gel electrophoresis. TUNEL positivity was prominently detected on keratinocytes and infiltrating mononuclear cells, but not endothelial cells and fibroblasts, in the lesional skin. DNA fragmentation revealed laddering at 3 to 4 wk following bleomycin treatment. Immunohistochemistry showed increased expression of Fas in infiltrating mononuclear cells at early phases following bleomycin exposure, whereas constitutive expression in fibroblasts. Fas ligand expression was increased in mononuclear cells as well as fibroblasts in the sclerotic skin. Results of reverse transcription–polymerase chain reaction analysis revealed that expression of Fas ligand mRNA was upregulated and reached a maximum at 3 wk, whereas Fas mRNA was continuously detected. mRNA expression as well as activity of caspase-3 was also enhanced at 3 wk. Administration of neutralizing anti-Fas ligand antibody together with local bleomycin treatment reduced the development of dermal sclerosis, associated with the reduction of TUNEL-positive mononuclear cells and also with the blockade of apoptosis. Caspase-3 activity in the lesional skin was also significantly reduced after anti-Fas ligand treatment. These findings suggest that excessive apoptosis, which is mediated by Fas/Fas ligand pathway and caspase-3 activation, is involved in the pathogenesis of bleomycin-induced scleroderma, possibly by playing an inflammatory role.

Key words: apoptosis/bleomycin/caspase-3/Fas/Fas ligand/mouse model/scleroderma.
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Systemic sclerosis is a connective tissue disorder characterized by excessive production and accumulation of extracellular matrix proteins in the affected skin, as well as various internal organs (Krieg and Meurer, 1988; LeRoy *et al*, 1991). In particular, type I, III collagen, fibronectin, and proteoglycans are produced by activated fibroblasts in the affected sites. Interactions among lymphoid cells, endothelial cells, and fibroblasts are likely to be central to the pathogenesis of systemic sclerosis; however, its etiology has not been fully elucidated as yet.

Recent studies suggest the involvement of apoptosis in several autoimmune disorders (Vaishnaw *et al*, 1997; Grodzicky and Elkon, 2000; Ricci-Vitiani *et al*, 2000). Apoptotic process is suggested to be involved in the pathogenesis of systemic sclerosis (Jelaska and Korn, 2000; Santiago *et al*, 2001). Apoptosis is a process regulated by numerous genes and factors. One possible mechanism is induction of apoptosis via death receptors such as CD95 (APO-1/Fas). Fas is a 45 kDa cell-surface receptor of the tumor necrosis factor/nerve growth factor receptor family. Fas is highly expressed in activated lymphocytes and in a variety of cells of lymphoid or nonlymphoid origin, as well as tissues and tumor cells (Itoh *et al*, 1991; Nagata and Golstein, 1995; Walczak and Krammer, 2000). Fas ligand (FasL) is a membrane protein belonging to the tumor necrosis factor family. Although expression of FasL was originally thought to be restricted to activated T cells and natural killer cells, FasL is also expressed in immune privileged sites (Suda *et al*, 1993; Nagata, 1996; Griffith and Ferguson, 1997). Fas and FasL are widely expressed and function in many tissues. When FasL binds to Fas on Fas-sensitive target cells, target cells die by apoptosis, triggering a cascade of caspases. The Fas/FasL system is a key molecular regulator of apoptosis and is also involved in the pathogenesis of various diseases (Fadeel *et al*, 1999; Siegel and Fleisher, 1999). Caspases are suggested to play an essential part in both the regulation and execution phases of apoptotic cell death and act upstream of DNA fragmentation (Los *et al*, 1995; Thornberry *et al*, 1997). Caspase-3 is considered to be key executor of apoptosis and is activated via the mitochondrial, death receptor such as Fas/FasL and tumor necrosis factor/tumor necrosis factor receptor, or endoplasmic reticulum routes.

We previously established a mouse model for scleroderma by the treatment with bleomycin (Yamamoto *et al*, 1999a,b, 2000; Yamamoto and Nishioka, 2001; Yamamoto and Nishioka, 2002). Repeated local injections of bleomycin induce dermal sclerosis that resemble human scleroderma both histologically and biochemically. Bleomycin is known to induce apoptosis *in vitro* (Omar *et al*, 1993; Hamilton *et al*, 1995; Ortiz *et al*, 1998). In this study, we examined the involvement of apoptosis as well as Fas and FasL system in

this *in vivo* model for scleroderma. Caspase-3 expression and activity was also examined following bleomycin exposure. We further investigated whether inhibition of FasL can suppress the induction of dermal sclerosis induced by bleomycin.

Results

Detection of apoptosis in the lesional skin following bleomycin treatment At first, bleomycin-treated skin was examined for the presence of TUNEL-positive cells. *In situ* detection of cell death by TUNEL assay showed only faint apoptotic signals on the hair follicles in the PBS-treated skin (Fig 1*A*). By contrast, positive signals for TUNEL were detected on the infiltrating mononuclear cells, hair follicles, and sebaceous glands in the dermis, and also a few positively labeled few keratinocytes were scattered following bleomycin treatment as early as at 1 wk (Fig 1*B*). The number of TUNEL-positive infiltrating cells were markedly increased at 3 wk following bleomycin exposure (Fig 1*C*), associated with bleomycin-induced inflammatory infiltrates in the skin. Also, positively labeled keratinocytes were prominently detected at 3 wk (Fig 1*D*). Negative controls without TdT did not show nuclear labeling (Fig 1*E*). On the contrary, positive labeling was not detected on the endothelial cells (Fig 1*F*), and positively labeled fibroblastic cells were few in the lesional skin after bleomycin treatment.

Results of gel electrophoresis of DNA extracted from skin tissues showed ladder patterns of DNA cleavage, confirming the development of apoptosis. DNA fragmentation in the lesional skin was prominently detected at 3 and 4 wk following bleomycin treatment (Fig 2). Here, we have demonstrated *in vivo* data that apoptosis was induced in the lesional skin following bleomycin treatment by a combination of TUNEL and gel electrophoretic analysis.

Expression of Fas and FasL in the lesional skin of bleomycin-induced scleroderma Fas/FasL system is a key molecular regulator of apoptosis. To address the potential involvement of Fas/FasL pathway in the pathogenesis of bleomycin-induced scleroderma, we examined the expression of these molecules. Immunohistochemical localization revealed that weak expression of Fas was detected in hair follicles, sebaceous glands, and epidermis in the PBS-treated skin (Fig 3*A*). On the other hand, Fas was detected on the cell membranes of some mononuclear cells in the dermis at 1 wk following bleomycin treatment, and the positive signals for Fas on the infiltrating mononuclear cells were highest at 2 wk (Fig 3*B*). Fas was also expressed on fibroblastic cells constitutively during 1 to 4 wk after bleomycin exposure (Fig 3*C*). FasL was not detected in the PBS-treated skin (Fig 3*D*), whereas positive on the inflammatory cells as early as at 1 wk after bleomycin treatment (Fig 3*E*). Furthermore, FasL was also detected on fibroblastic cells at 3 to 4 wk (Fig 3*F*). Negative controls for Fas and FasL are shown in Fig 3(*G*) and Fig 3(*H*), respectively. Reverse transcription–PCR analysis showed that Fas mRNA was clearly detectable in all tissues during 1 to 4 wk following bleomycin treatment, whereas faintly detected in the PBS-treated skin (Fig 3*I*). FasL mRNA expression was upregulated and peaked at 3 wk following bleomycin treatment (Fig 3*I*). Quantification of the signal indicated up to a 7.5-fold increase of FasL mRNA, as compared with the level detected in the PBS-treated skin.

Expression and activity of caspase-3 in the lesional skin of bleomycin-induced scleroderma As caspase-3 is believed to be one of the most commonly involved in the execution of apoptosis in various cell types, and the Fas/FasL pathway is an upstream signal for caspase-3 activation, we examined the expression and activity of caspase-3.

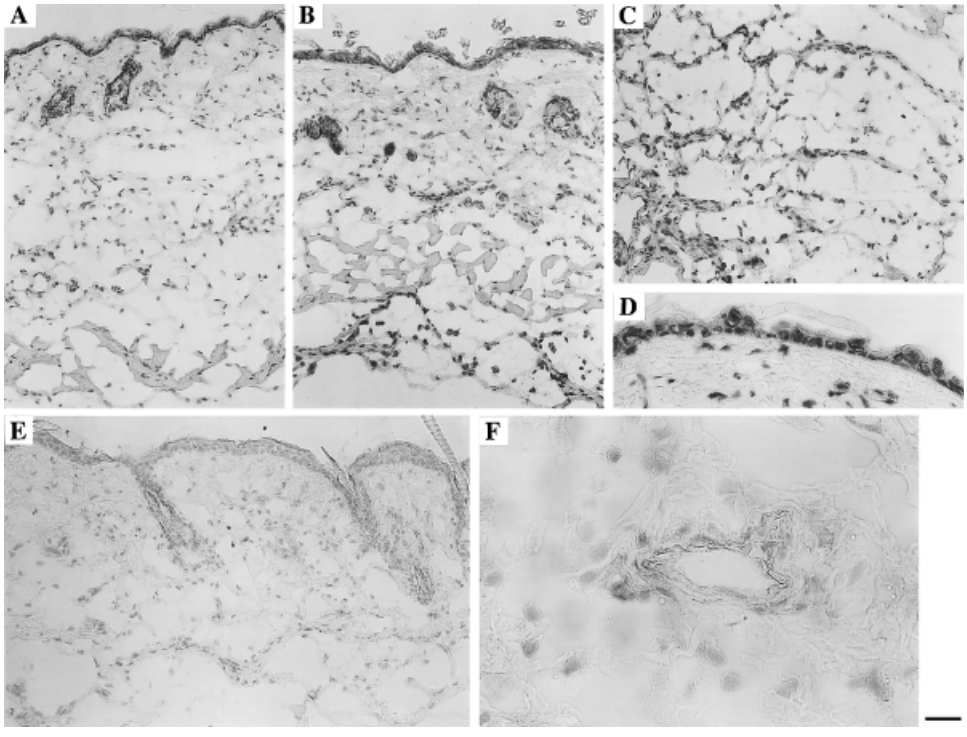

Figure 1
TUNEL staining of the lesional skin. Positive signals were faintly detected in the hair follicles in the PBS-treated skin (*A*). TUNEL-positive cells were detectable on the cellular infiltrates in the dermis at 1 wk after bleomycin treatment (*B*), and further increased at 3 wk (*C*). TUNEL-positive cells were also prominently detected on keratinocytes (*D*). No positive signals were observed in negative controls without TdT (*E*). On the contrary, TUNEL was negative on the endothelial cells even in the sclerotic skin, although positive on perivascular mononuclear cells (*F*). Original magnification: (*A,B*) × 100; (*C*) × 200; (*D*) × 300; (*E*) × 120; (*F*) × 400. *Scale bars:* (*A,B*) 100 μm; (*C*) 50 μm; (*D*) 33 μm; (*E*) 83 μm; (*F*) 25 μm.

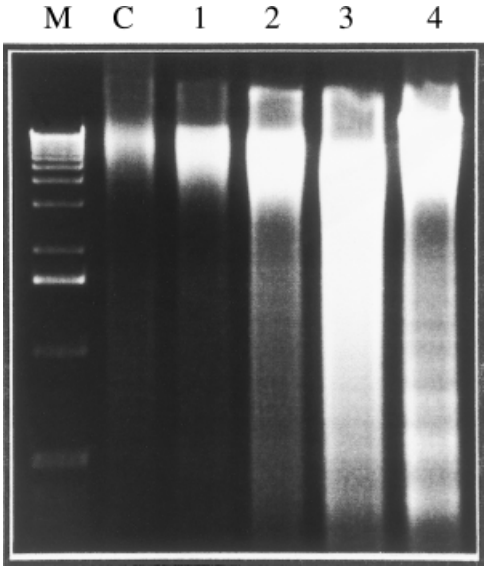

Figure 2
Electrophoretic analysis of DNA extracted from the lesional skin after either PBS or bleomycin treatment. Marker (M) and control (C) skin treated with PBS for 4 wk. *Lane 1*: 1 wk after bleomycin treatment. *Lane 2*: 2 wk after bleomycin treatment. *Lane 3*: 3 wk after bleomycin treatment. *Lane 4*: 4 wk after bleomycin treatment. Results shown are representative of three independent experiments.

Results of immunohistochemistry showed that caspase-3 expression was detected in the epidermis, hair follicles, and sebaceous glands, but not detected on cellular infiltrates scattered in the PBS-treated skin (Fig 4*A*). On the contrary, mononuclear cells infiltrating in the lower dermis were positive for caspase-3 after bleomycin exposure, in which immunoreactivity was detected in both the nucleus and the cytoplasm (Fig 4*B,C*). A small number of fibroblastic cells were also detectable. Reverse transcription–PCR analysis showed that caspase-1 and caspase-3 mRNA were concurrently upregulated in the lesional skin of the bleomycin-treated mice, compared with those of the PBS-treated mice (Fig 4*D*). Both caspase-1 and caspase-3 mRNA levels peaked at 3 wk following bleomycin treatment. Colorimetric enzymatic assay showed a 2-fold increase in the caspase-3 activity in the bleomycin-treated skin, as compared with the PBS-treated skin (Fig 4*E*). Caspase-3 expression and activity were correlated with an increased level of FasL, an upstream signals for caspase-3 activation.

Blockade of dermal sclerosis by anti-FasL Finally, to determine whether neutralization of FasL can suppress the induction of dermal sclerosis, we examined the effect of neutralizing antibody against FasL on the development of bleomycin-induced scleroderma. Mice were subcutaneously injected with bleomycin and intravenously treated with neutralizing anti-FasL antibody MFL3 every other day for 4 wk. Mice treated with bleomycin and normal hamster IgG showed definite dermal sclerosis with thickened collagen bundles and deposition of homogeneous materials in the thickened dermis (Fig 5*A*). On the contrary, treatment with anti-FasL antibody partially prevented the development of dermal sclerosis, as compared with mice treated with normal hamster IgG (Fig 5*B*). Collagen content in the skin was significantly reduced up to 50% after treatment with anti-FasL antibody ($140.6 \pm 8.5\%$ of control), as compared with mice treated with normal hamster IgG ($181.4 \pm 6.1\%$ of control) ($p < 0.01$) (Fig 5*C*). In mice treated with PBS, collagen content in the skin was not altered following anti-FasL treatment (not shown). Neutralizing anti-FasL antibody significantly decreased the number of TUNEL-positive mononuclear cells in the sclerotic skin (Fig 5*D*), as well as

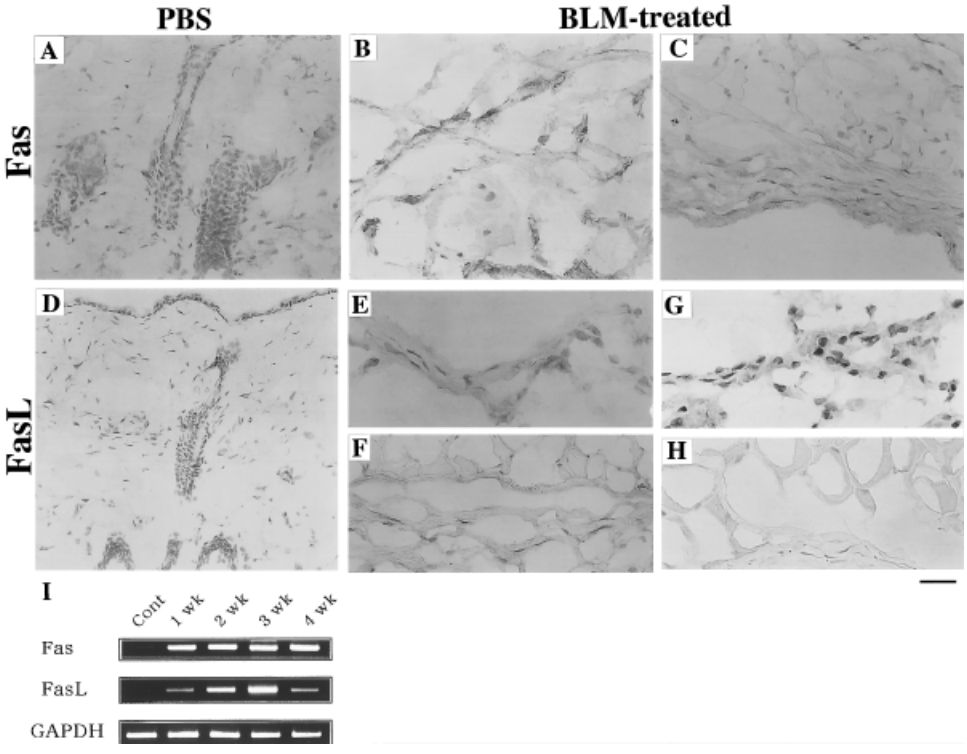

Figure 3
Expression of Fas and FasL in the lesional skin by immunohistochemistry (*A–H*) and reverse transcription–PCR (*I*). Fas was faintly detected on the hair follicles, sebaceous glands, and epidermis in the PBS-treated skin (*A*). After exposure with bleomycin (BLM), Fas was detected on the cellular infiltrates (*B*) as well as fibroblasts (*C*) in the dermis (*B*; at 2 wk and *C*; at 3 wk). FasL was negative in the PBS-treated skin (*D*). On the contrary, FasL was detectable on the cellular infiltrates as early as at 1 wk after bleomycin treatment (*E*), and also expressed on fibroblasts at 4 wk (*F*). Negative controls for Fas (*G*) and FasL (*H*) are shown. Original magnification: (*A,D*) $\times$ 200; (*B,C,E–H*) $\times$ 400. *Scale bars:* (*A,D*) 50 μm; (*B,C,E–H*) 25 μm. (*I*) Expression of Fas and FasL mRNA in skin tissues. Total RNA was extracted from skin samples treated with either PBS for 4 wk (Cont) or bleomycin for 1 to 4 wk, and analyzed by reverse transcription–PCR with specific primers. Results shown are representative of three independent experiments.

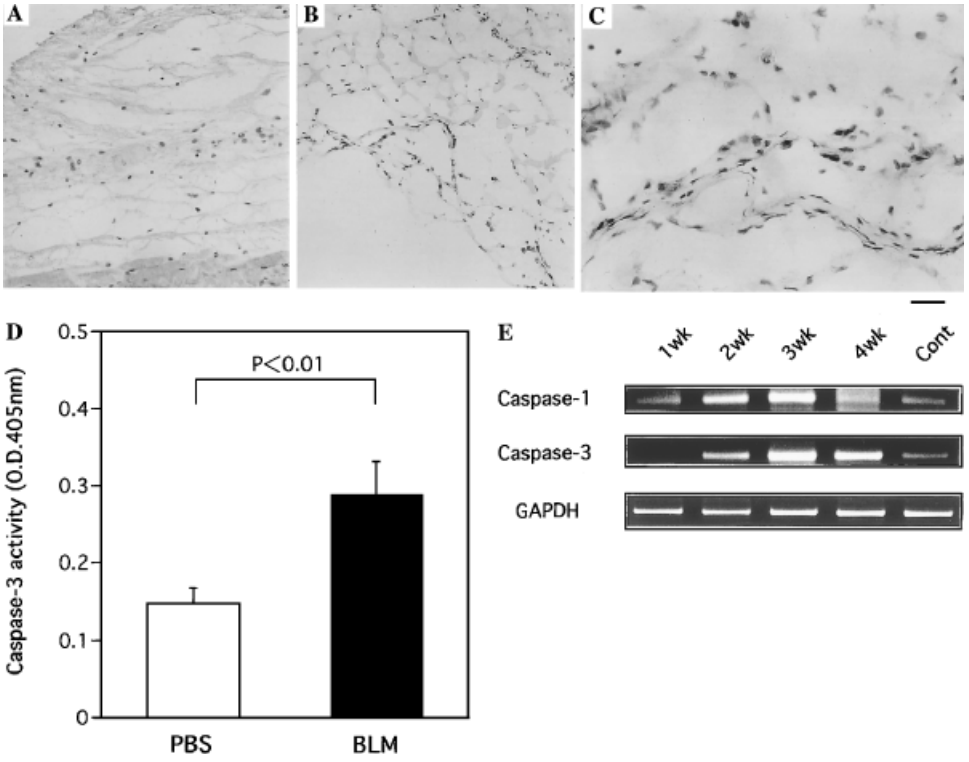

Figure 4
Expression and activity of caspase-3. Immunohistologic expression of caspase-3 in the lesional skin following PBS or bleomycin treatment. Immunoreactivity for caspase-3 is not detected on the scattered cellular infiltrates in the PBS-treated skin (*A*), whereas detected on the infiltrating mononuclear cells in the bleomycin-treated skin at 3 wk (*B,C*). (*D*) Representative results of reverse transcription–PCR analysis for caspase-1 and caspase-3 mRNA levels in the skin following either bleomycin (1–4 wk) or PBS (3 wk) (Cont) treatment. (*E*) Colorimetric assay of caspase-3 activity in the mice skin treated with either bleomycin or PBS for 3 wk. Original magnification: (*A*) × 200; (*B*) × 100; (*C*) × 400. *Scale bars:* (*A*) 50 μm; (*B*) 100 μm; (*C*) 25 μm.

dramatically suppressed the apoptotic condition indicated by DNA fragmentation (Fig 5*E*). Moreover, neutralization of FasL suppressed caspase-3 activation in the lesional skin (Fig 5*F*), suggesting a relationship between Fas/FasL system and caspase-3 activation-mediated apoptosis.

Discussion

In this study, we first demonstrate that apoptosis is induced in the lesional skin following local bleomycin treatments by TUNEL assay and DNA gel electrophoretic analysis. Apoptosis was clearly detected at later stages, and TUNEL reactive cells were mainly infiltrating mononuclear cells and also keratinocytes in the bleomycin-treated skin. Recent studies suggest that endothelial cell apoptosis is an early event in the pathogenesis of scleroderma. Endothelial cell apoptosis was first noted in the UCD 200/206 chickens that develop hereditary systemic connective tissue disease resembling human systemic sclerosis (Sgonc *et al*, 1996). This phenomenon is observed before perivascular mononuclear cell infiltration occurs. Also, TUNEL was shown to be positive on the endothelial cells in human scleroderma skin (Santiago *et al*, 2001). On the contrary, not only in Tsk 1 but also in Tsk 2 mice, alterations in endothelial apoptosis induction were not involved in the disease development (Sgonc *et al*, 1999). Thus, the mechanism and significance of endothelial cell apoptosis in scleroderma is still uncertain. Our results showed no TUNEL labeling on endothelial cells, but was positive in cellular infiltrates around the vessels, suggesting that endothelial apoptosis is not involved in the pathogenesis of bleomycin-induced scleroderma.

Transforming growth factor-β plays a crucial part in scleroderma, and also in this murine model (Yamamoto *et al*, 1999a,c). In addition to the variety of functions of transforming growth factor-β, including the chemotactic attraction of macrophages and fibroblasts, the stimulation of fibroblast proliferation and production of extracellular matrix proteins, the downregulation of extracellular matrix proteinases, and complementary upregulation of proteinase inhibitors, transforming growth factor-β protects myofibroblasts against apoptotic stimuli (Arora and McCulloch, 1999; Zhang and Phan, 1999). Scleroderma fibroblasts derived from affected dermis, which had a higher ratio of α-smooth muscle actin-positive cells, are shown to be resistant to anti-Fas-induced apoptosis (Jelaska and Korn, 2000). We have recently reported the phenotypic changes of sclerotic fibroblasts into α-smooth muscle actin-positive myofibroblasts in the lesional skin in this model (Yamamoto and Nishioka, 2002). Our results in this study showed that TUNEL-positive cells were mainly mononuclear cells and keratinocytes, and there was little or no TUNEL positivity on fibroblasts in the lesional skin following bleomycin treatment. Thus, sclerotic fibroblasts are suggested to be resistant to bleomycin-induced apoptosis.

Fas/FasL system is a key molecular regulator of apoptosis. In this model, immunohistologic examination showed that Fas as well as FasL was strongly expressed in the infiltrating inflammatory cells and fibroblastic cells after bleomycin treatments. The overlapping expression of Fas and FasL during bleomycin treatment was accompanied by apoptosis in the lesional skin. Fas signaling is suggested to be proinflammatory, because it results in activation of caspases, which cleaves and activates the proinflammatory cytokines (Restifo, 2000). Also, it is shown that FasL expression links with inflammation, and *in vivo* studies show that membrane-bound FasL effectively induces an inflammatory response (Hohlbaum *et al*, 2000). Although FasL is expressed mainly in active T lymphocytes, FasL expression can be induced in nonlymphoid tissues (Bonfoco

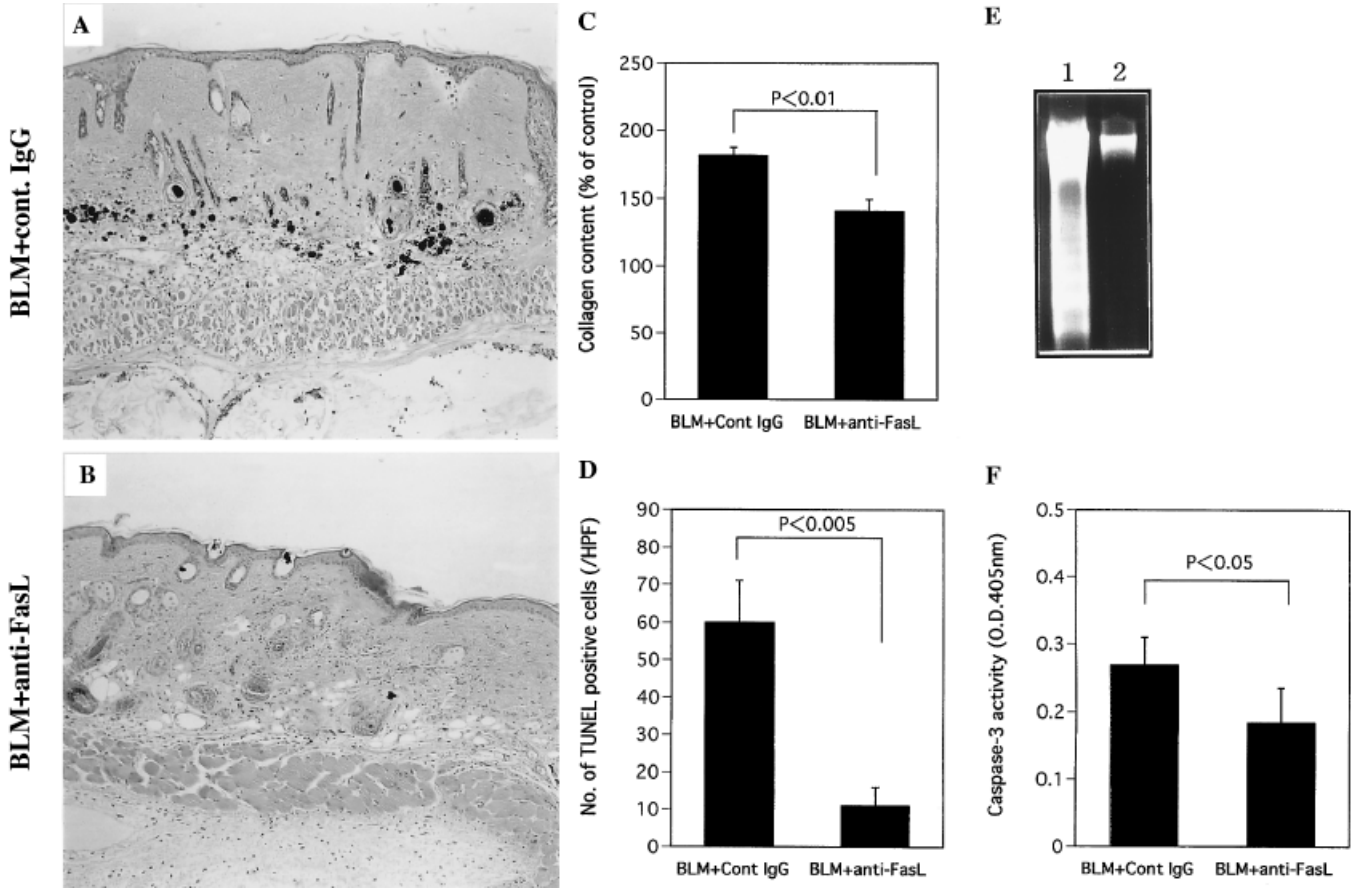

Figure 5
Effect of neutralizing anti-FasL antibody. (*A,B*) Histology of the lesional skin (hematoxylin and eosin stain). Mice were treated with bleomycin (BLM) 1 mg per mL together with intravenous administration of either anti-FasL antibody or control normal hamster IgG. Mice treated with bleomycin and normal hamster IgG for 4 wk demonstrate definite dermal sclerosis with thickened collagen bundles (*A*). Mice treated with bleomycin and anti-FasL neutralizing antibody for 4 wk show reduction of dermal sclerosis (*B*). Original magnification: × 100. *Scale bar:* 100 μm. (*C*) Collagen content in the skin. Collagen content was significantly suppressed in mice treated with intravenous administration of anti-FasL antibody together with local bleomycin, compared with those treated with control normal hamster IgG and bleomycin. Data represent the mean ± SD of six mice ($p < 0.01$). (*D*) Morphometric analysis of the number of TUNEL-positive cells per high-power field (HPF) in microscopy. The number of TUNEL-positive mononuclear cells was significantly decreased in the skin of mice treated with anti-FasL neutralizing antibody compared with that in mice treated with control normal hamster IgG, together with local bleomycin treatment. (*E*) Electrophoretic analysis. DNA fragmentation was clearly detected in DNA extracted from the skin treated with bleomycin and normal hamster IgG for 4 wk (*lane 1*), which diminished in DNA extracted from the skin treated with bleomycin and anti-FasL antibody (*lane 2*). Results shown are representative of three independent experiments. (*F*) Suppression of caspase-3 activity. Intravenous injections of FasL antibody together with local bleomycin treatment for 4 wk significantly inhibited the caspase-3 activity, compared with mice treated with normal hamster IgG and bleomycin.

et al, 1998), and is shown in dermal fibroblasts following activation (Saitoh *et al*, 2000) or renal fibroblasts (Ortiz *et al*, 1997). Our data suggest that dermal fibroblasts can be one of the sources of FasL. Induction of FasL on dermal fibroblasts may be upregulated by proinflammatory or fibrogenic cytokines in the lesional skin.

At present, the precise role of apoptosis in the development of bleomycin-induced dermal sclerosis is unclear. As apoptosis plays an important part in the normal resolution process, its alteration may result in pathologic conditions. It is speculated that at sufficiently high levels of apoptosis, the skin clearance system may be impaired or overwhelmed by the need to remove apoptotic cells. This disturbance of the resolution of the inflammation may develop secondary necrosis of apoptotic cells, which induce skin tissue damage by releasing proinflammatory cytokines and cytotoxic contents, leading to a fibroproliferative response.

Systemic administration of neutralizing anti-FasL antibody suppressed the induction of dermal sclerosis, in association with a significant reduction of the number of TUNEL-positive mononuclear cells as well as inhibition of DNA fragmentation in the skin. These effects of anti-FasL antibody are considered to be specific, because treatment with normal hamster IgG had no effect. Furthermore, caspase-3 activity was also significantly suppressed, indicating that caspase-3 is the key mediator of bleomycin-induced apoptosis in the skin. A recent report demonstrated that injection of anti-FasL antibody ameliorated the development of bleomycin-induced pulmonary fibrosis (Kuwano *et al*, 1999). They suggested that the inhibition of apoptosis induced by the Fas/FasL pathway may be of primary importance in preventing pulmonary fibrosis induced by bleomycin. On the other hand, as anti-FasL antibody could not provide complete protection against the induction of dermal sclerosis, other pathways are also supposed to activate mechanisms of the development of dermal sclerosis.

In conclusion, this study demonstrates an excessive apoptosis of mononuclear cells infiltrating in the bleomycin-

treated skin, which may induce an inflammatory process and then lead to fibrosis, suggesting a pivotal role of Fas/FasL signaling and furthermore Fas/FasL-mediated caspase-3 activation in the pathogenesis of bleomycin-induced scleroderma. The continuous and extensive expression of FasL may participate in the development of cutaneous fibrosis/sclerosis by inducing excessive apoptosis or by modulating inflammatory mediators.

Materials and Methods

Mice Specific pathogen-free, female C3H/HeJ mice (6 wk old) were purchased from Sankyo Labo Service Co. (Tokyo, Japan), and kept under standard conditions with food and water *ad libitum*. Mice were handled in accordance with institutional animal care and use committee protocols approved by the animal facility of Tokyo Medical and Dental University.

Development of dermal sclerosis by bleomycin Dermal sclerosis was induced by local injections of bleomycin (Nippon Kayaku Co., Tokyo), which was dissolved in phosphate-buffered saline (PBS) and sterilized by filtration (0.2 μm). One hundred microliters of bleomycin (1 mg per mL) was subcutaneously administered into the shaved back skins every other day for 4 wk.

Immunohistochemical analysis Immunohistochemical examination was performed with avidin–biotin peroxidase technique using 5 μm thick cryostat sections prepared on poly L-lysin coated slides. The sections were incubated with primary monoclonal antibodies against Fas (Jo2), FasL (MFL3) (both purchased from BD Pharmingen, San Diego, California) and polyclonal antibody against caspase-3 (L-18) (purchased from Santa Cruz Biotechnology Inc., Santa Cruz, California) (1:250, diluted in PBS) for 2 h at room temperature, followed by incubation with biotin-conjugated secondary antibodies. The sections were developed with 3,3′-diaminobenzidine solution as chromogen, counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were prepared by omission of the primary antibodies and by the substitution with control IgG.

Reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR) analysis Total RNA was isolated from biopsied skins using RNeasy kit (Qiagen, Tokyo, Japan). Complementary single-stranded DNA was synthesized from total RNA by reverse transcription as described previously (Yamomoto et al, 1999a). The cDNA was amplified by PCR using Ready-to-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, New Jersey) with specific primers for Fas (upstream; 5′-GAGAATTGCTGAAGACATGACAATCC-3′, downstream; 5′-GTAGTTTTCACTCCAGACATTGTCC-3′), FasL (upstream; 5′-AAGCTTCAGCTCTTCCACCTG-3′, downstream; 5′-ATGAATTCCTGGTGCCCATG-3′), caspase-1 (upstream; 5′-GGGACCTATGTGATCATGTCTC-3′, downstream; 5′-CAGTCAGTCCTGGAAATGTGCC-3′), caspase-3 (upstream; 5′-CTGACTGGAAAGC-CGAAA-3′, downstream; 5′-GCAAAGGGACTGGATGAA-3′), and GAPDH (upstream; 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, downstream; 5′- CATGTAGGCCATGAGGTCCACCAC-3′). The PCR conditions were consisted of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, for a total of 25 cycles (for GAPDH), and 30 cycles (for otherwise). 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.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) staining To detect DNA strand breaks, which are associated with the apoptotic response, an *in situ* cell death detection kit (Roche Diagnostics GmbH Maanheim, Germany) was used using cryostat sections, based on the TUNEL assay. Negative controls were obtained without TdT.

DNA fragmentation by agarose gel analysis Skin samples were cut into small pieces by scissors in sample buffer, and DNA was extracted using the apoptosis ladder kit (Roche Diagnostics GmbH). DNA samples were loaded on a 1% agarose gel containing ethidium bromide and visualized under ultraviolet light.

Activity of caspase-3 The level of caspase-3 activity was determined using a commercial assay kit (Caspase Colorimetric Protease assay kit; MBL, Nagoya, Japan). The skin extracts were prepared by homogenization of skin tissues in a lysis buffer. Homogenates were centrifuged at 7,000 g for 10 min, and the supernatants were assayed. Caspase-3 activity was measured by cleavage of the fluorogenic substrates DEVD-pNA. Fifty microliters of 2 × reaction buffer with 10 mmol per liter dithiothreitol and 5 μL of 4 mmol per liter DEVD-pNA substrate were added to 50 μL of supernatants containing a total of 50 μg of extracted proteins, followed by 1 h of incubation in a water bath at 37°C. Optical density for each specimen was determined at 405 nm using a plate reader.

Administration of anti-FasL To examine whether blockade of FasL leads to prevention of dermal sclerosis, 5 μL of anti-FasL antibody MFL3 (BD Pharmingen) (0.2 mg per mL) or normal hamster IgG were injected via the tail vein every other day for 4 wk together with local bleomycin (1 mg per mL) treatment.

Collagen content For collagen assay, 8 mm punch biopsy specimens were excised from the shaved back skins, and stored at −80°C. Collagen deposition was estimated using the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland). 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 mice that received only PBS injections.

Statistical analysis In each experiment, six mice were examined. Results were expressed as mean ± SD. Significance testing was analyzed using Mann–Whitney U test. A p value less than 0.05 was considered to be significant.

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