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Involvement of mast cell chymase in bleomycin-induced pulmonary fibrosis in mice

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Abstract

The possible role of mast cell chymase in organ fibrosis was examined using a bleomycin-induced pulmonary fibrosis model in mice. Intratracheal injection of bleomycin to mice significantly increased not only hydroxyproline content but also chymase activity in the lung. Administration of a chymase inhibitor SUN C8077 (7-chloro-3-(3-amynophenyl) quinazoline-2, 4-dione methanesulfonate) dose-dependently reversed the bleomycin-induced increase in hydroxyproline content as well as chymase activity in the lung. Human chymase digested latent transforming growth factor-β1 (TGF-β1) to form mature TGF-β1 in vitro, which was inhibited by SUN C8077. Human chymase, on the other hand, failed to stimulate DNA synthesis of human lung fibroblasts CCD-8Lu and LL97A. Taken together, it is suggested that mast cell chymase might participate in the pathogenesis of pulmonary fibrosis, and that the chymase-induced fibrosis might be mediated at least in part by TGF-β1. Chymase inhibitor may be promising for treatment of pulmonary fibrosis in humans.

Keywords: Chymase; Mast cell; Pulmonary fibrosis; Collagen synthesis; TGF-β1 (transforming growth factor-β1)

1. Introduction

Pulmonary fibrosis is a consequence of various severe lung injuries and characterized by increased proliferation of fibroblasts and excessive deposition of extracellular matrix collagen (Du Bois, 1993). Although there are many possible etiologies for pulmonary fibrosis (Lingos et al., 1991; Wagner, 1997), a clear cause for each patient cannot usually be ascertained. A chemotherapeutic agent bleomycin is known to develop lung fibrosis in humans as well as experimental animals, and is thereby used widely for studying the pathogenesis of this disorder (Lazo et al., 1990). The fibrotic process includes chronic inflammation, generally accompanied by accumulation of inflammatory cells, such as eosinophils (Noguchi et al., 1992; Gharaee-Kermani et al., 1998) and mast cells (Pesci et al., 1993; Inoue et al., 1996).

Chymase is a chymotrypsin-like serine protease stored in mast cell granules (Schwartz et al., 1980). Chymase hydrolyses a variety of physiological substrates, e.g. it converts angiotensin I to angiotensin II (Urata et al., 1990), and cleaves membrane bound stem cell factor to leading its release from cells (Longley et al., 1997; Tomimori et al., 2002a). However, its pathophysiological role is not completely clear. Recently, it has been reported that purified human chymase activates matrix metalloproteinase-1 (Saarinen et al., 1994; Fang et al., 1997), suggesting that mast cell chymase might prevent collagen accumulation by promoting matrix degradation. On the contrary, human chymase cleaves type I procollagen to form collagen fibril in vitro (Kofford et al., 1997). In addition, chymase activates transforming growth factor-β1 (TGF-β1) (Lindstedt et al, 2001), a fibrogenic cytokine (Ling and Robinson, 2002). Thus, it is also possible that mast cell chymase functions as a fibrogenic factor in organ fibrosis.

The aim of the present study is to examine the pathophysiological role of mast cell chymase in pulmonary fibrosis in vivo, and we examined the effect of a chymase inhibitor on bleomycin-induced pulmonary fibrosis in mice.

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Our data showed that mast cell chymase might promote collagen accumulation in the pulmonary fibrosis model, and that TGF-β1 might participate in this process.

2. Materials and methods

2.1. Mice

Male ICR mice were purchased from Charles River Japan (Yokohama, Japan) and used at the age of 10 weeks old. All animal experiments were performed according to the Guidelines for Animal Experimentation (Japan Association for Laboratory Animal Science, 1987), and the experimental protocol was approved by the Committee for Ethics in Animal Experiments of Suntory Biomedical Research.

2.2. Recombinant human chymase and chymase inhibitor

Recombinant human chymase was expressed and purified as described previously (Tomimori et al., 2002b). A chymase inhibitor SUN C8077, 7-chloro-3-(3-amynophenyl) quinazoline-2, 4-dione methanesulfonate, was synthesized as described (Fukami et al., 2000). SUN C8077 has an IC_{50} value of 0.36 μM for human chymase but shows no effect on bovine pancreas trypsin (Nacalai Tesque, Kyoto, Japan) and human neutrophil elastase (Calbiochem, San Diego, CA) even at 10 μM. SUN C8077 was suspended in 0.5% of hydroxy propyl cellulose (Nippon Soda, Tokyo, Japan), when administered to mice.

2.3. Pulmonary fibrosis induced by bleomycin

Bleomycin (Nippon Kayaku, Tokyo, Japan) dissolved in saline was injected intratracheally to anesthetized ICR mice (10 mice per group) at 0.04 or 0.08 mg/mouse using Hamilton's syringe (100 μl, Hamilton, Reno). Control mice (sham group) were treated with saline instead of bleomycin. The mice were sacrificed 14 days after the bleomycin treatment, and the lungs were extirpated to determine hydroxyproline content in the tissues. SUN C8077 was administered i.p. five times a week (Monday to Friday) for 14 days (10 times in all); the first administration was done just after the bleomycin treatment.

2.4. Hydroxyproline measurement

Hydroxyproline, the specific marker for collagen content in tissues, was measured colorimetrically by the method of Lindenschmidt and Witschi (1985) with a slight modification. Briefly, lung samples were hydrolyzed in 6 M HCl by heating 110 °C for 18 h, followed by neutralization with KOH. The solution was subsequently oxidized with chloramine T (Sigma-Aldrich, St. Louis, MO) for 25 min and the reaction was stopped by adding sodium thiosulphate to the solution. The mixture was then extracted with toluene, and the toluene layer was discarded. The aqueous phase was boiled for 30 min and extracted again with toluene. The hydroxyproline-derived pyrrole in the second toluene extract was then reacted with *p*-dimethylaminobenzaldehyde (Sigma-Aldrich) for 30 min and the absorbance at 560 nm was measured.

2.5. Measurement of chymase activity in the lung

The lung was homogenized in 20 mM Tris–HCl buffer, pH7.5 (10 times volume of the tissue weight), and the homogenate was centrifuged at $10{,}000 \times g$ for 10 min. The chymase activity in the resulting supernatant was measured using Suc-Ala-Ala-Pro-Phe-MCA (Peptide Institute, Osaka, Japan) as a substrate according to the method of Pasztor et al. (1991). To block the serine proteases other than chymase, 10 μM of aprotinin was added to the solution of the enzyme reaction, which does not affect chymase activity (Takai et al., 1996, Kakizoe et al., 2001).

2.6. Histological analyses

Lungs were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 5 μm, and then stained with hematoxylin and eosin.

2.7. Mitogenic ability of human chymase to human fibroblasts

CCD-8Lu (human normal lung fibroblasts) and LL97A (human fibroblasts from idiopathic pulmonary fibrosis) were obtained from American Type Culture Collection (Manassas, VA). The cells of passage 5–10 were seeded at a density of 5000 cells per well in 96-well plates and cultured in RPMI1640 containing 10% fetal calf serum for 3 days. The cells were then washed once with serum-free RPMI1640 and starved for 72 h in serum-free RPMI1640 medium. After removing the medium, human chymase in serum-free RPMI1640 medium or RPMI1640 containing 10% fetal calf serum was added. As a positive control, human lung tryptase (Sigma-Aldrich) was used instead of human chymase, as it is known to stimulate fibroblast proliferation (Ruoss et al., 1991; Brown et al., 2002). DNA synthesis of the cells was qualified by measuring bromodeoxyuridine (BrdU) incorporation into the cells for 24 h using Cell Proliferation ELISA (enzyme-liked immunosorbent assay) (Roche Diagnostics, Basel, Switzerland).

2.8. Ability of human chymase to activate latent form of TGF-β1

Human latent TGF-β1 (R&D Systems, Minneapolis, MN) (8 μg/ml) was incubated with human chymase (0.1–100 nM), human plasma plasmin (1 unit/ml, Sigma-Aldrich), and human lung tryptase (1 unit/ml) at 37 °C for 15 min, and the reaction was terminated by addition of fetal calf serum.

When the effect of SUN C8077 on the reaction was examined, human chymase (100 nM) was preincubated with the inhibitor at 37 °C for 15 min. The formation of mature TGF-β1 in the reaction was examined by Western blotting. Briefly, latent and mature TGF-β1 molecules were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis with 15–25% gradient gel (15/25 Multi gel, Daiichi Pure Chemicals, Tokyo, Japan) under non-reducing condition; the reaction mixture including originally 0.1 nmol latent TGF-β1 was applied to the gel. The separated proteins were subsequently transferred onto polyvinylidene fluoride membrane (Bio-Rad Laboratories, Tokyo, Japan) and probed with a mouse anti-human TGF-β1 antibody (R&D Systems), followed by incubation with peroxidase-conjugated second antibody, goat anti-mouse immunogloblin G-HRP (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody bound to TGF-β1 on the membrane was then visualized using Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). The light produced by the chemiluminescent reaction was detected by exposure to Fuji IX industrial X-ray film (Fujifilm, Tokyo, Japan).

2.9. Statistical analysis

The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t*-test using Super-ANOVA (analysis of variance) (Abacus Concepts, Berkeley, CA) or Statview (SAS Institute, Cary, NC), respectively. The *P*-value less than 0.05 were considered significant.

3. Results

3.1. Change in hydroxyproline content and chymase activity in the lung of bleomycin-treated mice

As shown in Fig. 1A, intratracheal injection of bleomycin to mice significantly increased hydroxyproline content in the lung. The effect of bleomycin was dose-dependent, and the increases in hydroxyproline content were 1.15- and 1.25-fold at doses of 0.04 and 0.08 mg/mouse, respectively. No mouse has died during the experiments even at 0.08 mg/mouse. Bleomycin was therefore used at 0.08 mg/mouse in the following experiments. The bleomycin treatment also increased the level of chymase activity in the lung (Fig. 1B). This result is in agreement with the finding that mast cells accumulate in fibrotic lung (Pesci et al., 1993; Inoue et al., 1996), and indicates that mast cell chymase may be related to the pathogenesis of bleomycin-induced lung fibrosis.

3.2. Effect of chymase inhibitor on hydroxyproline content in the lung of bleomycin-treated mice

When a chymase inhibitor SUN C8077 was injected i.p. five times a week for 14 days to the bleomycin-treated mice, the increase in hydroxyproline content as well as chymase activity in the lung was inhibited at a dose of 50 mg/kg (Fig. 2). The inhibition by SUN C8077 at 50 mg/kg was approximately 46% and 67% for hydroxyproline content and chymase activity, respectively, though little effect was observed at 10 mg/kg for both.

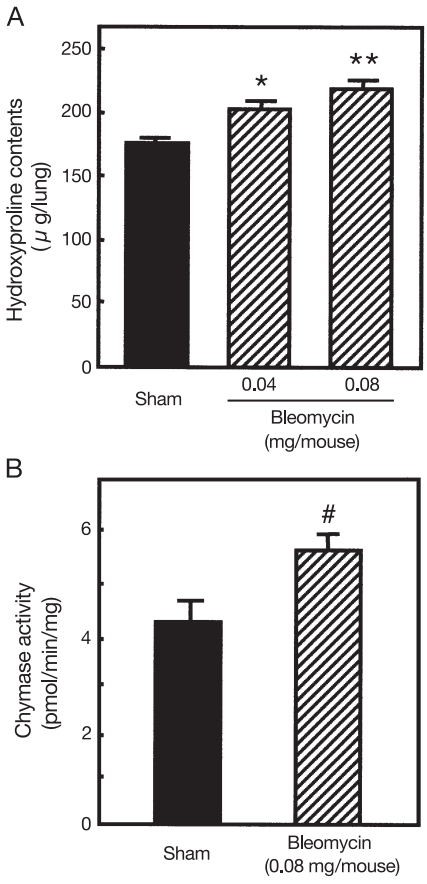

Fig. 1. Change in hydroxyproline content and chymase activity in the lung of bleomycin-treated mice. Bleomycin was injected intratracheally to ICR mice, and the lung was extirpated 14 days after the bleomycin treatment. Control mice were treated with saline instead of bleomycin (sham group). Hydroxyproline content (A) and chymase activity (B) in the extirpated lung were measured as is described in Materials and methods. Data are mean ± S.E.M. ($N=10$). *$P<0.05$; **$P<0.01$ (Dunnett's test compared with the sham group), $^{\#}P<0.05$ (Student's *t*-test compared with the sham group).

It is known that bleomycin treatment decreases body weight, depending severity of lung fibrosis (Oury et al., 2001). Consistently, the mean of body weight of the bleomycin-treated mice on day 14 was significantly decreased as compared with the sham group (37.7 ± 1.8 and 43.7 ± 0.4 g for bleomycin-treated and sham groups, respectively; $P<0.05$, Student's *t*-test). In addition, SUN C8077 treatment inhibited the bleomycin-induced body weight loss, i.e., the means of body weigh of the bleomycin-treated mice that had been administered with 10 and 50 mg/kg of SUN C8077 were 36.6 and 40.9 g, respectively, though statistical significance was not observed between the SUN C8077-treated and bleomycin-treated control groups. The data of body weight support the idea that chymase inhibition reduces the severity of pulmonary fibrosis.

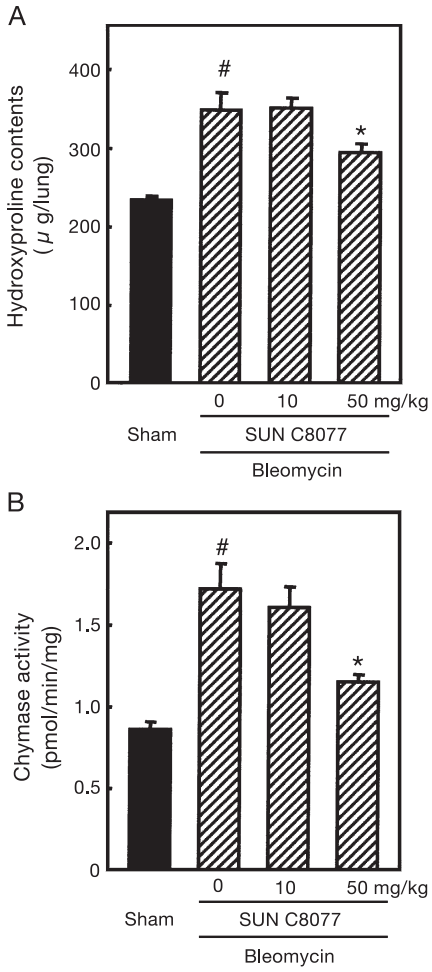

Fig. 2. Effect of chymase inhibitor SUN C8077 on hydroxyproline content and chymase activity in the lung of bleomycin-treated mice. Bleomycin (0.08 mg/mouse) was injected intratracheally to ICR mice, and the lung was extirpated at day 14. Control mice were treated with saline instead of bleomycin (sham group). SUN C8077 was administered intraperitoneally five times a week for 14 days (10 times in all). Hydroxyproline content and chymase activity in the extirpated lung were measured as is described in Materials and methods. Data are mean ± S.E.M. ($N=10$). *$P<0.05$ (Dunnett's test compared with the vehicle-treated mice); $^{\#}P<0.01$ (Student's *t*-test compared with the sham group).

3.3. Effect of chymase inhibitor on histological changes in the lungs of bleomycin-treated mice

Fig. 3 shows the representative results of histological observations of the lung of the bleomycin-treated mice. On day 14 after the bleomycin injection, the alveolar wall and septa were considerably thicker, and prominent cellular infiltration was observed in the lung interstitium, compared with the sham group (Fig. 3A and B). In the lung of the mice treated with 50 mg/kg of SUN C8077, the fibrotic changes in interalveolar septa were much less in degree, while some lobes still showed a mild interstitial fibrosis (Fig. 3C). The infiltrated cells were little, if any, in the interstitium of the lung of the SUN C8077-treated mice.

3.4. Effect of chymase on proliferation of human lung fibroblasts

To elucidate the mechanism by which chymase is involved in pulmonary fibrosis, an ability of human chymase

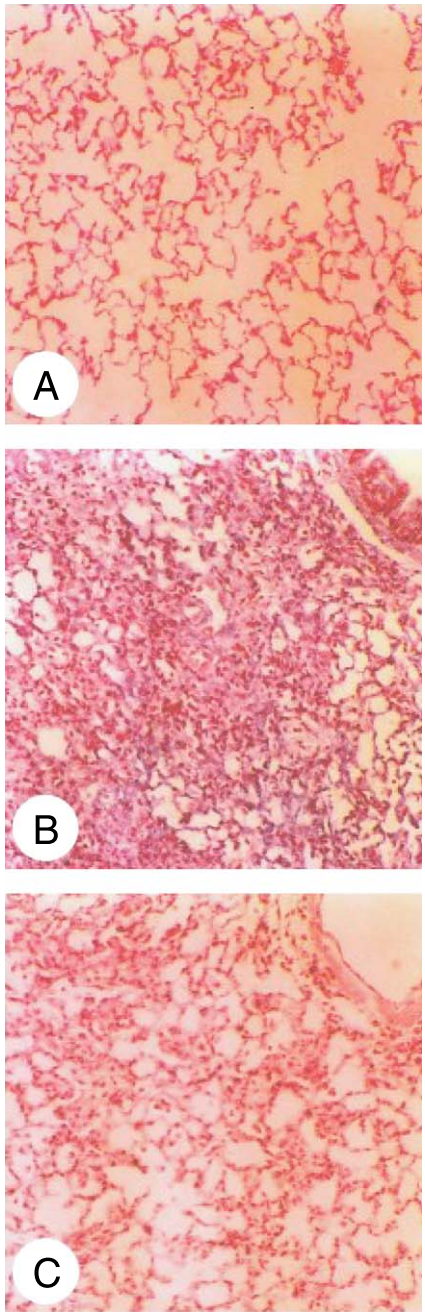

Fig. 3. Effect of a chymase inhibitor SUN C8077 on bleomycin-induced histological change of the lung. Bleomycin was injected intratracheally to ICR mice, and the lung was extirpated on day 14 for histological examination. The histological samples were prepared and stained with hematoxylin and eosin as is described in Materials and methods. (A) Sham group in which saline was injected instead of bleomycin; (B) bleomycin-treated mouse to which vehicle was administered instead of SUN C8077; (C) bleomycin-treated mouse to which SUN C8077 was administered. Photographs are the representatives of the results.

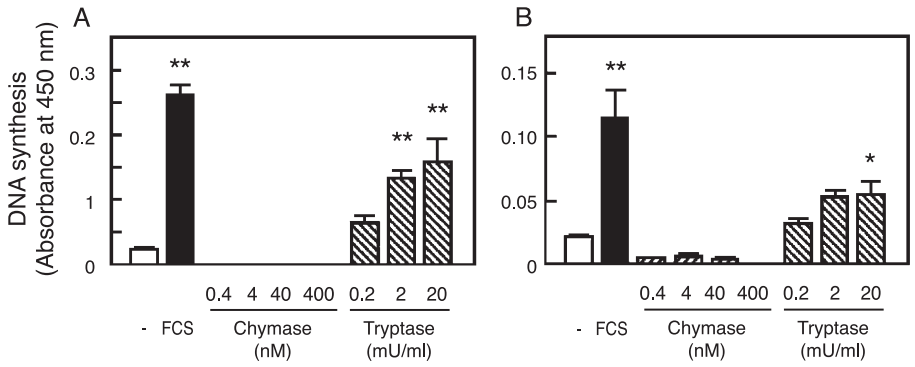

Fig. 4. Effect of human chymase on proliferation of human lung fibroblasts. Human fibroblasts CCD-8Lu (A) and LL97A (B) were seeded at 5000 cells per well in 96-well plates and cultured in RPMI1640 containing 10% fetal calf serum for 3 days. The cells were then starved for 72 h in serum-free medium and stimulated with 10% fetal calf serum (FCS), human chymase or human tryptase. DNA synthesis of the cells was qualified as is described in Materials and methods. Data are mean ± S.E.M. ($N=3$). $^{*}P<0.05$; $^{**}P<0.01$ (Dunnett's test compared with the control).

to stimulate proliferation of fibroblasts was examined using human lung fibroblasts. As shown in Fig. 4A, addition of 10% fetal calf serum stimulated DNA synthesis of human normal lung fibroblasts CCD-8Lu. Human tryptase increased DNA synthesis in a concentration-dependent manner, consistent with the data reported by others (Ruoss et al., 1991; Brown et al., 2002). In contrast, human chymase did not stimulate the growth of CCD-8Lu at 0.4–400 nM. Similar result was obtained when LL97A cells, the fibroblasts derived from idiopathic pulmonary fibrosis, was used instead of CCD-8Lu (Fig. 4B). These results suggest that chymase-induced fibrosis may not be due to mitogenic activity of chymase to fibroblasts.

3.5. Effect of chymase on activation of latent TGF-β1

TGF-β1 is thought to be one of the major cytokine involved in organ fibrosis. In addition, rat chymase has been shown to activate large latent TGF-β1 (Lindstedt et al., 2001). Thus, it is possible that chymase-induced activation of TGF-β1 is associated with the improvement of the lung fibrosis by SUN C8077. Next, therefore, this point was investigated by Western blotting. As shown in Fig. 5A, incubation of human latent TGF-β1 with human plasmin resulted in formation of mature TGF-β1 (lane 2). Human chymase also formed mature TGF-β1 (lanes 3–6), while human tryptase did not (lane 7). SUN C8077 inhibited the

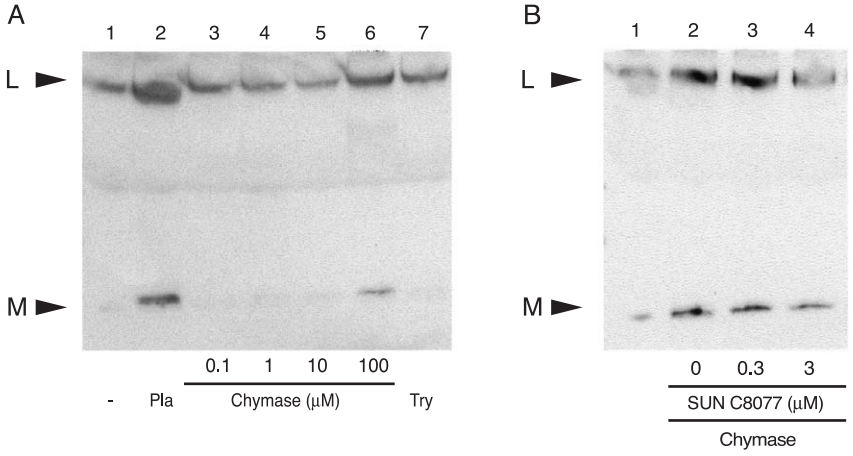

Fig. 5. Ability of human chymase to activate human latent TGF-β. Human latent TGF-β1 (8 μg/ml) was incubated with human chymase at 37 °C for 15 min, and the reaction was terminated by addition of fetal calf serum. The formation of mature TGF-β1 was analyzed by SDS polyacrylamide gel electrophoresis under non-reducing condition followed by Western blotting as is described in Materials and methods. (A) Concentration-dependency of the effect of chymase (lanes 3–6). Plasmin (Pla, lane 2, 1 unit/ml) and human tryptase (Try, lane 7, 1 unit/ml) was added instead of human chymase as positive and negative control, respectively. (B) Effect of SUN C8077 (lanes 3, 4) on the ability of chymase to active latent TGF-β. SUN C8077 was incubated with human chymase at 37 °C for 15 min prior to the reaction. The arrowhead indicates the band corresponding to latent TGF-β (L; ~ 100 kDa) and mature TGF-β1 (M; ~ 23 kDa). This figure is the representative of three experiments.

chymase-induced mature TGF-β1 generation in a concentration-dependent manner (Fig. 5B, lanes 3, 4). These results suggest that chymase may contribute to TGF-β1 activation.

4. Discussion

The data in the present study showed that a chymase inhibitor SUN C8077 significantly suppresses the bleomycin-induced collagen accumulation in the lung (Fig. 2). Since SUN C8077 is specific for chymase (not effective on trypsin and elastase even at 10 μM), our data suggest that mast cell chymase may contribute to the pathogenesis of pulmonary fibrosis.

It is known that the number of mast cells increases in fibrotic organ including the lung (Pesci et al., 1993; Inoue et al., 1996), raising the possibility that mast cells are implicated in the pathogenesis of pulmonary fibrosis. In addition, O'Brien-Ladner et al. (1993) have reported that fibrotic response induced by bleomycin injection is much less in the mast cell-deficient mi/mi mice compared with normal mice. However, Okazaki et al. (1998) have shown that injection of bleomycin successfully induces lung fibrosis in the mast cell-deficient Ws/Ws rats as well, and that the fibrosis in the mutant rats is severer than that in their control rats. The mi/mi mice but not Ws/Ws rats have several abnormalities including disfunction of natural killer cells and osteopetrosis. The authors concluded therefore that mast cells may be associated but not a cause of the lung fibrosis. If so, our data in this study may show that induction of lung fibrosis in the Ws/Ws rats is the result of compensation by other chymotrypsin-like enzyme, as chymase is expressed exclusively in mast cells.

Recently, Kakizoe et al. (2001) have shown that mast cell chymase may be involved in skin fibrosis using the tight-skin (TSK) mice, a model for scleroderma, i.e., chymase activity is significantly higher in the skin of TSK mice than that of the pallid mice, and the activity in TSK mice augmented in accordance with increase in thickness of subcutaneous fibrous layers. Thus, chymase might also participate in collagen accumulation in a variety of organs other than the lung.

The mechanism under the pathogenesis for tissue fibrosis is not fully understood at present, but it is conceivable that TGF-β1 plays a crucial role. For instance, the expression of TGF-β1 is induced in animal models of lung fibrosis (Broekelmann et al., 1991), liver cirrhosis (Czaja et al., 1989) and skin fibrosis (Border and Ruoslahti, 1992). Furthermore, Smad7, the antagonist of TGF-β1 signaling, as well as neutralizing antibodies to TGF-β1 prevent lung fibrosis in mouse models (Giri et al., 1993; Nakao et al., 1999). Interestingly, our data (Fig. 5) and others (Lindstedt et al., 2001) have shown that human chymase activates human latent TGF-β1. It is therefore probable that TGF-β1 is associated with the SUN C8077-mediated amelioration of lung fibrosis.

Mast cell tryptase has been shown to possess mitogenic activity to several types of cells, such as fibroblasts (Ruoss et al., 1991) and airway smooth muscle cells (Brown et al., 2002). Likely, chymase is known to induce proliferation of dermal fibroblasts (Algermissen et al., 1999) and cultured myocardial cells (Hara et al., 1999). In the present study, however, human chymase did not at all stimulate DNA synthesis of human lung fibroblasts (Fig. 4), which suggests that the effect of SUN C8077 in bleomycin-induced fibrosis model may not be related to fibroblast proliferation. Consistent with our data for the effect of chymase on fibroblast are those of Ruoss et al. (1991), i.e., dog tryptase stimulated thymidine uptake of Rat-1 fibroblasts in a concentration-dependent manner with an EC_{50} of approximately 5 nM, while a similar effect was not observed for 3 nM of chymase purified from dog mastocytoma. The difference in the ability of chymase to stimulate cell growth between the studies appears depend on sources of the cells, experimental conditions, and purity of chymase.

It has been reported that purified human chymase cleaves type I procollagen to form collagen-size chains, although the cleavage site of chymase is 20 amino acids carboxyl to the known C-proteinase cleavage site (Kofford et al., 1997). Turbidity studies revealed that chymase-generated collagen is able to form collagen fibrils, which are similar to those generated by procollagen proteinases. Accordingly, it is possible that mast cell chymase augments collagen synthesis in the lung by promoting procollagen processing in bleomycin-induced lung fibrosis, and that chymase inhibitor SUN C8077 reduces the collagen accumulation in the lung by blocking this process. Further studies are needed to clarify this point.

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