Plasminogen activator inhibitor-1 is elevated, but not essential, in the development of bleomycin-induced murine scleroderma

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Summary
Accumulative data have demonstrated that plasminogen activator inhibitor-1 (PAI-1) plays an important role in the extracellular matrix metabolism; however, the involvement of PAI-1 in scleroderma has not been fully elucidated. In this study, we investigated the role of PAI-1 in bleomycin-induced murine scleroderma. 100 μg of bleomycin was injected subcutaneously to the back skin of C3H/HeJ mice on alternate day for 4 weeks. Histopathological findings revealed that PAI-1 was positive in macrophage-like cells and fibroblastic cells in the dermis, in parallel with the induction of dermal sclerosis. PAI-1 mRNA expression in the whole skin was up-regulated at 1 and 4 weeks. The production of active PAI-1 protein in the lesional skin was significantly increased 3 and 4 weeks after bleomycin treatment. Next, we examined whether dermal sclerosis is induced by bleomycin in PAI-1-deficient (PAI-1–/–) mice. 10 μg of bleomycin was subcutaneously injected to PAI-1–/– and wild type (WT) mice 5 days per week for 4 weeks. Histological examination revealed that dermal sclerosis was similarly induced even in PAI-1–/– as well as WT mice. Dermal thickness and collagen contents in the skin were significantly increased by bleomycin injection in both PAI-1–/– and WT mice, and the rate of increase was similar. These data suggest that PAI-1 plays an important role, possibly via TGF-β pathway activation. However, the fact that PAI-1 deficiency did not ameliorate skin sclerosis suggest that PAI-1 is not the essential factor in the development of bleomycin-induced scleroderma, and more complex biochemical effects other than PA/plasmin system are greatly suspected.

Keywords: bleomycin, TGF-β, PAI-1, mouse model, scleroderma

Introduction
Systemic sclerosis (SSc) is characterized by excessive production of extracellular matrix (ECM) by activated fibroblasts and its deposition in the affected skin and in various internal organs. Although the pathogenesis of SSc remains incompletely understood, it is now evident that the multifunctional cytokine transforming growth factor-β (TGF-β) plays a central role in the fibrogenic process. TGF-β is a strong chemoattractant for fibroblasts [1] and increases the synthesis of collagen or fibronectin by many cell types in vitro [2–4]. In addition, TGF-β down-regulates ECM proteinases, and complementary up-regulates proteinase inhibitors. Thus, maintenance of increased TGF-β production may lead to progressive deposition of ECM, resulting in fibrosis.

Fibrosis is a consequence of perturbation of the normal balance between ECM synthesis and its degradation. The plasminogen activator (PA)/plasmin system is a key regulator of fibrinolysis and ECM degradation [5,6]. Tissue-type plasminogen activator (tPA) and urinary-type PA (uPA) are well-characterized serine proteases that catalyse the conversion of plasminogen to the broad-spectrum protease, plasmin, which is important for fibrinolysis. Plasmin can degrade ECM both directly by its own proteolytic activity and by activation of latent matrix metalloproteinases. PA activity is tightly regulated by specific, high-affinity inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2.

PAI-1 is a 50 kD glycoprotein belonging to the serine proteinase superfamily. In addition to stimulating the synthesis of most ECM proteins, TGF-β also regulates the production of proteins that can modify the ECM by proteolytic action, such as plasminogen activator, an inhibitor of plasminogen, or procollagenase [7–10]. Plasmin can degrade fibrin, fibronectin and laminin, and activates matrix metallopro-
teinases and latent collagenases. PAI-1 is strongly induced by TGF-β and its promoter contains Smad binding elements [11]. TGF-β activates transcription of the plasminogen activator type-1 gene through a major TGF-β-responsive region in the PAI-1 promoter. This process requires the Smad family of signalling molecules. Upon TGF-β receptor activation, Smad2 and Smad3 become phosphorylated and form heteromer complexes with Smad4. Smad3/Smad4 binds CAG boxes within the promoter of the human PAI-1 gene.

Recent studies show an associate with PAI-1 and fibrosis in the kidney [12], liver [13] and lung [5,14,15]. The lung fibrosis by the intratrachial administration of bleomycin was well suppressed with PAI-1−/− mice [5]. PAI-1 suppresses the dissolution of collagen and promotes their accumulation. It has been shown that bleomycin-induced pulmonary fibrosis is more severe in transgenic mice overexpressing PAI-1 or in mice deficient of plasminogen, uPA or tPA [5,6]. Administrations of uPA into the lungs of WT or PAI-1 transgenic mice alter bleomycin injury decreases lung fibrosis [16]. Accordingly, PAI-1−/− mice are protected against bleomycin-induced pulmonary fibrosis. Furthermore, plasminogen knockout mice exhibit delayed skin wound repair [17,18]. These observations suggest that members of the plasminogen activator system play an essential role in the metabolic process of ECM.

We have recently established a mouse model for scleroderma by repeated local injections of bleomycin [19–23]. Local injections of bleomycin induce dermal sclerosis in various strains of mice. In this study therefore we examined the mRNA expression of PAI-1 and the level of functionally active immunoreactive PAI-1 in the bleomycin-induced murine skin sclerosis. Also, we investigated whether the induction of dermal sclerosis is attenuated in PAI-1-deficient mice.

**Materials and methods**

**Mice**

Specific pathogen-free female C3H/HeJ mice purchased from Clea (Tokyo, Japan) at 4 weeks of age weighing 20–25 g were used. Mice were kept freely in separate clean rooms in our animal facility and with food and water *ad libitum*. In a separate experiment, C57BL/6 mice (purchased from Clea, Tokyo) and PAI-1−/− mice, which were back-crossed onto a C57BL/6 genetic background (purchased from The Jackson Laboratory, Bar Harbor, ME, USA) were also used. In each experiment, 5 mice were examined.

**Bleomycin treatment**

Bleomycin (Nippon Kayaku Co., Tokyo) was dissolved into phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and sterilized with filtration (0.2 µm filter). 100 µl of bleomycin (1 mg/ml) was subcutaneously injected to the shaved back skin of C3H/HeJ mice on alternate days for 4 weeks. For the experiments using PAI-1−/− mice, 100 µl of bleomycin (100 µg/ml) was injected to the shaved back skin of WT mice and PAI-1−/− mice five days per week for 4 weeks. The mice were killed by cervical dislocation under anaesthesia with diethyl ether on the day after the final treatment. Controls were injected with only PBS.

**Histological and histochemical examination**

The back skins were removed, and cut into two. The one was fixed in 10% formalin solution, and the other was snap-frozen in liquid nitrogen and immediately stored at −80°C. Paraffin-embedded or cryostat sections were stained. They were stained using standard avidin–biotin peroxidase kit (Nichirei Co., Tokyo) with primary antibodies against u-PA (sc-6831), t-PA (N-14), PAI-1 (M-20), PAI-2 (D-20), TGF-β1 (sc-146) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), type I collagen (Bigenesis Ltd), fibronectin and tenascin (Abcam Limited, Cambridge, UK). Antibodies against u-PA, t-PA, PAI-1, PAI-2 and TGF-β1 were used at a final concentration of 1 µg/ml and tenasin (1:200 dilution), type I collagen (1:250) and fibronectin (1:250) were used accordingly to the optimal suggestion of the manufacturers. Subsequently, they were incubated with biotinylated secondary antibody, followed by the streptavidin-peroxidase treatment. The sections were developed with DAB (3,3′-diaminobenzidine) or AEC (3-amin-9-ethylcarbazole) solution as chromogen, and then counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls were prepared by omission of the primary antibodies and by the substitution for corresponding IgG.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted weekly from biopsied skin tissues using RNeasy Mini Kit (QIAGEN K.K., Tokyo). RNA yield and purity was determined with spectrophotometry. RT-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. After adding 4 µl of 10×PCR buffer (500 mM KCl, 100 mM Tris–HCl buffer, pH 8.4, 15 mM MgCl₂, and 0.01% gelatin), 2 µl of 2.5 mM dNTP (Takara), 4 µl of 10X hexanucleotide mixture (Boehringer, Mannheim, Germany), 40 U of ribonuclease inhibitor (Takara), and 12 U of RAV-2 reverse transcriptase (Takara), the mixture was incubated at 42°C for 60min, 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 u-PA, t-PA, PAI-1, PAI-2, TGF-β1, type I collagen, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and connective tissue growth factor (CTGF). The upstream and downstream primers are: u-PA:
Results were expressed as mean ± SD. Significance testing was assessed by Mann–Whitney U-test. A P-value <0.05 was considered as significant.

Results

PAI-1 gene expression was up-regulated by bleomycin treatment

RT-PCR was performed to analyse PA system and inhibitor of PA during the development of dermal sclerosis. Results showed that mRNA level of PAI-1 in the lesional skin was increased at 1 week, and 4 week (Fig. 1a). Quantification by densitometry revealed that PAI-1 mRNA level showed 2.2-fold increase at 1 week and 4-week (Fig. 1a). mRNA expression of TGF-β1 was detected all through the course, and mildly up-regulated at 2–3-week (Fig. 1a). mRNA expression of u-PA and t-PA were not significantly altered by bleomycin treatment. PAI-2 mRNA expression was mildly increased (up to 1.5-fold) at 4 week (Fig. 1a,b). Enhanced TGF-β1 expression in the bleomycin-treated skin was confirmed by immunohistochemistry (Fig. 1c).

PAI-1 protein expression and synthesis was increased by bleomycin treatment

As previously reported [21], subcutaneous injections of 100 μg bleomycin three times per week for 4 weeks induced marked dermal sclerosis and inflammatory cell infiltration in C3H/HeJ mice, whereas dermal sclerosis was not induced by the PBS treatment. Only faint inflammatory cell infiltrates were observed and only keratinocytes were positive for PAI-1 in the PBS-treated skin (Fig. 2a). On the contrary, PAI-1 was prominently detected in the bleomycin-treated skin. After 2 weeks' treatment, PAI-1 was detected on macrophage-like cells in the dermis (Fig. 2b). PAI-1- positive inflammatory cells were increased in number, in parallel with the
induction of dermal sclerosis. After 4 weeks’ treatment, PAI-1 was detected on fibroblastic cells and macrophage-like cells (Fig. 2c,d). Positive staining for u-PA was only found in keratinocytes in the PBS-treated skin (Fig. 2e), but also found in the cellular infiltrates and keratinocytes after 3 weeks (Fig. 2f). t-PA and PAI-2 were expressed diffusely in the dermis all through the course (not shown).

ELISA revealed that functionally active immunoreactive PAI-1 levels in whole skin samples were increased after bleomycin injection, as compared with that in PBS-treated mice. PAI-1 production in the bleomycin-treated skin peaked at 3 week and increased up to 6.6-fold as compared with PBS treatment (Fig. 3). Immunoblot analysis also showed enhanced PAI-1 signals in the bleomycin-treated skin (Fig. 4).

Sclerotic skin was induced even in PAI-1−/− mice by bleomycin treatment

Since we confirmed that PAI-1 was increased at the mRNA and protein levels in the lesional skin following bleomycin treatment, we next examined whether PAI-1 deficiency prevents dermal sclerosis induced by bleomycin injection. We injected subcutaneously 10 μg of bleomycin to PAI-1−/− mice and WT mice 5 days per week for 4 weeks. Histological examination revealed that sclerotic skin was definitely
Fig. 2. Histological examination. (a) Immunohistochemical localization of PAI-1 was faintly detected in the PBS-treated skin. (b) In contrast, PAI-1 was detected on the macrophage-like cells in the bleomycin-treated skin at 2 week. (c, d) At 4 week, immunoreactive cells for PAI-1 are mainly infiltrating macrophage-like cells and fibroblastic cells in the bleomycin-treated skin. Positive staining for u-PA was only found in keratinocytes in the PBS-treated skin (e), but also found in the cellular infiltrates and keratinocytes after 3 weeks (f). Skin sections were developed with DAB (a–d) or AEC (e,f). Magnification; (a–c) ×60, (d) ×120, (e, f) ×50.

Fig. 3. Immunoreactive PAI-1 protein levels were assessed by ELISA. Mice (n = 5) were treated with either PBS or bleomycin. The skin samples were homogenized and after centrifugation, the supernatants were measured by ELISA. *P < 0.05.

Fig. 4. Detection of PAI-1 by immunoblot analysis. Bleomycin-treated skin (for 3 weeks) shows stronger signals as compared with PBS-treated skin.
induced even in PAI-1−/− mice as much as WT (Fig. 5). Type I collagen, fibronectin, tenasin were diffusely expressed in the sclerotic dermis in both control and PAI-1−/− mice following bleomycin treatment (Fig. 5). Dermal thickness was 344 ± 50 μm in WT, 414 ± 50 μm in PAI-1−/− mice after PBS treatment and 373 ± 40 μm in WT, 483 ± 127 μm in PAI-1−/− mice after bleomycin treatment. Collagen contents in the skin were significantly increased following bleomycin treatment as compared with PBS treatment in both WT (3·63 ± 1·20 μg/mg versus 1·87 ± 1·05 μg/mg, P < 0·05) and PAI-1−/− (3·91 ± 0·54 μg/mg versus 1·26 ± 1·36 μg/mg, P < 0·05) mice. There was no statistical difference of collagen contents in the skin following bleomycin treatment between WT and PAI-1−/− mice. These findings indicate that dermal sclerosis is not prevented by PAI-1 deficiency after 4 weeks’ bleomycin treatment.

Examination of TGF-β1 positive cells were increased in the lesional skin after bleomycin treatment, mainly on infiltrating mononuclear cells and fibroblasts, in both WT and PAI-1−/− mice (Fig. 6). By contrast, there were few immunoreactive cells in the PBS-treated skin (Fig. 6).

Gene expression of MMP-2, TIMP-1, PAI-2, CTGF, type I collagen and TGF-β1

Because TGF-β up-regulates MMP-2, TIMP-1, PAI-2 and CTGF and also plasmin can contribute to the proteolytic activation of pro-MMP-2 [24], we examined gene expression of MMP-2, TIMP-1, PAI-2, CTGF, type I collagen and TGF-β1 in skin tissues of PAI-1−/− and WT mice. mRNA levels of MMP-2 and CTGF were not significantly altered between 4 groups. TIMP-1 and PAI-2 mRNA expression was elevated (up to 1·9-fold and 1·5-fold) following bleomycin treatment in WT mice, whereas TIMP-1 mRNA level was rather down-regulated in PAI-1−/− mice (Fig. 7a). Quantification by densitometry revealed that TGF-β1 and type I collagen mRNA levels showed more than 2-fold increase in bleomycin-treated skins of both PAI-1−/− and WT mice (Fig. 7b).

Discussion

In addition to stimulating the synthesis of most ECM proteins, TGF-β regulates the homeostasis of ECM by decreasing ECM degradation. One of the mechanisms includes the synthesis of PAI-1, which prevents the conversion of plasminogen to plasmin, through inhibiting tPA and uPA. Plasmin can degrade fibrin, fibronectin, and laminin, and activates MMPs and latent collagenases. TGF-β stimulates the expressions of PAI-1 [25–27], fibronectin, and collagen [28], while simultaneously suppresses the expression of uPA [8].

Increased PAI-1 has been associated with thrombotic diseases and organ fibrosis in liver, lung, kidney, blood vessels, and skin [5,13,14,29]. PAI-1 overexpression has been found in fibroblasts of keloids [29,30], Werner’s fibrosis [29], and scleroderma [31]. In scleroderma fibroblasts, TGF-β signaling events, including phosphorylation of Smad-2 and -3, and transcription of the PAI-1 gene are increased as compared with normal fibroblasts [31]. The mean plasma level of prothrombin fragment and PAI were higher in patients with SSc than control [32]. Lee et al. [33] showed serum fibrin degradation products were increased in 44% of patients with SSc.

In this study, we examined the expressions of u-PA, t-PA, PAI-1, PAI-2 during the development of bleomycin-induced scleroderma. We found that mRNA level of PAI-1 is increased in the lesional skin at 1 week and 4 week. PAI-1 plays an important role in wound repair, fibrin degradation in PA system. Subcutaneous bleomycin injection induces local inflammation in early phase. The first peak of PAI-1 mRNA expression might be derived from early tissue inflammation. PAI-1 is strongly induced by TGF-β and its promoter contains Smad binding elements [11]. We showed that mRNA level of TGF-β1 is up-regulated at 2 and 3 week after bleomycin treatment. The second peak of PAI-1 at 4 week was suspected to be mediated by TGF-β. Immunoreactive active PAI-1 was increased at every week up to 3·4–6·6 folds as compared with PBS control. And immunoblot analysis revealed that PAI-1 signals were elevated following treatment with bleomycin as compared with PBS control. PAI-1 mRNA level was increased in the lesional skin at 1 week as much as at 4 week, whereas protein levels of PAI-1 increased gradually. At a glance, it seems like there is a discrepancy between mRNA and protein levels, but we detect immunoreactive active PAI-1 levels, which bounds to uPAs, in our ELISA method. PAI-1 binds to uPA and tPA at the lesion of inflammation to play a role to control fibrin degradation in early phase of bleomycin-induced scleroderma. Therefore, we could detect only resumed PAI-1 production at the lesional skin.

PAI-1 plays a role in the control of MMP activation through several different mechanisms. The functional association between the MMPs and the PA system, in particular the role of plasmin as a pro-MMP activator, has generated substantial attention in the context of both physiological and pathological tissue remodeling. Moreover, numerous studies have demonstrated that plasmin can contribute to the proteolytic activation of pro-MMP-1, -2, -3, -9, -10, -12, and -13 in vitro [34]. Furthermore, higher PAI-1 activity will lead to a dramatic reduction of plasmin-mediated MMP activation and subsequent matrix degradation. Hence, increased PAI-1 activity may act through several synergistic mechanisms to increase matrix accumulation and encourage fibrosis.

Definitive proof of PAI-1 involvement in ECM metabolism during tissue injury repair comes from targeted gene interruption or overexpression in mice. In this regard, it has been shown that bleomycin-induced pulmonary fibrosis is more severe in transgenic mice overexpressing PAI-1 and in mice deficient of plasminogen, uPA or tPA [5,6]. It was concluded
Fig. 5. Histological analysis. PAI-1−/− and WT mice treated with either PBS or bleomycin for 4 weeks. Sections were examined with HE, Masson staining, and immunostaining using antibodies against type I collagen, fibronectin, and tenascin. Magnification ×40.
Fig. 6. Histological analysis. PAI-1−/− and WT mice were treated with either PBS or bleomycin for 4 weeks. In both WT and PAI-1−/−, immunoreactive cells for TGF-β1 in the lesional skin after bleomycin treatment were mainly mononuclear cells and fibroblasts, whereas there were few positive cells in PBS-treated skin. Skin sections were developed with DAB. Magnification ×30.

Fig. 7. (a) RT-PCR analysis of MMP-2, TIMP-1, type I collagen, PAI-2, CTGF and TGF-β1 in the lesional skin. PAI-1−/− and WT mice were locally treated with either PBS or bleomycin for 4 weeks and total RNA was isolated from skin samples. Representative data are shown in three independent experiments. (b) Graph shows quantitative densitometric analysis. Relative amounts are expressed as arbitrary units after normalization of MMP-2, TIMP-1, type I collagen, PAI-2, CTGF and TGF-β1 for β-actin.
that increased PAI-1, which determined the extent of plasminogen activation, might result in fibrin accumulation at the site of lung injury and fibrosis. And PAI-1−/− mice are protected against bleomycin-induced pulmonary fibrosis [5]. Plasminogen knockout mice also exhibit delayed skin wound repair [17], whereas, PAI-1-deficient mice show accelerated wound closure [18]. These data suggest that plasminogen activator systems play an essential role in bleomycin-induced pulmonary fibrosis and wound repair. On the contrary, in this study, histopathology revealed that sclerotic skin was developed in PAI-1−/− mice as well. Collagen contents in whole biopsied skin were significantly increased following bleomycin treatment in PAI-1−/− mice as compared with PBS treatment, and the amount of collagen contents were as much as WT mice. Thus, PAI-1-deficient phenomenon could not prevent the development of dermal sclerosis. Although PAI-1 plays an important role in ECM metabolism, it is not the prerequisite factor in bleomycin-induced dermal sclerosis. From the early investigation, we at first hypothesized that PAI-1-deficient phenomenon prevent the development of bleomycin-induced scleroderma. Against our expectations, PAI-1−/− mice exhibited scleroderma as much as WT control by 4 weeks’ treatment with bleomycin. These results may contrast to those of previous reports in which fibrosis is prevented in PAI-1-deficient mice [5,6,14]. These bleomycin-induced lung fibrosis were developed by instilled bleomycin at doses of 0.075 µg, or 0.15 µg [5], slowly instilled 4 U/kg of bleomycin sulphate into tracheal lumen [14], intratracheal administration of a single dose of 0.075 U bleomycin [6]. There may be a few speculations about the difference between previous reports and our results. First is the different method of bleomycin treatment. Single injection of bleomycin can cause lung fibrosis in mice, whereas our method requires frequently repeated bleomycin treatment for much longer period to induce dermal sclerosis. Second, plasmin converts latent form of TGF-beta into the active form TGF-beta, which stimulates ECM production by fibroblasts. Thus, PAI-1 deficiency theoretically augments the extent of TGF-beta dependent process, leading to fibrosis. Hertig et al. [35], showed evidence that, converse to their initial hypothesis, PAI-1 deficiency dramatically worsens the course of experimental crescentic glomerulonephritis. And they found that lack of PAI-1 induces the overactivation of TGF-β [35] and immunohistochemical study showed that enhanced TGF-β1 expression was noted with similar intensity in both WT and knockout mice. Third, the mechanism of fibrosis is different between skin and lung. Unfortunately, a comparative study between skin and lung was not carried out in this study. And finally, even if PAI-1 is deleted, other fibrogenic cytokines work redundantly.

mRNA levels as well as protein expression of TGF-β1 were up-regulated after bleomycin treatment in both WT and PAI-1−/− mice. Type I collagen gene expression was up-regulated after bleomycin treatment but MMP-2 did not show significant difference among these 4 groups (WT and PAI-1−/− mice, bleomycin-treatment and PBS-treatment). TGF-β1 induced a strong up-regulation of TIMP-1 gene in pulmonary fibroblasts as well as in the whole lungs of C57BL/6 mice, in contrast to a weak induction in Balb/c mice [36]. Our results that TIMP-1 mRNA level is up-regulated in C57BL/6 WT mice are in consistent with their data, however, TIMP-1 mRNA level was rather down-regulated in PAI-1−/− mice. The mechanism of this down-regulation of TIMP-1 in PAI-1−/− mice is unknown at present. There might be an interaction between PAI-1 and TIMP-1, but further investigation will be necessary: mRNA level of PAI-2 and CTGF were not up-regulated after bleomycin treatment in PAI-1−/− mice. In vivo, plasminogen activator inhibitors (PAI-1, −2) play a crucial role in the regulation of serine protease activity. Even though, PAI-2 exhibits inhibitory activity toward t-PA and u-PA, its efficacy is 20- to 100-fold less than that of PAI-1 [37]. We thus considered that PAI-2 and CTGF were not major effectors in increased collagen contents in whole skin following bleomycin treatment in PAI-1−/− mice.

In conclusion, we demonstrate that PAI-1 is elevated in protein production and mRNA expression by bleomycin treatment but absence of PAI-1 does not affect the development of bleomycin-induced dermal sclerosis. Although PAI-1 plays an important role in ECM metabolism, PAI-1 does not play an essential role in this in vivo model of scleroderma.

References


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