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Short communication

A specific chymase inhibitor, NK3201, suppresses bleomycin-induced pulmonary fibrosis in hamsters

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Abstract

We evaluated whether a chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-*N*-[{3,4-dioxo-1-phenyl-7-(2-pyridyloxy)}-2-heptyl]acetamide (NK3201), suppressed bleomycin-induced pulmonary fibrosis. Hamsters were orally administered NK3201 (30 mg/kg per day) or placebo, beginning 5 days before intratracheal instillation of bleomycin (10 mg/kg). Four weeks after the instillation of bleomycin, pulmonary chymase activity in placebo-treated hamsters was significantly higher than in control hamsters, whereas the activity in NK3201-treated hamsters was significantly lower than in placebo-treated hamsters. The ratio of fibrotic area to total area in NK3201-treated hamsters was significantly decreased to 54.0% of the ratio in placebo-treated hamsters. Therefore, NK3201 may be useful in the prevention of pulmonary fibrosis.

Keywords: Chymase inhibitor; Fibrosis; Lung; Transforming growth factor-β

1. Introduction

Chymase is a chymotrypsin-like serine protease contained in the secretory granules of mast cells, and this enzyme may contribute to the activation of transforming growth factor (TGF)-β (Taipale et al., 1995). TGF-β is released from a latent TGF-β-binding protein in fibroblasts (Kanzaki et al., 1990). The latent TGF-β-binding protein is cleaved as latent TGF-β, and the latent form of TGF-β is activated to TGF-β by extremes of pH and by plasmin (Lawrence et al., 1985; Lyons et al., 1990; Miyazono and Heldin, 1989). Taipale et al. (1995) suggested that chymase could contribute to the release of latent TGF-β from latent TGF-β-binding proteins of the extracellular matrix of human epithelial and endothelial cells. Recently, in cardiomyopathic hamsters, we demonstrated that chymase induced the growth of fibroblasts via the activation of TGF-β and that inhibition of chymase could suppress cardiac fibrosis (Takai et al., 2003).

In idiopathic pulmonary fibrosis in patients and animals, TGF-β is known to be increased (Broekelmann et al., 1991; Zhang et al., 1996). In animal models of bleomycin-induced pulmonary fibrosis, TGF-β may also play an important role in the development of pulmonary fibrosis. For example, both administration of anti-TGF-β antibodies and an antagonist of TGF-β signaling reduced bleomycin-induced pulmonary fibrosis via a reduction in collagen mRNA level (Giri et al., 1993; Nakao et al., 1999). However, it is unclear whether chymase plays an important role in the development of pulmonary fibrosis. Here, we investigated the effect of a specific chymase inhibitor on the development of bleomycin-induced pulmonary fibrosis in hamsters.

2. Methods

2.1. Drugs and animals

A specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-*N*-[{3,4-dioxo-1-phe-

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nyl-7-(2-pyridyloxy)}-2-heptyl]acetamide (NK3201), was synthesized as an orally active chymase inhibitor (Nippon Kayaku, Tokyo, Japan) (Takai et al., 2001). The cDNA probes used were as follows: mouse collagen III cDNA (a 1.8-kb *Eco*RI/*Eco*RI fragment) (Liau et al., 1985) and rat GAPDH (a 1.3-kb *Pst*I/*Pst*I fragment) (Fort et al., 1985).

Male Syrian 6-week-old hamsters were obtained from Japan SLC (Shizuoka, Japan) and were divided into three groups: placebo- and NK3201-treated groups and a control group ($n=6$ for each group). Each hamster was given NK3201 (30 mg/kg per day, oral administration) or placebo, beginning 5 days before the instillation of bleomycin. The bleomycin-induced hamster model of pulmonary fibrosis was established using a modified procedure of Iyer et al. (1999). Under pentobarbital anesthesia (50 mg/kg, i.p.), hamsters were intratracheally instilled with saline or bleomycin (10 mg/kg) in a volume of 0.3 ml/kg. Age-matched hamsters were used as control hamsters. The experimental procedures for animals were conducted in accordance with the guidelines of Osaka Medical College.

2.2. Measurement of enzyme activities

The lung was homogenized in 10 volumes (w/v) of 20 mM Na phosphate buffer, pH 7.4. The homogenate was centrifuged at $20,000 \times g$ for 30 min. The supernatant was discarded, and the pellet was re-suspended and homogenized in 5 volumes (w/v) of 10 mM Na phosphate buffer, pH 7.4, containing 2 M KCl and 0.1% Nonidet P-40. The homogenate was stored overnight at 4 °C, and centrifuged at $20,000 \times g$ for 30 min. The supernatant was used for measurement of chymase activity.

Chymase activity was measured by incubating tissue extracts for 30 min at 37 °C with 4 mM angiotensin I in 150 mM borax-borate buffer, pH 8.5, containing 8 mM dipyridyl, 770 μM diisopropyl phosphorofluoridate, which does not inhibit hamster chymase at this concentration, and 5 mM ethylenediaminetetraacetic acid, as described previously (Takai et al., 1996). The reaction was terminated by addition of 15% trichloroacetic acid, and then the mixture was centrifuged at $20,000 \times g$ for 5 min at 4 °C. One unit of chymase activity was defined as the amount of enzyme that cleaved 1 μmol angiotensin I/min.

Protein concentration was assayed with bicinchoninic acid Protein Assay Reagents (Pierce, Rockford, IL), using bovine serum albumin as a standard.

2.3. Assessment of fibrotic area

After trunk blood collection, the lungs were collected. For the determination of fibrotic area, four transverse slices approximately 2 mm thick were cut from the lung and these slices were fixed in methanol–Carnoy's fixative and embedded in paraffin. Four 5-μm sections were cut from each slice. Each section from the four slices was stained with azan stain and the blue area stained with azan stain was determined as the fibrotic area. The fibrotic area/total ratio area was measured by using a computerized morphometry system, MacSCOPE Ver 2.2 (Mitani, Fukui, Japan).

2.4. RNA isolation and Northern blot hybridization

Total RNA was extracted from the lung, as previously described (Kim et al., 1994). The RNA concentration was spectrophotometrically determined at 260 nm. Twenty micrograms of total RNA from the lung was denatured by incubation with 1 M deionized glyoxal and 50% dimethyl sulfoxide at 50 °C for 1 h, electrophoresed on a 1% agarose gel at 50 V, and transferred to a nylon membrane. Each cDNA probe was labeled with ^{32}P-deoxycytidine 5′ -triphosphate by the random primer extension method with a Random Primer DNA Labeling Kit (Takara Shuzo, Shiga, Japan). To evaluate tissue mRNA levels, we used an optical scanner to digitize the autoradiograms. The density of the autoradiogram bands in the digitized image was measured with the use of the public domain National Institutes of Health Image Program. For all RNA samples, the density of an individual mRNA band was normalized to that of glyceraldehyde phosphate dehydrogenase (GAPDH), to correct for differences in RNA loading and/or transfer.

2.5. Statistical analysis

All numerical data shown in the text are expressed as means ± standard error of the mean (S.E.M.). Significant differences among the mean values of multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by a post-hoc analysis (Fisher's test). $P<0.05$ was used as the threshold for statistically significant differences.

3. Results

The chymase activity in lung extracts from control and placebo-treated hamsters was 1.80 ± 0.36 and 5.02 ± 1.66 mU/mg protein, respectively, and this difference was significant (Fig. 1). In NK3201-treated hamsters, the chymase activity from lung extract was 1.85 ± 0.08 mU/mg protein and this value was significantly lower than the value of placebo-treated hamsters (Fig. 1). The mRNA level of collagen III in placebo-treated hamsters was also significantly reduced by treatment with NK3201 (Fig. 1).

Typical photographs of azan-stained lungs from bleomycin-instilled hamsters treated with placebo or NK3201 and control hamsters are shown in Fig. 2. The ratio of fibrotic area to total area in pulmonary tissues in control hamsters and placebo-treated hamsters was $28.9 \pm 1.21\%$ and

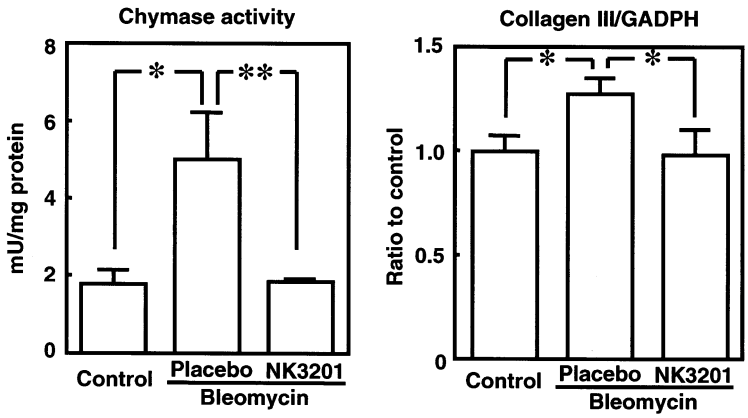

Fig. 1. Effects of a chymase inhibitor, NK3201, on chymase activity (left) and gene expression of collagen III (right) in lung extract from bleomycin-instilled hamsters treated with placebo or NK3201 and control hamsters. Values are means ± S.E.M. ($n = 6$). *$P < 0.05$ and **$P < 0.01$ vs. placebo-treated group.

61.9 ± 1.37%, respectively, and the ratio in placebo-treated hamsters was significantly higher than in control hamsters (Fig. 2). The ratio of fibrotic area to total area in pulmonary tissues from NK3201-treated hamsters was 33.4 ± 0.38%, and this ratio was significantly lower than that in placebo-treated hamsters (Fig. 2).

4. Discussion

Chymase is contained in granules of mast cells, and mast cells are found in increased numbers in the pulmonary tissues of mice with bleomycin-induced pulmonary fibrosis (Goto et al., 1984). A previous report demonstrated that this fibrosis was suppressed by treatment with a mast cell stabilizer (Mori et al., 1991). Therefore, mast cells are thought to contribute to the development of pulmonary fibrosis (Goto et al., 1984; Tomioka et al., 1989). However, mast cells release a large number of inflammatory mediators, such as histamine, serotonin, chemotactic factors, cytokines and serine proteases, during inflammation and repair processes (Sperr et al., 1994; Marone et al., 1995), and it is unclear whether chymase is involved in the development of pulmonary fibrosis. In the present study, both chymase activity and fibrotic area in pulmonary tissues were significantly increased after bleomycin treatment. Treatment with a specific chymase inhibitor, NK3201, significantly decreased not only chymase activity but also the fibrotic area. These findings clearly suggest that an increase in chymase activity may be involved in the development of bleomycin-induced pulmonary fibrosis in hamsters.

In the present study, we used NK3201, which was developed recently as an orally active specific chymase inhibitor (Takai et al., 2001). NK3201 inhibits human, dog and hamster chymases with IC_{50} concentrations of 2.5, 1.2

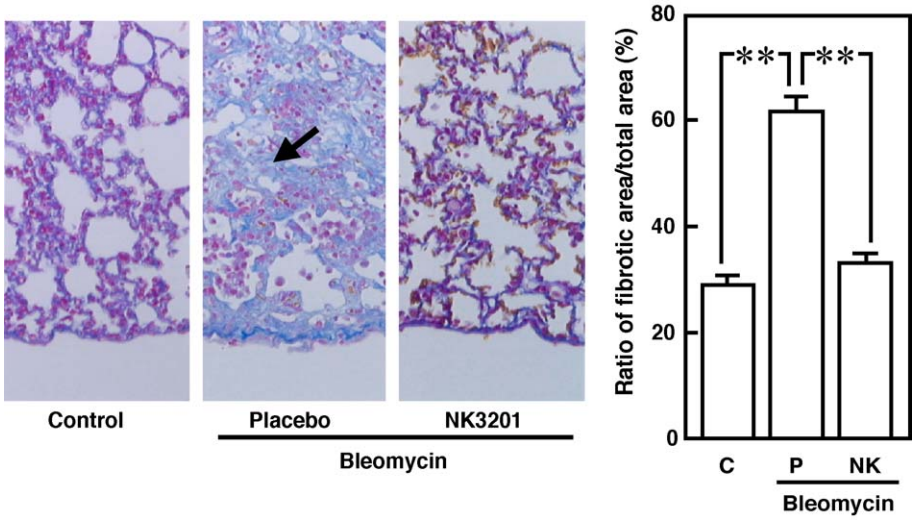

Fig. 2. Effects of a chymase inhibitor, NK3201, on bleomycin-induced pulmonary fibrosis. Typical photographs of azan-stained lungs from bleomycin-instilled hamsters treated with placebo or NK3201 and control hamsters. Arrows show the fibrotic lesions (blue) stained with azan stain. The ratio of fibrotic area to total pulmonary area in the bleomycin-instilled hamsters treated with placebo (P) or NK3201 (NK) and control hamsters (C). Values are means ± S.E.M. ($n = 6$). **$P < 0.01$ vs. placebo-treated group.

and 28 nM, respectively, whereas this inhibitor does not inhibit the activity of other types of serine proteases, tryptase, thrombin, elastase, plasmin and plasminogen activator. In a hamster peritoneal adhesion model, chymase activity was significantly increased at the adhesion site but was significantly reduced by treatment with NK3201, at the same dose (30 mg/kg per day) as that used in the present experiments, along with a reduction in adhesion formation, which is closely related to fibrotic formation (Okamoto et al., 2002b). Furthermore, in this adhesion model, the TGF-β concentrations in the peritoneal fluid were significantly increased, and the increased TGF-β concentrations were reduced by treatment with a chymase inhibitor (Okamoto et al., 2002a). Although we could not determine the effect of NK3201 on TGF-β concentrations in lung extract, we could demonstrate that NK3201 reduced the mRNA level of collagen III, which is induced by TGF-β (Giri et al., 1993). Thus, reduction of TGF-β by chymase inhibition may be involved in its anti-fibrotic action in bleomycin-induced pulmonary fibrosis.

In this study, we demonstrated that increased chymase activity in pulmonary tissues may be related to the development of bleomycin-induced pulmonary fibrosis and that a chymase inhibitor may be useful for the prevention of pulmonary fibrosis.

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