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Release of neutrophil elastase and its role in tissue injury in acute inflammation: effect of the elastase inhibitor, FR134043

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Abstract

Neutrophil elastase degrades extracellular matrix components and is involved in tissue destruction in several inflammatory states. We examined the inhibition of the elastase activity derived from activated neutrophils in vitro and in vivo by FR134043, disodium-(*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo[16.13.6.1^{24,28}.0^{3,8}]octatriconta-3,5,7-trien-5,6-diyl disulfate, an elastase inhibitor with broad specificity, and elucidated the role of neutrophil elastase in pathogenesis of acute inflammation. In a culture of human neutrophils, phorbol myristate acetate (PMA) and calcium ionophore increased elastase activity in the supernatants, which was amplified by co-existing mononuclear leukocytes. Formyl–Met–Leu–Phe stimulated elastase release in the presence of, not without, mononuclear leukocytes. Intratracheal injection of lipopolysaccharide elevated the elastase activity in bronchoalveolar lavage fluid of rats. These elastase activities were significantly inhibited by FR134043. Intratracheal treatment with FR134043 in rats also inhibited the enzyme induced by lipopolysaccharide, though the maximum inhibition was 52%. Ear edema elicited by topical application of PMA in mice was significantly suppressed by pretreatment with FR134043 (38% inhibition at 1 mg/ear). In carrageenan-induced joint injury in rats, plasma extravasation into the synovial cavity was partially and significantly inhibited by FR134043 at 1 mg/knee, while an elastase-specific inhibitor showed no effect. These results suggest that neutrophil elastase is partially involved in tissue damage in acute inflammation provoked by irritants, but not in carrageenan-induced hyperpermeability.

Keywords: Neutrophil elastase; FR134043; Bronchoalveolar lavage fluid; Ear edema; Carrageenan arthritis

1. Introduction

Neutrophil elastase, a granule serine proteinase, is a member of the proteinase family that hydrolytically degrades connective tissue components such as elastin, proteoglycan, fibronectin and collagen types I, II, III, and IV (Havemann and Gramse, 1984). Normally the activity of neutrophil elastase released to extracellular region is strictly regulated by endogenous macromolecular inhibitors, including α1-proteinase inhibitor, α2-macroglobulin and secretory leukoproteinase inhibitor. However, in certain inflammatory states where large numbers of polymorphonuclear leukocytes are infiltrated and activated, enormous amounts of the elastase are released and the endogenous inhibitors are inactivated by neutrophil oxidative products, which results in excessive elastase activity and crucial tissue damage. Such a local elastase–antielastase imbalance has been hypothesized to be involved in the pathogenesis of several acute and chronic inflammatory diseases including pulmonary emphysema (Carp et al., 1982; Janoff, 1985; Cox and Levison, 1988; Gadek and Pacht, 1990), adult respiratory distress syndrome (Lee et al., 1981; McGuire et al., 1982), septic shock (Uchida et al., 1995), cystic fibrosis (O'Conner et al., 1993; Hansen et al., 1995), chronic bronchitis (Llewellyn-Jones et al., 1996), rheumatoid arthritis (Mohr and Wessinghage, 1978; Virca et al., 1984), myocarditis (Lee et al., 1998) and other inflammatory states (Adeyemi et al., 1985; Fric et al., 1985). These findings point to the likely therapeutic advantage of elastase inhibitors in these pathogenic conditions.

We have previously reported the discovery of FR901277, an elastase inhibitor of microbial origin, and its

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biochemical and pharmacological properties (Fujie et al., 1993, 1999). FR901277 is a competitive inhibitor of human neutrophil elastase with a K_i of 12 nM that inhibits chymotrypsin-type serine proteinases as well, additionally the compound attenuates the elastase-induced inflammatory responses in vivo. FR901277 has relatively low solubility in water so that it is difficult to administer the compound in animals. Therefore we modified FR90 1277 to the more water-soluble disulfonated derivative, FR134043, disodium (*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo [16.13.6.1^{24,28}.0^{3,8}]octatriconta-3,5,7-trien-5,6-diyl disulfate (Fig. 1), and confirmed that FR134043 has almost the same biochemical and pharmacological profiles as its original form (Shinguh et al., 1998). These results indicated that FR134043 could be a useful agent for studying the contribution of neutrophil elastase in several pathological conditions. However, both FR901277 and FR134043 have so far been evaluated only in inflammation models induced by administering elastase itself, while the role of the enzyme in certain inflammatory states is still controversial.

In the present study, the release of elastase activity from human neutrophils stimulated by various irritants and the inhibitory effect of FR134043 on elastase were examined. Bacterial lipopolysaccharide has been reported to cause airway hypersensitivity and inflammatory responses in the bronchopulmonary region (Nagai et al., 1991). Therefore, we attempted to measure directly the elastase activity induced by lipopolysaccharide in the bronchoalveolar lavage fluid of rats. We then evaluated the inhibitory effects of FR134043 on two well-studied acute inflammation models in rodents, phorbol myristate acetate (PMA)-induced ear edema and carrageenan-induced plasma extravasation in the knee joint. Another inhibitor, FK706, sodium 2-[4-[[(*S*)-1-[[(*S*)-2-[[(*RS*)-3,3,3-trifluoro-1-isopropyl-2-oxopropyl]aminocarbonyl]pyrrolidin-1-yl]carbonyl]-2-methylpropyl]aminocarbonyl]benzoylamino]acetate, is an elastase-specific inhibitor (Shinguh et al., 1997). FK706 was also evaluated in these inflammation models and compared with FR134043 regarding their inhibitory potencies in order to determine whether neutrophil elastase is involved in these inflammatory states.

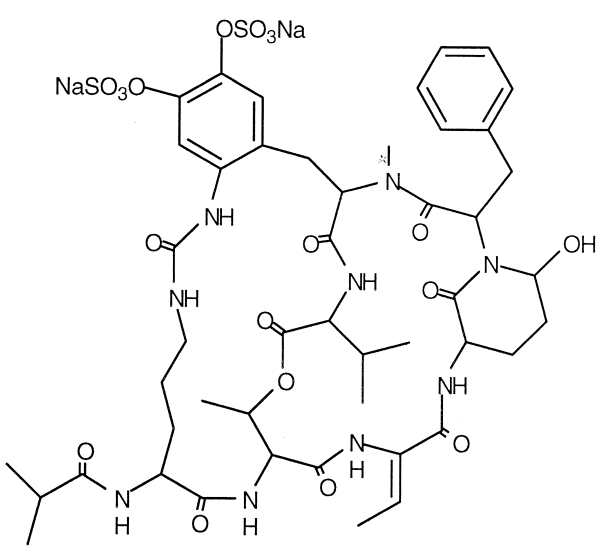

Fig. 1. Chemical structure of FR134043, a water-soluble derivative of FR901277, disodium (*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo[16.13.6.1^{24,28}.0^{3,8}]octatriconta-3,5,7-trien-5,6-diyl disulfate, $C_{47}H_{61}N_9Na_2O_{19}S_2$.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from commercial sources: lipopolysaccharide (from *Escherichia coli* Serotype O127:B8), carrageenan (Type IV), PMA, formyl–Met–Leu–Phe (FMLP) (Sigma, St. Louis, MO, USA), A23187 (Calbiochem, La Jolla, CA, USA), human sputum elastase (EC 3.4.21.37) (Elastin Products, Owensville, MO, USA), Hank's balanced salt solution (HBSS) (Nissui Pharmaceutical, Tokyo, Japan), S-2484 (L-pyroglutamyl-prolyl-valyl-*p*-nitroanilide) (Chromogenix, Molndal, Sweden). FR134043 was isolated and FK706 was synthesized in our laboratory. Other chemicals used in the present experiments were of reagent grade.

2.2. Animals

Male Sprague–Dawley rats (6 weeks old), male Wistar rats (8 weeks old) and female BDF1 mice (8 weeks old) were obtained from Japan SLC (Shizuoka, Japan), and allowed free access to standard pellet diet and water under controlled conditions. All animal experiments in the present study were conformed to the guidelines for animal experiments at Fujisawa Pharmaceutical.

2.3. Cell separation and culture of human neutrophils

Heparinized venous blood was obtained from healthy male volunteers. Mononuclear cells (monocytes/lymphocytes) were isolated by centrifugation on Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) density gradient. Polymorphonuclear neutrophils were separated by dextran sedimentation from the pellet after isolation of mononuclear cells. Contaminating erythrocytes were removed by hypotonic lysis. Both cell types were washed twice and resuspended in HBSS, then dispensed into 24-well tissue culture plates (Corning Costar, Corning, NY, USA) in a final volume of 1 ml. Neutrophils (2×10^6 cells/ml) were preincubated for 30 min at 37°C in CO_2 incubator in the

presence or the absence of mononuclear cells (1×10^6 cells/ml). Various stimuli were then added and the incubation was continued. After 2 h of incubation with stimuli, the culture supernatants were collected and centrifuged. These cell-free supernatants were stored at −20°C for testing elastase activity.

2.4. Collection of bronchoalveolar lavage fluid from lipopolysaccharide-treated animals

Male Sprague–Dawley rats were anesthetized by intraperitoneal injection of 40 mg/kg of pentobarbital. The trachea was exposed in the ventral region, then 1 ml/kg of saline or lipopolysaccharide (10 mg/kg) dissolved in saline was instilled intratracheally through a small diameter tube inserted earlier. For ex vivo studies of elastase release, a saline solution of drug was administered intratracheally in a volume of 1 ml/kg, 5 min before and 3.5 h after lipopolysaccharide treatment. Four hours after lipopolysaccharide instillation, the rats were killed by CO_2 asphyxiation and the lungs were lavaged with a single 7-ml volume of saline by infusing and withdrawing 10 times, yielding a final volume of 5 ml of bronchoalveolar lavage fluid.

2.5. Assay of neutrophil elastase activity

The activity of neutrophil elastase was measured according to a method previously reported (Tanaka et al., 1990), with some modifications. Briefly, 200 μl of sample was mixed with 200 μl of reaction buffer (0.1 M Tris containing 0.96 M NaCl, pH 8.3), then the reaction was started by the addition of 200 μl of 2 mM S-2484 (a synthetic substrate for neutrophil elastase, dissolved in 25% dimethyl sulfoxide). In the case of the supernatants of human neutrophils, the reaction was carried out at room temperature and cleavage of the substrate was continuously monitored spectrophotometrically at 410 nm. To examine the inhibitory activity of FR134043, the reaction was performed in 96-well plates (Sumitomo Bakelite, Tokyo, Japan) by scaling down the volume, and the optical density at 405 nm was measured after incubation for 60 min using a microplate reader. For measurement of the elastase activity in bronchoalveolar lavage fluid from rats, the reaction mixture was incubated at 37°C for 3 h and, at the end of the reaction, 500 μl each of 5.8 mM sodium nitrite (dissolved in 100 mM HCl), 26.3 mM ammonium sulfamide and 2.7 mM *N*-1-naphtyl ethylenediamine dihydrochloride were added to diazotize the *p*-nitroaniline. The amount of diazo dye yielded was assayed from the optical density at 545 nm. Elastase activity was calculated from a standard curve obtained with commercially available standard elastase (human sputum elastase, 875 units/mg protein) and expressed as U/l. Human sputum elastase was used as human neutrophil elastase without further purification (Skiles et al., 1984; Green et al., 1991).

2.6. PMA-induced ear edema in mice

The ability of elastase inhibitors to prevent PMA-induced ear edema was evaluated in female BDF1 mice. Drugs were dissolved in acetone–ethanol (1:1) and applied in a volume of 20 μl to both the inner and outer surfaces of the right ear of mice (total 40 μl/ear). After 10 min, 2 μg/ear of PMA dissolved in 40 μl of acetone–ethanol (1:1) was similarly applied to the right ear. The left ear was treated with the same volume of vehicle. The mice were killed by CO_2 asphyxiation 6 h after PMA treatment, and a circular tissue specimen was cut from the middle part of each ear with a 6-mm diameter punch and weighed. Ear edema was expressed as the difference in weight between the right and the left ear. The dose required to get 50% inhibition compared to the PMA control was determined as ED_{50}.

2.7. Carrageenan-induced joint inflammation in rats

The effects of elastase inhibitors on carrageenan-induced joint injury were examined by measuring the exuded dye in the cartilage extracts according to the method previously described (Lam and Ferrel, 1989; Hirayama et al., 1993), with some modification. Briefly, male Wistar rats were anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital. Drugs were suspended in 0.1 (w/v)% methylcellulose and injected into the synovial cavity of the right knee in a volume of 50 μl/knee, while the left knee received the same volume of 0.1% methylcellulose. Evans blue, 20 mg/kg, dissolved in saline was administered intravenously just before carrageenan. Thereafter, 15 min after the drug injection, 50 μl/knee of 2 (w/v)% carrageenan was injected into the synovial cavity of both knees. The animals were killed 4 h after carrageenan treatment, then the whole knee joint capsules (about 1.5 cm length) of both sides without skin were dissected. Evans blue in these samples was extracted with 3 ml of 1% Na_2SO_4–acetone mixture (3:7) for 24 h at room temperature. Each preparation was centrifuged and the optical density of the supernatant was measured at 620 nm. The amount of exuded dye was calculated by comparing the optical density with that of a standard curve prepared with known concentrations of Evans blue, and the difference in dye amount between right and left knees was taken as the effect of drugs.

2.8. Statistic analysis

All results were expressed as the means ± S.E.M. In vitro IC_{50} and in vivo ED_{50} values were calculated by using a non-weighted least-square fit of the data. Statistical significance of differences was determined by Student's *t*-test with significance at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Release of elastase activity from human neutrophils and its inhibition by FR134043

Human neutrophils stimulated by various irritants were investigated for their ability to release elastase activity, using a synthetic substrate, S-2484 (Fig. 2). Lipopolysaccharide (100 ng/ml, final concentration) did not enhance the elastase release from human neutrophils under the present experimental conditions. However, neutrophils exposed to PMA (20 nM) or A23187 (1 μM) for 2 h released a marked amount of elastase activity into the culture supernatants: 181.3 ± 8.2 and 243.4 ± 16.0 U/l, respectively (means ± S.E.M., $n = 4$). These increases in elastase activity caused by PMA and A23187 were amplified significantly when neutrophils were cultured with mononuclear cells: 258.6 ± 6.8 U/l ($P < 0.001$ vs. without mononuclear cells, $n = 4$) and 670.6 ± 26.4 U/l ($P < 0.001$ vs. without mononuclear cells, $n = 4$), respectively. FMLP (0.1 μM) had no effect on the elastase activity when added to neutrophils alone, but in the presence of mononuclear cells FMLP increased elastase release significantly: 93.8 ± 5.0 U/l without mononuclear cells vs. 121.7 ± 3.2 U/l with mononuclear cells ($P < 0.01$, $n = 4$).

The inhibitory activity of FR134043 against elastase released from human neutrophils was studied. Fig. 3 shows the concentration–inhibition relationships of FR134043, using standard human neutrophil elastase (289 U/l, final concentration) and the culture supernatants of neutrophils stimulated for 2 h with PMA or A23187 in the presence of mononuclear cells as elastase preparations. These three inhibition curves almost completely overlapped with IC_{50} values of 2.2–2.7 nM, and nearly 100% inhibition was obtained at 100 nM and higher concentrations of FR134043. This result suggests that the degrading activity against S-2484 in the supernatants of co-culture of human neutrophils and mononuclear cells was mostly due to neutrophil elastase.

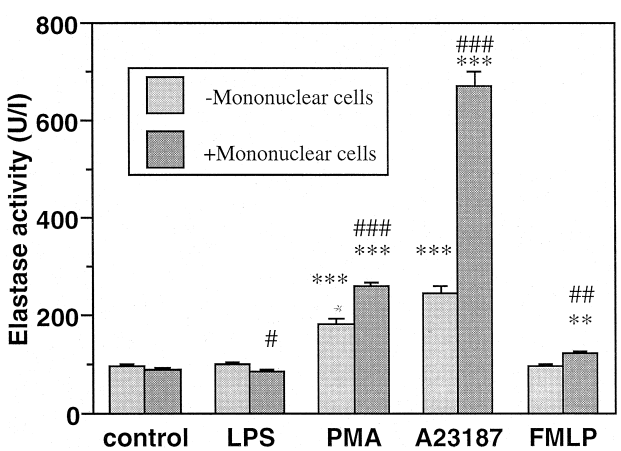

Fig. 2. Elastase release in the culture supernatants of human neutrophils in response to several stimuli. Neutrophils (2×10^6 cells/ml) were incubated for 2 h at 37°C with lipopolysaccharide (LPS) (100 ng/ml), phorbol myristate acetate (PMA) (20 nM), A23187 (1 μM) or formyl–Met–Leu–Phe (FMLP) (0.1 μM), in the absence or presence of mononuclear cells (1×10^6 cells/ml). Elastase activity was assessed by monitoring the degradation of S-2484 and shown as U/l compared with the activity of standard human neutrophil elastase (875 units/mg). Results are expressed as means ± S.E.M. for four experiments. Significant differences are: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control (without or with mononuclear cells). Additionally, $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ vs. corresponding neutrophils cultured without mononuclear cells.

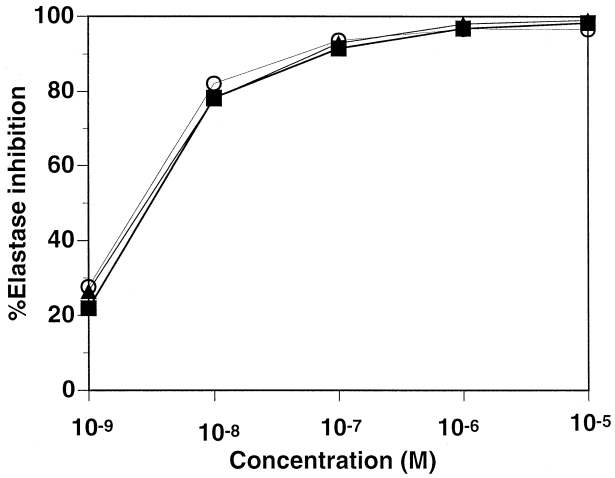

Fig. 3. Inhibitory effects of FR134043 on the elastase activity released from human neutrophils. Neutrophils (2×10^6 cells/ml) were mixed with mononuclear cells (1×10^6 cells/ml) and stimulated with PMA (20 nM) or A23187 (1 μM) for 2 h at 37°C. The cell-free supernatants from PMA- (■) or A23187- (▲) treated cultures, and standard human neutrophil elastase (0.33 μg/ml, ○) were investigated for their ability to degrade S-2484 in presence of various concentrations of FR134043. Elastase activity in the absence of FR134043 was defined as 0% inhibition. Results are means of two experiments.

3.2. FR134043 and FK706 inhibited the elastase activity in bronchoalveolar lavage fluid from lipopolysaccharide-treated rats

Lipopolysaccharide-treated animals have been reported to show elevated local elastase activity. We tested the ability of lipopolysaccharide to release elastase activity in rats and observed that intratracheal instillation of lipopolysaccharide provoked an evident elastase release into bronchoalveolar lavage fluid after 4 h of exposure: 1.30 ± 0.05

Table 1
Inhibitory effects of FR134043 and FK706 on elastase activity induced by lipopolysaccharide in rat bronchoalveolar lavage fluid

Drugs	IC_{50}s (nM)	
	Rat BALF	Standard HNE
FR134043	2000	2.8
FK706	5900	17.9

Bronchoalveolar lavage fluid (BALF) from lipopolysaccharide-treated rats and standard human neutrophil elastase (HNE) were studied for the effects of elastase inhibitors. Drugs dissolved in reaction buffer, elastase preparation and 2 mM S-2484 were mixed and incubated for 3 h at 37°C, then the optical density at 545 nm was measured after diazotization.

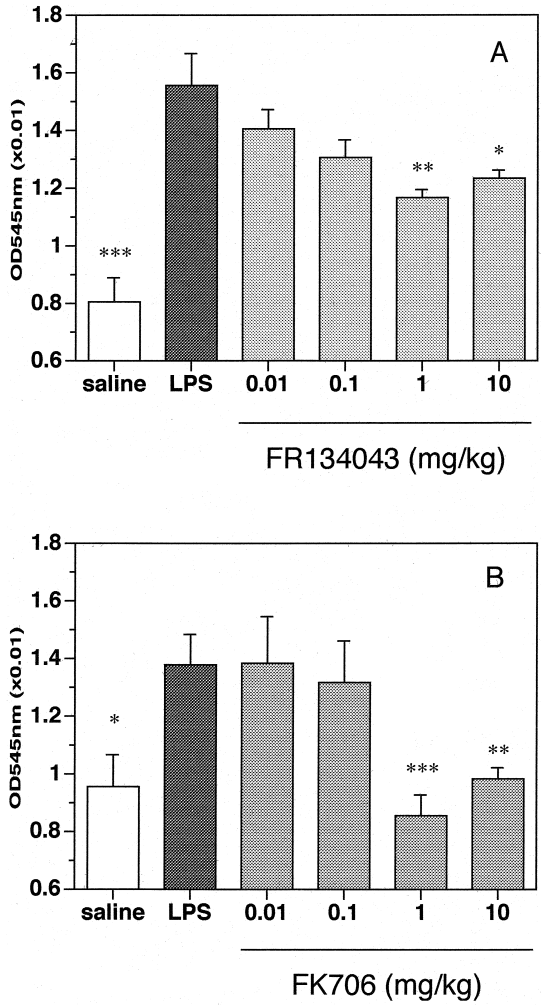

Fig. 4. Ex vivo study for the effects of FR134043 (A) and FK706 (B) on elastase activity in lipopolysaccharide-treated rat bronchoalveolar lavage fluid. lipopolysaccharide (10 mg/kg) was instilled in rat trachea, and the drugs were administered intratracheally 0.5 h before and 3.5 h after lipopolysaccharide instillation. Four hours after lipopolysaccharide treatment, bronchoalveolar lavage fluid was collected and elastase activity was measured by diazotization method. Results are expressed as OD at 545 nm (means ± S.E.M. for 10 animals). The control elastase activities calculated from the standard curve were: 0.85 ± 0.05 (A) and 0.94 ± 0.08 U/l (B) in saline-treated rats vs. 1.34 ± 0.07 (A) and 1.22 ± 0.07 U/l (B) in lipopolysaccharide-treated rats. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. lipopolysaccharide.

U/l in lipopolysaccharide-treated rats vs. 0.81 ± 0.07 U/l in saline-treated controls ($P < 0.001$, $n = 5$). Table 1 indicates that FR134043 and FK706 inhibited the elastase activity in bronchoalveolar lavage fluid in vitro with IC_{50} values of 2.0 and 5.9 mM, respectively. These IC_{50} values were 700- and 300-fold greater than those obtained with standard human neutrophil elastase, respectively, although higher concentrations of the inhibitors of the order of 100 μM could show more than 90% inhibition against elastase in bronchoalveolar lavage fluid (data not shown).

Effects of intratracheally administered FR134043 and FK706 on lipopolysaccharide-induced increase of elastase activity in bronchoalveolar lavage fluid were also examined (Fig. 4). FR134043 reduced the elastase activity in a dose-dependent manner up to 1 mg/kg, but the dose of 10 mg/kg produced no greater inhibition than did 1 mg/kg (52.0% inhibition). On the other hand, FK706 inhibited the increase of elastase activity completely at the dose of 1 mg/kg, with an ED_{50} value of 0.21 mg/kg.

3.3. FR134043 and FK706 attenuated PMA-induced ear edema in mice

In order to examine the involvement of neutrophil elastase in an acute inflammation model, we evaluated the ability of elastase inhibitors to suppress ear edema in mice (Fig. 5). PMA application on the ear surface elicited marked swelling, which was indicated by the increase in dissected tissue weight (5.44 ± 0.61 mg in PMA control, $n = 10$). Pretreatment with FR134043 10 min before PMA application had no effect on ear swelling at doses of 0.01 and 0.1 mg/ear, while FR134043 inhibited the edema significantly at 1 mg/ear (38.4% inhibition, $P < 0.05$ vs. PMA control). FK706 similarly inhibited the ear swelling at 1 mg/ear, but the potency of attenuation was greater than that of FR134043 (60.7% inhibition, $P < 0.01$ vs. PMA control). The ED_{50} values were > 1.0 and 0.73 mg/ear, respectively.

3.4. Carrageenan-induced joint inflammation in rats was improved by FR134043 but not by FK706

Another acute inflammation model, plasma extravasation in the rat knee joint induced by carrageenan was

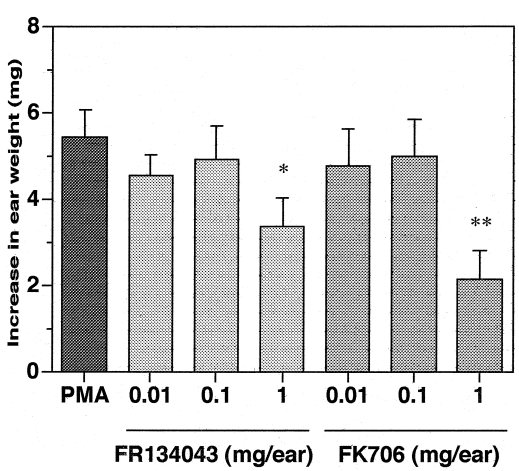

Fig. 5. Effects of FR134043 and FK706 on PMA-induced ear edema in mice. The drugs dissolved in acetone–ethanol (1:1) were applied on the right ear 10 min before PMA treatment (2 μg/site). Circular specimens (6 mm diameter) of both ears were obtained and weighed 6 h after PMA application. Results are expressed as the difference in weight between right and left ears (means ± S.E.M. for 10 animals). The control right and left ear weights were 11.9 ± 0.6 and 6.5 ± 0.3 mg, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. PMA.

Table 2
Effects of elastase inhibitors on intra-articular dye exudation induced by carrageenan

Drugs	Dose (μg/knee)	Knee	Dye leakage (μg)	% Inhibition
Negative control			(4.6 ± 0.6)	
Carrageenan control		R	19.8 ± 1.5	5.7
		L	21.0 ± 0.9	
FR134043	100	R	20.1 ± 2.0	−12.3
		L	17.9 ± 1.2	
	1000	R	15.1 ± 0.9[a]	25.6
		L	20.3 ± 0.5	
FK706	100	R	26.9 ± 3.8	−14.0
		L	23.6 ± 3.5	
	1000	R	32.0 ± 3.6	−23.6
		L	25.9 ± 2.4	

Carrageenan (1 mg/knee) was injected into the synovial cavity of both knee joints in male Wistar rats after topical pretreatment with drugs. Four hours after carrageenan injection, the animals were killed and knee joint capsules were dissected to extract the exuded dye. Amount of the dye leakage in intra-articular region was expressed as substraction of basal leakage in negative control (treated with saline and 0.1% methylcellulose) from total amount of dye leakage. The results are means ± S.E.M. for 5–6 animals. Inhibition rate was calculated by comparing the dye amount of right joint (R) to that of left (L). [a] $P < 0.01$ vs. left joint.

employed to study the role of elastase. Table 2 indicates the amounts of Evans blue dye exuded into the intra-articular region of right (drug-treated) and left (0.1% methylcellulose-treated) knees, both of which were injected with 1 mg carrageenan. Approximately 20 μg of dye was recovered in the extract of left cartilage after 4 h stimulation with carrageenan. The amount of dye was significantly reduced by 1000 μg/knee injection of FR134043 into the right joint ($P < 0.01$), but not by 100 μg/knee. FK706, however, had no suppressive effect on the plasma extravasation in the carrageenan-treated rat joint, but tended to facilitate it.

4. Discussion

Neutrophils are activated and release superoxide, cytokines and chemokines, and tissue destructive proteinases including elastase in response to various stimuli in acute and chronic inflammatory states. Not only neutrophils but also mononuclear cells migrate to the location of the actual inflammation, and activated, producing a complicated interaction. Therefore, the effect of co-existing mononuclear cells on the elastase release from neutrophils is worth examining. As shown in Fig. 2, phorbol ester PMA and calcium-ionophore, A23187, promoted the elastase activity in the culture supernatant of human neutrophils, which was amplified in the presence of mononuclear cells. These results suggest that those stimuli directly affect both cell types by activating protein kinase C or elevating intracellular calcium, respectively, and that some factors facilitating neutrophil activation are secreted by mononuclear cells. Tumor necrosis factor-α (TNF-α) derived from mononuclear cells is a major candidate for such a factor, since it has been reported that TNF-α stimulates neutrophil function via p38 mitogen-activated protein kinase activation followed by nuclear translocation of nuclear factor-κB (NF-κB) (McDonald et al., 1997; Zu et al., 1998). No detectable amount of elastase was observed with the neutrophils stimulated by chemoattractant FMLP, but elastase activity was significantly increased in the presence of mononuclear cells. Based on the previous report that FMLP can induce elastase release in lipopolysaccharide-primed neutrophils (Ottonello et al., 1997), co-existence of mononuclear cells is thought to have a priming effect against neutrophils. Lipopolysaccharide had no effect on elastase release regardless of the presence or absence of mononuclear cells. It has been reported that lipopolysaccharide inhalation by guinea pigs increases elastase activity in bronchoalveolar lavage fluid 2 h after exposure (Nagai et al., 1991). Nevertheless, in the present in vitro study, elastase activity was not enhanced even by 24 h incubation of neutrophils with lipopolysaccharide (data not shown). This result suggests that lipopolysaccharide has only a priming effect on neutrophils concerning release of elastase, which agrees with results of the study indicating neutrophil priming by lipopolysaccharide for enhanced superoxide release (DeLeo et al., 1998). Lipopolysaccharide-stimulated mononuclear cells are thought to produce various factors, but their amounts might be insufficient for enhanced elastase exocytosis in the primed, not activated, neutrophils.

S-2484 is a granulocyte elastase-specific synthetic substrate with high sensitivity and supposed to be suitable for detecting elastase activity in biological materials (Kramps et al., 1983; Tanaka et al., 1990). In our study, we focused on neutrophil elastase, a serineproteinase, and used S-2484 for the analysis of elastase activity in order to ignore the activity of macrophage elastase, a metalloproteinase. The degrading activity against S-2484, released from co-culture of neutrophils and mononuclear cells stimulated by PMA or A23187, was fully inhibited by a neutrophil elastase inhibitor, FR134043, with a potency similar to that of standard human neutrophil elastase (Fig. 3). This result also demonstrates that the degrading activity observed in the culture supernatants in the present study mostly originated from neutrophil elastase, and that inhibitors like FR134043 have inhibitory activity against elastase even in crude biological materials. We also tested the activity of the neutrophil supernatants to degrade a natural substrate, elastin, using a method previously described (Shinguh et al., 1997). However, no degradation was observed, probably due to low sensitivity of elastin against elastase (data not shown).

Lipopolysaccharide, intratracheally instilled in rats, provoked an increased elastase release in bronchoalveolar lavage fluid 4 h after exposure. This result, compared with those obtained in vitro, suggests that pulmonary compo-

nents other than neutrophils and mononuclear cells may influence and increase elastase release in lipopolysaccharide-induced lung injury. The elastase activity was inhibited by FR134043 and FK706, another elastase inhibitor, though the IC_{50} values of each inhibitor were 700- and 300-fold greater, respectively, than those found against standard human neutrophil elastase. These greater IC_{50}s may result from: (1) inactivation of these drugs in the reaction mixture including bronchoalveolar lavage fluid; (2) degradation of the substrate, S-2484, by some proteinases other than elastase. Since both drugs exerted more than 90% inhibition at higher concentrations and the activity of neutrophil elastase was thought to be measured specifically under the present experimental conditions as stated above, the former possibility is probably applicable. In an ex vivo study, intratracheal administration of FR134043 reduced the elastase activity in rat bronchoalveolar lavage fluid significantly but only partially (approximately 50% maximum), while FK706 inhibited it completely at 1 mg/kg. This observation suggests that FR134043 is more sensitive to inactivating attack by the components of bronchoalveolar lavage fluid (oxidative compounds or metabolic enzymes, probably) than FK706. Additionally, the difference in the mode of inhibition might cause this result; FR134043 is a reversible and competitive inhibitor while FK706 is a slow binding inhibitor. It has been reported that S-2484 hydrolytic activity in bronchoalveolar lavage fluid from human patients with adult respiratory distress syndrome or pneumonia, is moderately reduced by EDTA or *o*-phenanthroline as well as α1-proteinase inhibitor (Tanaka et al., 1990). Therefore, the contribution of other proteinases, especially metalloproteinases, to the currently observed elastase activity is not excluded.

We evaluated the ability of elastase inhibitors to ameliorate inflammatory states in two acute inflammation animal models, both of which reflect the elevated vascular permeability based on the tissue damage following the activation of peripheral blood cells. In PMA-induced ear edema, topical treatment with FR134043 and FK706 had significant and dose-dependent sparing effects on ear swelling with ED_{50} values of >1 and 0.73 mg/site, respectively. Treatment with the drugs at doses higher than 1 mg/ear was impossible due to the limited solubility in acetone–ethanol and detachment from the application site, resulting in a substantially lower dose. PMA directly activates protein kinase C, which is thought to be involved in various neutrophil responses such as superoxide generation by NADPH oxidase (Castagna et al., 1982), release of granule enzymes (White et al., 1984) and activation of Na^+/H^+ antiporter (Grinstein and Furuya, 1986). Other investigators have reported that PMA-induced ear edema is mainly mediated by leukotrienes and prostaglandins, and 5-lipoxygenase inhibitors can strongly attenuate the edema (Carlson et al., 1985; Yamazaki et al., 1998). Our present results suggest that neutrophil elastase may play a partial role in ear swelling in this model, but possibility of a contribution of other factors such as leukotrienes and prostaglandins is not negligible.

The effect of elastase inhibitors on carrageenan-induced inflammation was also evaluated by measuring plasma extravasation into the synovial cavity of the knee. It has been reported that this acute arthritis model involves both the neurogenic components and other non-neurogenic mediators of the inflammatory response, as seen in a the study using a denervation procedure and an antagonist of substance P (Lam and Ferrel, 1989). Carrageenan is, additionally, known to strongly recruit inflammatory cells, especially neutrophils. This evidence increases the interest in the role of neutrophil-derived proteolytic enzymes in the joint inflammation caused by carrageenan. In our experiments, plasma extravasation in the knee joint was weakly but significantly reduced by the treatment with FR134043, however, it was not affected by FK706. FR134043 inhibits chymotrypsin-type serine proteinases (Shinguh et al., 1998), while FK706 is an elastase-specific inhibitor (Shinguh et al., 1997). Therefore, it is hypothesized that neutrophil elastase is little involved in carrageenan-induced joint inflammation, but some other serine proteinases are possibly involved.

Neutrophil elastase elicits the tissue-destructive activity directly or indirectly. Matrix proteins such as elastin, collagen and proteoglycan are readily degraded by neutrophil elastase. Neutrophil elastase has also been shown to activate matrix metalloproteinases (MMP) (Nagase et al., 1990; Ferry et al., 1997), and inactivate the endogenous inhibitor, tissue inhibitor of metalloproteinases (TIMP), resulting in an imbalance between MMP and TIMP (Itoh and Nagase, 1995). Some investigators have reported that the elastolytic activity in synovial fluids from patients with osteoarthritis or rheumatoid arthritis is mainly due to metalloproteinases (Chevalier et al., 1996). Others have suggested that neutrophils elicit local release of cathepsin G and elastase in synovial fluid of patients with reactive and rheumatoid arthritis (Nordstrom et al., 1996), and that those proteinases are potent degraders of cartilage proteoglycan (Janusz and Doherty, 1991). Though some of these observations regard subjects with chronic joint inflammation, they are suggestive of acute inflammatory states. Based on the above evidence, the reduction of plasma extravasation by FR134043 in the present study was likely to have been caused by inhibition of neutrophil-derived cathepsin G, since FR134043 has no inhibitory effect on metalloproteinases, including human collagenase (MMP-1), even at 10 μM in vitro (data not shown). It has been indicated that the inhibitory effect of an elastase inhibitor, MDL 101,146, on cartilage degradation depends on the choice of animal model, that is, the inhibitor is effective on collagen-induced arthritis but not on adjuvant arthritis (Janusz and Durham, 1997). The joint inflammation model used in the present study does not seem to involve neutrophil elastase, however, it shows the advantage of

FR134043, an inhibitor with broad specificity, for the treatment of cartilage destruction.

In summary, neutrophils activated in response to certain stimuli release significant amount of elastase, a granule proteolytic enzyme, both in vitro and in vivo. This phenomenon is affected by mononuclear cells and/or other co-existing components, suggesting the construction of an inflammatory network. FR134043 is a potent neutrophil elastase inhibitor, and almost fully inhibits the neutrophil-derived elastase activity in crude biological materials. Judged by this result, FR134043 is a useful agent for studying the pathogenic role of neutrophil elastase in inflammatory disorders. The significant attenuation of PMA-induced ear edema by FR134043 and FK706 suggests the partial involvement of neutrophil elastase in the elevated vascular permeability in this model. The observation that carrageenan-induced plasma extravasation is barely ameliorated by FR134043 and not at all by FK706, indicates the very small contribution of neutrophil elastase and possible involvement of neutrophil cathepsin G in the acute arthritis model.

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