We previously reported a mouse model for scleroderma by repeated local injections of bleomycin. In this study, we investigated the level of transforming growth factor-β1 (TGF-β1) in various mice strains, in order to determine whether the expression of TGF-β1 correlates with the susceptibility to bleomycin-induced scleroderma. Histological examination revealed prominent dermal sclerosis with increased collagen deposition in the bleomycin-treated skin in B10.A and C3H/HeJ strains as compared with BALB/c, C57BL/6J and DBA/2 strains. Collagen contents in the skin were also increased in B10.A and C3H/HeJ strains. Analysis of skin lesions from B10.A and C3H/HeJ exhibited the increased mRNA expression and protein synthesis of TGF-β1. TGF-β1 concentrations in culture supernatants of skin fibroblasts and spleen macrophages were significantly increased by bleomycin stimulation in B10.A and C3H/HeJ strains, and TGF-β1 gene expression in fibroblasts derived from B10.A and C3H/HeJ strains was significantly increased by bleomycin stimulation. Thus we conclude that C3H/HeJ and B10.A mice are susceptible to bleomycin-induced scleroderma, which may be, in part, due to increased TGF-β1 gene expression and protein production.

Key words: scleroderma; model mouse; transforming growth factor-β; bleomycin; mouse strain.

Abbreviations: TGF-β1: Transforming growth factor-β1
ECM: extracellular matrix, SSc: Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a connective tissue disorder characterized by extensive fibrosis associated with increased collagen synthesis and accumulation, and affects the skin as well as various internal organs. Fibroblasts derived from affected skin of SSc are considered to be activated and synthesize increased amounts of extracellular matrix (ECM) proteins.

It is particularly important to establish animal models of SSc for understanding the pathogenesis and improving the therapeutic procedure, however, animal models which exhibit all the aspects of SSc are not yet established by now. It is known that there is a clear association between the exposure to several chemical agents and the development of SSc. Bleomycin is produced by Streptomyces verticillus, and is a frequently used antitumor antibiotic for various kinds of cancers. Lung fibrosis is a well-known side effect of bleomycin. In addition, cutaneous changes including fibrosis, hyperpigmentation, alopecia, gangrene, edema, Raynaud’s phenomenon, and “flagellate” erythema (scratch dermatitis) have been described. Scleroderma is also reported to be developed in malignancy-bearing patients after bleomycin therapy. We have recently reported a mouse model for scleroderma by repeated local injections of bleomycin.
Histological examination demonstrated prominent dermal sclerosis showing thickened collagen bundles and deposition of homogenous material in the dermis, which resembles human scleroderma. Also, we confirmed that dermal sclerosis could be inducible in different mice strains\textsuperscript{14}, however, the intensity of dermal sclerosis varied to some extent among strains. In humans, previous studies have suggested an association of particular HLA alleles with SSc\textsuperscript{17-25}, however, no data indicate whether a specific aberration among general chromosomal abnormalities is common to all patients with SSc. For the better understanding of the susceptibility for scleroderma, detail investigations for the difference among these strains with the different genetic background are aimed in this study.

Cytokines are important in the immune and inflammatory systems. Although a variety of cytokines and growth factors are involved in the pathogenesis of SSc, transforming growth factor-\(\beta\) (TGF-\(\beta\)) has been suggested to play a key role. TGF-\(\beta\) has a strong chemoattractant for fibroblasts, stimulates the production of collagen, fibronectin, and proteoglycans, and downregulates ECM proteinases and complementary upregulates proteinase inhibitors\textsuperscript{26,27}. In addition to the role of TGF-\(\beta\) in promoting ECM deposition, TGF-\(\beta\) modulates cell-matrix adhesion protein receptors\textsuperscript{28}. Thus, maintenance of increased TGF-\(\beta\) production may lead to the progressive deposition of ECM, resulting in fibrosis. Indeed, TGF-\(\beta\) mRNA level is elevated in the lesional skin of SSc and other fibrotic conditions\textsuperscript{29-31}, and also shown to co-localize with type \(\alpha\) collagen in scleroderma skin lesions\textsuperscript{29}. In the current study, we investigate to compare the synthesis and expression of TGF-\(\beta\)1 in the lesional skin among various strains of mice.

Materials and Methods

All experimental procedures were performed in accordance with the guidelines of Institutional Review Board of the Tokyo Medical and Dental University.

Mice

Specific pathogen-free female, B10.A, C3H/HeJ, C57BL/6J, DBA/2, and BALB/c mice at 6 weeks of age weighing 20 to 25g were used. B10.A strain was purchased from Sankyo Labo Service (Tokyo, Japan). C3H/HeJ and C57BL/6J strains were purchased from Japan Clea (Tokyo). DBA/2 and BALB/c strains were purchased from Oriental Yeast Co., Ltd (Tokyo). Mice were kept in separate clean rooms and fed \textit{ad libitum}.

Bleomycin treatment

Mice were subcutaneously injected with 100 \(\mu\)l of bleomycin (Nippon Kayaku Co., Tokyo) (1 mg/ml in phosphate-buffered saline (PBS)) to the shaved back skin every day or on alternate day. Mice (\(n=6\)) of each strain were sacrificed with diethylether on the next day after the final treatment, and back skins were harvested with 8-mm punch biopsy. Control groups (\(n=6\)) were injected with PBS.

Histological, histochemical and immunohistochemical examination

Formalin-fixed, paraffin-embedded sections were prepared on poly-L-lysin coated slides, and examined histologically with hematoxylin and eosin (HE) and Elastica van Gieson (EVG) stain. They were also stained with the staining ENVISION kit (Dako, Carpinteria, CA, USA) using rabbit polyclonal antibodies against TGF-\(\beta\)1 (Santa Cruz Biotechnology, Santa Cruz, CA) and type \(\alpha\) collagen (Biogenesis, Poole, UK). The sections were counterstained with hematoxylin, dehydrated, cleared and mounted. Negative controls were prepared by omission of primary antibodies, and by the substitution with rabbit IgG. The dermal thickness of bleomycin-injected and PBS-injected skin was compared after 4 weeks' treatment.

Collagen content in the sclerotic skin

Eight mm-punch biopsies were taken from the shaved back skins in each strain, cut into small pieces and homogenized in serum-free Dulbecco’s modified Eagle’s medium (DMEM). After centrifugation, the supernatants were analyzed with collagen assay kit (Silcol Collagen Assay, Biocolor Ltd, Newtownabbey, Northern Ireland).

Determination of cytokine levels

The supernatants of homogenized skin samples in serum-free DMEM were used. TGF-\(\beta\)1 and interleukin-4 (IL-4) concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kit (Genzyme Technie, Minneapolis, MN) according to the recommendations of the manufacturer. Since the samples are activated with acid, the results indicate total concentrations of TGF-\(\beta\)1 including both active and latent forms.
Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis
Total RNA was extracted from biopsied skin samples with RNeasy kit (Qiagen, Tokyo). Complementary single-stranded DNA was synthesized from total RNA by reverse transcription, as previously described. The cDNA was amplified by PCR with the use of the specific reverse transcription, as previously described. The upstream and downstream primers are: TGF-β1, 5'-AAGTGGATCCAGGAGCCCAA-3' and 5'-CTGCACTTGCAGGAGCGAC-3', α1(I) collagen, 5'-GGACCTCTGTACACCACGTTCACC-3' and 5'-GTGTCATGGATGACCTTGGCC-3'. 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. Cycle number for amplification was 35, except for GAPDH (30 cycles). After amplification, PCR products were subjected to electrophoresis on 1.5% agarose gel (BioWittaker Molecular Applications, Rockland, ME) and detected by ethidium bromide under UV illuminator (Bio-Rad Laboratories, Helcules, CA), diluted to a final concentration of 1 mg per ml. Sodium dodecyl sulfate (15%) polyacrylamide gel electrophoresis was performed with constant amounts (10 µg per lane) of samples and proteins were transferred to PVDF membranes. Immunoblot analyses were performed in independent three experiments.

Immunoblotting
For immunoblotting, skin specimens were homogenized in Laemmli sample buffer. The protein concentration of each sample was determined with a commercially available kit (Bio-Rad protein assay kit, Bio-Rad Laboratories, Helcules, CA), diluted to a final concentration of 1 mg per ml. Sodium dodecyl sulfate (15%) polyacrylamide gel electrophoresis was performed with constant amounts (10 µg per lane) of samples and proteins were transferred to PVDF membranes. Immunoblots were preincubated in TTBS containing 0.3% nonfat dry milk, incubated with primary anti-TGF-β1 antibody (Santa Cruz Biotechnology) for 1 hr. Membranes were incubated in a 1:2000 dilution of peroxidase-conjugated anti-rabbit polyclonal antibodies in TTBS. Antibody staining was revealed by enhanced chemiluminescence. The probed membranes were stripped with stripping buffer (2% SDS, 62.5 mM Tris HCl, pH 6.8, 100 mM 2-mercaptoethanol) at 60°C for 30 min and then reprobed with anti-actin (AC-2668, Sigma, St. Louis, MO).

Fibroblast cultures
Fibroblasts were established by outgrowth method from untreated back skins. The each skin was diced into small pieces and placed onto culture dishes. DMEM containing 10% fetal calf serum (FCS), 1% L-glutamine, and ampicillin (100 U/ml), streptomycin (25 mg/ml) and amphotericin B (5 µg/ml) was added and incubated at 37°C in 95% air-5% CO2. Cells at passage 2 were used for the experiments. Cells were seeded at 105 cells /3.5cm-diameter dish. Twenty-four hrs later, the medium was exchanged for serum-free DMEM. Fibroblasts were further incubated in the presence or absence of 100 nM bleomycin for 24 hrs. In preliminary experiments, we confirmed that the TGF-β1 levels in culture supernatants from mouse fibroblasts were dose-dependently increased after incubations with bleomycin between 1-1000 nM for 24 hrs, and trypan blue dye exclusion revealed that bleomycin at the concentrations 1-100 nM did not decrease the viability of fibroblasts up to 24 hrs (cell viabilities >95% under these conditions). Thus we chose this condition.

Preparation of macrophage
Macrophages were separated from spleen cells according to the method previously described. Cells were stimulated with 100 nM bleomycin for 18 hrs in serum-free medium at 106 cells /3.5cm-diameter dish. As TGF-β1 amounts in culture supernatants from macrophages peaked at 18 hrs' stimulation in preliminary experiments, macrophages from each strain were examined following 18 hrs' stimulation with or without bleomycin (100 nM).

Statistical analysis
Results were expressed as means± SD. Significance was tested by Mann-Whitney U-test. A P value <0.05 was considered as significant.

Results
Bleomycin Induces Dermal Sclerosis Particularly in B10.A and C3H/HeJ Strains
Results of histochemical and immunohistochemical examination are shown in Fig. 1. EVG stain and type I collagen stain revealed intense deposition of ECM in the dermis after bleomycin-treatment in all strains examined, as compared with PBS-treatment (Fig. 1). In particular, dermal sclerosis was strongly induced in B10.A and C3H/HeJ strains. On the contrary, there were mild changes in BALB/c and DBA/2 strains. As
previously reported\textsuperscript{14}, the ratio of the mean thickness of bleomycin-treated dermis to that of PBS-treated dermis in C3H/HeJ and B10.A strains were higher than that in C57BL/6J and DBA/2 strains.

Cutaneous fibrosis was also quantified by analysis of collagen content. Collagen contents in the punch-biopsied skins were increased up to 2.5-fold after bleomycin treatment in B10.A strain and 1.8-fold in C3H/HeJ strain as compared with PBS treatment, whereas less increase was observed in BALB/c and DBA/2 strains (Fig. 2). Combining the histopathological and biochemical results, we concluded that B10.A and C3H/HeJ strains are ‘susceptible’ to bleomycin-induced scleroderma, while BALB/c, C57BL/6J and DBA/2 strains are more refractory to bleomycin treatment (relatively ‘resistant’).

\textbf{Increased TGF-\(\beta\)1 Levels in Skin Tissues after Bleomycin Treatment in Bleomycin-‘susceptible’ Strains}

Since we confirmed that dermal sclerosis can be induced by alternate day’s injections of bleomycin\textsuperscript{33}, mice were injected with either bleomycin or PBS on alternate day for 4 weeks for the following experiments. Assessment with ELISA showed that TGF-\(\beta\)1 levels in the bleomycin-treated skin were significantly higher than those of PBS-treated skin in B10.A and C3H/HeJ strains (\(\* P<0.05\)) (Fig. 3). TGF-\(\beta\)1 protein levels in the bleomycin-treated skin in C3H/HeJ was significantly increased than that in DBA/2 strain (\(\* P<0.05\)) (Fig. 3). We confirmed that there were only little amounts of TGF-\(\beta\)1 left in the re-extracted skin tissues by 10 mM HCl (data not shown). Since IL-4 is also a representative fibrogenic cytokine, we measured the level of IL-4 in the lesional skin of C3H/HeJ strain. Bleomycin treat-
ment for 4 weeks enhanced both TGF-β1 and IL-4 production, but there was no significant difference in IL-4 level between mice treated with bleomycin and PBS (data not shown).

Immunoblot analysis using the skin homogenates showed higher signals of TGF-β1 in B10.A and C3H/HeJ strains (Fig. 4). Densitometric quantification revealed that the signal intensity in C3H/HeJ strain was highest, which showed a nearly 4-fold increase as compared with BALB/c strain and a 7-fold increase as compared with DBA/2 strain.

To determine whether the enhancement of TGF-β1 protein production is also seen at the gene level, we examined TGF-β1 expression in the skin tissues by RT-PCR analysis. Three mice of each strain were sacrificed after 4 weeks’ treatment, and the punch-biopsied skins were examined for TGF-β1 mRNA expression. Results of RT-PCR showed that TGF-β1 mRNA expression was enhanced after bleomycin treatment in all strains (Fig. 5). Densitometric quantification revealed that a nearly 2-fold increase in TGF-β1 mRNA level is seen in the B10.A strain compared with that in BALB/c strain. These data indicated that the TGF-β1 levels were increased at both mRNA and protein levels in the bleomycin-susceptible B10.A and C3H/HeJ strains.

Increased Secretion and mRNA Expression of TGF-β1 by Macrophages after Bleomycin Stimulation in vitro

Immunohistochemical examination showed that TGF-β1 was strongly detected on the fibroblastic spindle cells and macrophage-like round large nucleolar cells in the sclerotic dermis following bleomycin treatment in C3H/HeJ strain (Fig. 6). Since fibroblasts and macrophages are suggested to be major sources
of TGF-β1, we compared TGF-β1 production and expression after bleomycin stimulation in both macrophages and fibroblasts derived from different strains.

First, we obtained spleen macrophages from C3H/HeJ mice, and TGF-β1 concentrations in the cultured supernatants with or without bleomycin stimulation were assessed. In a preliminary experiment, TGF-β1 concentrations peaked following 18 hrs’ stimulation with bleomycin (100 nM). The amounts of TGF-β1 in the supernatants of macrophages are increased in a dose-dependent effect of bleomycin, and significantly increased at a dose of more than 100 nM of bleomycin (P<0.05) (Fig. 7A). The TGF-β1 concentrations in the culture supernatants of bleomycin-stimulated macrophages of B10.A (48.4 ± 8.3 pg/ml) and C3H/HeJ (54.3 ± 1.5 pg/ml) were significantly higher than those of BALB/c (26.4 ± 4.5 pg/ml), C57BL/6J (31.4 ± 1.0 pg/ml) and DBA/2 (30.4 ± 6.8 pg/ml) strains (P<0.05) (Fig. 7B). These data suggest that macrophages derived from bleomycin-susceptible mice strains produce significantly higher levels of TGF-β1 than relatively resistant strains. There was no significant difference in the basal TGF-β1 levels of unstimulated macrophages in each strain.

Total RNA was isolated from spleen macrophages following 18 hrs’ treatment with bleomycin (100 nM) in each strain. Results of RT-PCR showed that TGF-β1 mRNA expression was enhanced in B10.A and C3H/HeJ strains (Fig. 7C). Densitometric quantification revealed a nearly 2.4-fold increase in the B10.A strain and a 1.8-fold increase in C3H/HeJ strain respectively, as compared with BALB/c strain.
Increased Secretion and mRNA Expression of TGF-β1 by Fibroblasts after Bleomycin Stimulation in vitro

The TGF-β1 concentrations in the culture supernatants of skin fibroblasts were significantly increased after bleomycin stimulation as compared with those of unstimulated fibroblasts in B10.A (268.2±27.9 vs. 87.1±15.3 pg/ml), C3H/HeJ (286.3±46.4 vs. 88.7±9.8 pg/ml), BALB/c (153.8±44.4 vs. 75.0±33.2 pg/ml) and C57BL/6J (234.8±44.4 vs. 53.4±23.8 pg/ml) strains (*P<0.05) (Fig. 8A). The TGF-β1 levels in bleomycin-stimulated fibroblasts of B10.A and
Secretion and mRNA expression of TGF-β1 by macrophages.

(A) Spleen macrophages were obtained from C3H/HeJ mice and TGF-β1 concentrations in culture supernatants were measured by ELISA following stimulation with bleomycin (0-1 nM) for 18 hrs. TGF-β1 concentrations were significantly increased by incubation with bleomycin (≥100 nM) (**P<0.05). LPS stimulation (100 ng/ml) is performed as positive control.

(B) TGF-β1 concentrations in culture supernatants were measured by ELISA. Macrophages obtained from spleen of each strain of mice were harvested after 18 hrs’ stimulation with bleomycin (100 nM). TGF-β1 concentrations in C3H/HeJ strain were significantly higher than those in BALB/c, C57BL/6J and DBA/2 strains (**P<0.05).

(C) Total RNA was isolated from cultured macrophages with or without stimulation with bleomycin (100 nM) for 18 hrs. PCR analysis was performed as described in Materials and Methods. Representative data are shown in three independent experiments. Graph shows quantitative densitometric analysis. Relative amounts are expressed as arbitrary units after normalization with corresponding GAPDH mRNA levels.

Fig. 7C

Fig. 7  Secretion and mRNA expression of TGF-β1 by macrophages.
(A) Spleen macrophages were obtained from C3H/HeJ mice and TGF-β1 concentrations in culture supernatants were measured by ELISA following stimulation with bleomycin (0-1 nM) for 18 hrs. TGF-β1 concentrations were significantly increased by incubation with bleomycin (≥100 nM) (**P<0.05). LPS stimulation (100 ng/ml) is performed as positive control.

(B) TGF-β1 concentrations in culture supernatants were measured by ELISA. Macrophages obtained from spleen of each strain of mice were harvested after 18 hrs’ stimulation with bleomycin (100 nM). TGF-β1 concentrations in C3H/HeJ strain were significantly higher than those in BALB/c, C57BL/6J and DBA/2 strains (**P<0.05).

(C) Total RNA was isolated from cultured macrophages with or without stimulation with bleomycin (100 nM) for 18 hrs. PCR analysis was performed as described in Materials and Methods. Representative data are shown in three independent experiments. Graph shows quantitative densitometric analysis. Relative amounts are expressed as arbitrary units after normalization with corresponding GAPDH mRNA levels.
C3H/HeJ strains were significantly higher than those of BALB/c and DBA/2 (125.4 ± 31.3 pg/ml) strains (*P<0.05) (Fig. 8A).

Collagen synthesis in the culture supernatants of fibroblasts was assessed using Silcol Collagen Assay Kit, which showed significant increases after bleomycin stimulation in B10.A (20.0 ± 2.3 µg/ml) and C3H/HeJ (22.9 ± 4.2 µg/ml) strains, as compared with that of unstimulated fibroblasts (9.8 ± 1.2 µg/ml, 11.1 ± 4.3 µg/ml, respectively) (*P<0.05) (Fig. 8B). There was no significant difference in basal levels among all strains.

Results of RT-PCR showed that basal mRNA levels of both α1(1) collagen and TGF-β1 were similar in cultured fibroblasts derived from mice of all strains (Fig. 8C). Following bleomycin treatment, α1(1) collagen and TGF-β1 mRNA expressions were concurrently upregulated in fibroblasts of B10.A, C3H/HeJ and C57BL/6J strains (Fig. 8C). Densitometric quantification showed a significant difference of TGF-β1 mRNA levels between bleomycin-stimulated and unstimulated fibroblasts in B10.A (3.5-fold increase) and C3H/HeJ (2.8-fold increase) strains (Fig. 8D). mRNA expression of α1(1) collagen was also increased in B10.A (2.6-fold increase) and C3H/HeJ (2.8-fold increase) strains.

Discussion

Recently we have reported the model of bleomycin-induced scleroderma in various mice strains. Dermal sclerosis was induced by local bleomycin treatment in various strains of mice, however, the intensity of dermal sclerosis somewhat varied among
strains. This study was undertaken to determine whether growth factors are involved in the different development of dermal sclerosis induced by bleomycin. Since TGF-β is known to play a key role in fibrosis, we compared TGF-β levels in the lesional skin following bleomycin treatment among various strains of mice.

EVG and type I collagen stain revealed the compact collagen bundles in the affected dermis particularly in B10.A and C3H/HeJ strains. In these strains, the thickness of the dermis was more than doubled after bleomycin treatment as compared with PBS treatment. Analysis of collagen contents in the lesional skin revealed that both B10.A and C3H/HeJ strains showed significant increase of collagen contents after bleomycin treatment. Combining the histopathological and biochemical results, we concluded that B10.A and C3H/HeJ mice are relatively ‘susceptible’ strains to bleomycin-induced scleroderma.

TGF-β1 is a pleiotropic cytokine with multiple effects on various cells, and sustainment of the increased production of TGF-β1 leads to the progressive deposition of ECM and tissue fibrosis. In vitro studies showed that bleomycin exposure to rat lung fibroblast cultures results in elevated TGF-β mRNA expression as well as TGF-β protein levels, and bleomycin increases TGF-β1 mRNA expression in human skin fibroblasts. We previously demonstrated that TGF-β1 has an important role in the pathogenesis of scleroderma induced by bleomycin. Systemic in vivo neutralization of TGF-β led to the suppression of the development of dermal sclerosis in this model. In this study, we demonstrated that TGF-β1 mRNA levels were increased in the bleomycin-induced sclerotic skin in B10.A and C3H/HeJ strains. Although we did not examine active form of TGF-β1, total TGF-β1 production was elevated B10.A and C3H/HeJ strains. In addition, in vitro studies showed that TGF-β1 synthesis after bleomycin stimulation in cultured macrophages of B10.A and C3H/HeJ strains were significantly high, compared with other strains. mRNA levels of TGF-β1 in bleomycin-stimulated macrophages of B10.A and C3H/HeJ strains were also significantly increased. mRNA expression and synthesis of TGF-β1 was significantly increased in skin fibroblast derived from B10.A and C3H/HeJ strains than in BALB/c and DBA/2 strains, but not in C57BL/6J strain. One possible explanation is that C57BL/6J is not decided as either bleomycin-susceptible or resistant, which indicating that this strain has the characteristic of intermediate. In parallel, collagen synthesis by cultured fibroblasts from B10.A and C3H/HeJ strains were also increased. Thus, in vitro studies provided evidence for the hypothesis that the differences in susceptibility to bleomycin-induced scleroderma may be due to differences in TGF-β1 gene expression and protein production by fibroblasts and macrophages.

Taken together, our results suggest that B10.A and C3H/HeJ strains are ‘susceptible’ strains to bleomycin-induced scleroderma, which may be, in part, due to the increased TGF-β1 gene expression and protein production in response to bleomycin.

Acknowledgements

We thank Mrs. Motoko Sekiya for the excellent technical assistance.

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