

Potency of Truncated Secretory Leukoprotease Inhibitor Assessed in Acute Lung Injury Models in Hamsters

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ABSTRACT

We evaluated the potency of truncated secretory leukoprotease inhibitor (truncated SLPI) in a human sputum elastase (HSE)-induced lung injury model and in a specific neutrophil-mediated acute lung injury model in hamsters. Intratracheal administration of HSE induced acute lung hemorrhage that could be measured by determination of the hemoglobin content in the bronchoalveolar lavage fluid. Intratracheal administration of truncated SLPI 1 hr before HSE administration inhibited acute lung hemorrhage in a dose-dependent manner ($ED_{50} = 46.8 \mu\text{g}/\text{kg}$), as did i.v. injection of the inhibitor given 2 min before HSE administration ($ED_{50} = 14.7 \text{ mg}/\text{kg}$). Intratracheal admin-

istration of endotoxin (lipopolysaccharide) induced pulmonary neutrophilia. Twenty-four hours after lipopolysaccharide administration, the addition of formyl-methionyl-leucyl-phenylalanine resulted in a neutrophil-dependent acute lung injury that expressed an increase in hemoglobin content and in elastase-like activity in bronchoalveolar lavage fluids. In this model, lung injury was significantly attenuated by i.v. and intratracheal administration of truncated SLPI. These results suggest that truncated SLPI appears to be a good candidate inhibitor for the treatment of destructive lung diseases due to neutrophils.

It has been suggested that neutrophils play a role in the pathogenesis of lung injury in many chronic respiratory diseases such as chronic bronchitis, diffuse panbronchiolitis, emphysema and cystic fibrosis (McCusker and Hoidal, 1988; Fick and Reynolds, 1983; Mikami, 1991). However, the mechanism by which neutrophils promote lung injury remains incompletely understood.

Elastase is a neutral serine protease that occurs in the azurophilic granules of the neutrophil. This enzyme can digest many components of the extracellular matrix, including elastin, collagen types I, III, and IV, fibronectin and proteoglycans, and it has been suggested to play important roles in the metabolism of connective tissue and in the resolution of foreign substances. It is likely that excessive release of elastase, accompanying neutrophil lysis and phagocytosis, induces many pathological events and results in functional disorders as a result of the broad specificity and proteolytic intensity of this enzyme. Indeed, it has been reported that neutrophil elastase is significantly increased in sputum and bronchoalveolar lavage fluids of patients with various chronic respiratory diseases (Mikami, 1991; Piccioni *et al.*, 1992; Stockley, 1987; Goldstein and Döring, 1986). Therefore, attention has been focused on neutrophil elastase in lung

injury. However, because neutrophils release a number of other toxic factors, such as lipid metabolites, oxidants and proteases, the participation of neutrophil elastase in the lung injury promoted by neutrophils remains unclear.

SLPI is a 11.7-kDa serine protease inhibitor of 107 amino acids. It inhibits a wide range of proteases, including neutrophil elastase, cathepsin G, chymotrypsin and trypsin (Kramps *et al.*, 1981). It has been localized in the granules of serous cells of bronchial glands (Kramps *et al.*, 1981) and in Clara cells and goblet cells of the bronchial epithelium (Mooren *et al.*, 1982; Kramps *et al.*, 1987). SLPI consists of two domains. Recently, Barbara *et al.* (1990) indicated that domain 2 (C-terminal domain) contains neutrophil elastase, chymotrypsin and trypsin inhibitory sites and suggested that domain 1 (N-terminal domain) might also be necessary to express trypsin inhibitory activity. Therefore, Masuda *et al.* developed truncated SLPIs, corresponding to the C-terminal domain (Asn⁵⁵-Ala¹⁰⁷, Arg⁵⁸-Ala¹⁰⁷, Arg⁵⁹-Ala¹⁰⁷), by recombinant DNA technology. As they expected, truncated SLPI was shown to be more specific for elastase than for trypsin, compared with native SLPI (Masuda *et al.*, 1992; Masuda *et al.*, 1994). We proposed that these truncated SLPIs might be promising as therapeutics for the treatment of lung diseases mediated by neutrophils.

Accordingly, we undertook this study to evaluate the po-

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ABBREVIATIONS: truncated SLPI, truncated secretory leukoprotease inhibitor; HSE, human sputum elastase; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; FMLP, formyl-methionyl-leucyl-phenylalanine; CMK, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethyl-ketone; SOD, superoxide dismutase; DFP, diisopropyl fluorophosphate; EDTA, disodium ethylenediaminetetraacetate; α 1-PI, α 1-protease inhibitor; PBS, phosphate-buffered saline.

tency of truncated SLPI, an inhibitor of serine proteases, in acute lung injury models in hamsters and to investigate the role of neutrophil proteases in lung injury. We showed that the combination of LPS and FMLP was capable of making a specific neutrophil-mediated lung injury model and that this lung injury was mediated mainly by neutrophil proteases.

Materials and Methods

Materials. The reagents used in this study and their sources were the following: FMLP, catalase, α 1-PI, indomethacin and dexamethasone (Sigma Chemical Co., St. Louis, MO), CMK (Bachem Feinchemikalien AG, Budendorf, Switzerland), LPS (*E. coli* 004) (Difco Laboratories, Detroit, MI) and SOD (Wako Chemical Co., Osaka, Japan). FMLP was dissolved in dimethylsulfoxide and diluted to the appropriate concentration in saline.

Exogenous elastase-induced acute lung injury model in hamsters. Male Golden Syrian hamsters, 8 to 10 weeks old, were anesthetized with halothane and administered HSE (50 μ g/animal) intratracheally and transorally. Three hours after HSE administration, BAL was performed, according to the method of Barry *et al.* (1990). Briefly, hamsters anesthetized with urethane (1 g/kg intraperitoneally). Then they were intubated endotracheally, and BAL was performed 3 times by the rewash lavage method (6 ml of PBS). As a marker of lung injury, the hemoglobin content of the BAL fluid was determined by the cyanmethemoglobin method (Matsubara and Shibata, 1969). Inhibition of HSE-induced lung hemorrhage was determined by dosing the hamster with truncated SLPI either i.v. 2 min before HSE treatment or intratracheally 1 hr before HSE treatment.

Combination of LPS and FMLP-induced acute lung injury model in hamsters. Hamsters anesthetized with halothane were administered LPS (0.42 mg/kg) intratracheally, and this treatment was followed 24 hr later by intratracheal administration of FMLP (0.25 mg/kg). Ninety minutes after FMLP administration, BAL was performed. Inhibition of lung hemorrhage was determined by dosing the hamster with truncated SLPI either i.v. 2 min before FMLP treatment or intratracheally at the same time as FMLP treatment.

Administration of drugs. In LPS and FMLP-induced lung injury, we investigated the effects of several drugs. CMK, a specific elastase inhibitor, and human plasma-derived α 1-protease inhibitor were intratracheally administered simultaneously with FMLP treatment. Truncated SLPIs were administered i.v. 2 min before FMLP treatment. Dexamethasone and indomethacin, the latter a cyclooxygenase inhibitor, were administered p.o. 1 hr before FMLP treatment. SOD (50,000 U/kg) and catalase (90,000 U/kg) were given s.c. 2 hr before FMLP treatment and were followed by SOD infusion (6000 U/kg/30 min) from 10 min after FMLP treatment, under the conditions described by Yoshikawa *et al.* (1990).

Assay for elastase-like activity. Elastase-like activity in BAL fluid was measured by use of a synthetic substrate of elastase, methoxysuccinyl-alanyl-alanyl-prolyl-valine-7-amido-4-methylcoumarin, according to Mario *et al.* (1979). Briefly, 75 μ l of the supernatant of centrifuged BAL fluid was added to 1.4 ml of 0.05 M Tris buffer (pH 7.5) containing 0.5 M NaCl and 1 mM CaCl₂. This solution was incubated with 25 μ l of 6 mM substrate at 37°C for 60 min. The fluorescence intensity was measured at excitation 370 nm and emission at 460 nm. A standard curve was constructed to convert relative fluorescence intensity into concentration of 7-amido-4-methylcoumarin released by enzymatic hydrolysis. By reference to a standard curve constructed with known concentrations of HSE, elastase-like activity in BAL fluid was presented as the concentration of HSE producing the same level of fluorescence. The source of elastase-like protease recognized in LPS-induced inflammation was suspected to be the neutrophil or the macrophage. Neutrophil elastase is a serine protease that is inhibited by DFP. On the other hand, macrophage elastase is a metallo protease inhibited by EDTA. So, to identify the

source of protease, we measured elastase-like activity in the presence of DFP (5 mM) or EDTA (10 mM) (Abrams *et al.*, 1984).

Histopathology. For histopathological study, the lungs were inflated with 10% formaldehyde solution at a constant pressure of 25 cm H₂O for 48 hr. The lungs were then removed, and sections were prepared and stained with hematoxylin-eosin.

Production of recombinant truncated SLPI. The recombinant truncated SLPI was produced by Teijin Limited, Tokyo, Japan. Briefly, DNA fragments encoding the C-terminal domain (Asn⁵⁵-Ala¹⁰⁷) and (Arg⁵⁸-Ala¹⁰⁷) of SLPI were synthesized chemically by use of appropriate codons from *E. coli*. The human growth hormone gene was fused to this domain to optimize the expression size by linkage with the DNA sequence for Leu-Val-Pro-Arg, which is cleavable by thrombin. The expression vector was constructed and introduced into *E. coli* HB 101. After the transformed cells had been cultured, the fusion protein was obtained as an inclusion body. It was then dissolved and cleaved with thrombin. The active inhibitor was purified by chromatography after S-S refolding (Masuda *et al.*, 1992).

Statistical analysis. The data were expressed as the mean \pm S.E.M. Groups were compared by Student's standard two-tailed *t* test and by one-way analysis of variance in conjunction with Dunnett's multiple comparison *t* test when appropriate. Groups were considered different if the *P* value was less than .05.

Results

Exogenous elastase-induced acute lung injury model in hamsters. First we evaluated the potency of truncated SLPI to inhibit HSE-induced acute lung injury when administered i.v. and intratracheally. The assessment of HSE-induced acute lung injury was performed by measurement of the hemoglobin content in BAL fluid (Durham *et al.*, 1994; Fletcher *et al.*, 1990; Gillissen *et al.*, 1993; Herbert *et al.*, 1992). Intratracheal administration of truncated SLPI (0.01, 0.03 and 0.1 mg/kg) 1 hr before HSE administration inhibited acute lung hemorrhage in a dose-dependent manner (fig. 1). Pulmonary hemorrhage was inhibited by 31.4, 52.1 and 61.8%, respectively, with an ED₅₀ of 34.7 μ g/kg.

The i.v. administration of truncated SLPI (10 and 30 mg/kg) 2 min before HSE administration also inhibited acute lung hemorrhage in a dose-dependent manner (fig. 2). In this

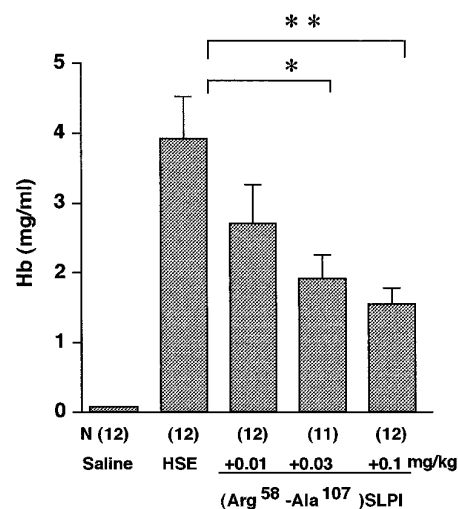


Fig. 1. Effect of intratracheal treatment with truncated SLPI, (Arg⁵⁸-Ala¹⁰⁷)SLPI, on exogenous elastase-induced acute lung injury in hamsters. Each value is the mean \pm S.E.M. (Arg⁵⁸-Ala¹⁰⁷)SLPI was administered intratracheally 1 hr before HSE administration. **P* < .05 and ***P* < .01, significantly different from HSE-treated group.

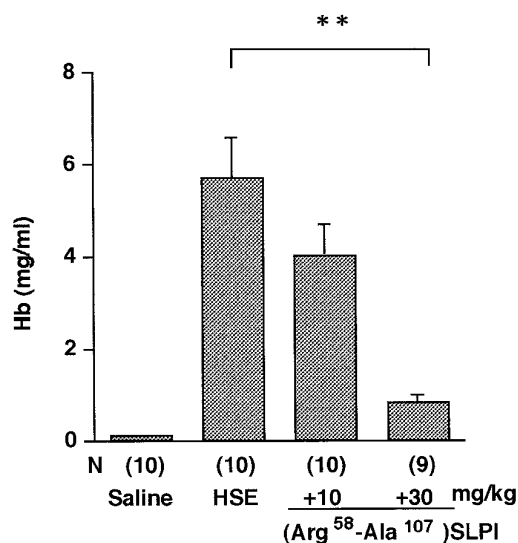


Fig. 2. Effect of i.v. treatment with truncated SLPI, (Arg⁵⁸-Ala¹⁰⁷)SLPI, on exogenous elastase-induced acute lung injury in hamsters. Each value is the mean \pm S.E.M. (Arg⁵⁸-Ala¹⁰⁷)SLPI was administered i.v. 2 min before HSE administration. ** $P < .01$, significantly different from HSE-treated group.

case, pulmonary hemorrhage was inhibited by 29.7 and 87.1%, respectively, with an ED₅₀ of 14.7 mg/kg.

Combination of LPS and FMLP-induced acute lung injury model in hamsters. To make a specific neutrophil-dependent model, we tried intratracheal administration of FMLP, a neutrophil stimulator, 24 hr after LPS administration. In the LPS-treated animals, the number of cells in the BAL fluid was significantly increased compared with that of intact animals. But the elastase-like activity was not changed. From the differential cell count analysis, the cells found in BAL fluid 24 hr after LPS administration were mostly neutrophils. The cellular proportions of neutrophils, alveolar macrophages, lymphocytes and eosinophils in BAL fluid were 86.9, 7.8, 4.6 and 0.8%, respectively. The additional treatment with FMLP at 24 hr after LPS treatment caused a very significant increase in elastase-like activity ($P < .01$), as well as a significant increase in hemoglobin concentration, compared with those levels for LPS-treated animals ($P < .01$, fig. 3).

In this model, we investigated the effects of i.v. and intratracheal administrations of truncated SLPI. Truncated SLPI administered intratracheally (0.1, 0.5 and 2.5 mg/kg) simultaneously with FMLP administration inhibited acute lung hemorrhage and attenuated the increase of elastase-like activity in a dose-dependent manner (fig. 3). Pulmonary hemorrhage was inhibited by -46.5, 40.2 and 70.9%, respectively, with an ED₅₀ of 0.85 mg/kg.

The i.v. administration of truncated SLPI (10 and 30 mg/kg) 2 min before FMLP administration also inhibited acute lung hemorrhage dose-dependently (fig. 4). Pulmonary hemorrhage was inhibited by 29.0 and 59.1%, respectively, with an ED₅₀ of 21.5 mg/kg.

Histopathological study. LPS-treated animals showed a remarkable diffuse infiltration of neutrophils around the bronchium and in the alveolar spaces, as well as perivascular edema. Addition of FMLP induced severe lung hemorrhage in the alveolar spaces and distortion of alveolar septa. Trun-

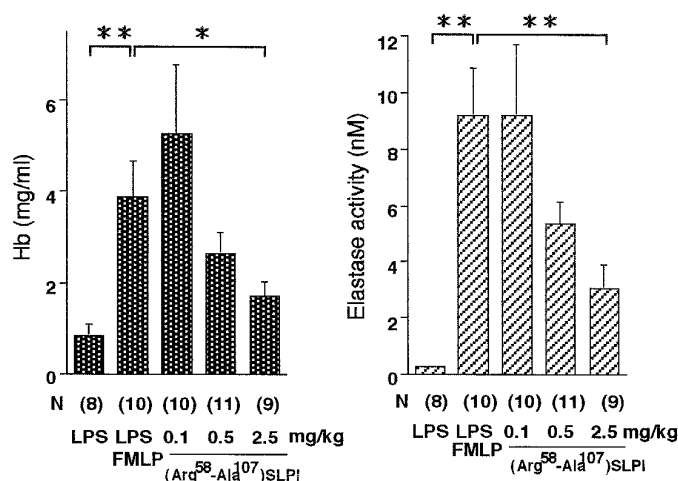


Fig. 3. Effect of intratracheal treatment with truncated SLPI, (Arg⁵⁸-Ala¹⁰⁷)SLPI, on the LPS and FMLP-induced acute lung injury in hamsters. Each value is the mean \pm S.E.M. (Arg⁵⁸-Ala¹⁰⁷)SLPI was administered intratracheally simultaneously with FMLP administration. Truncated SLPI inhibited dose-dependently the lung hemorrhage and attenuated the increase in elastase activity. * $P < .05$ and ** $P < .01$, significantly different from LPS and FMLP-treated group.

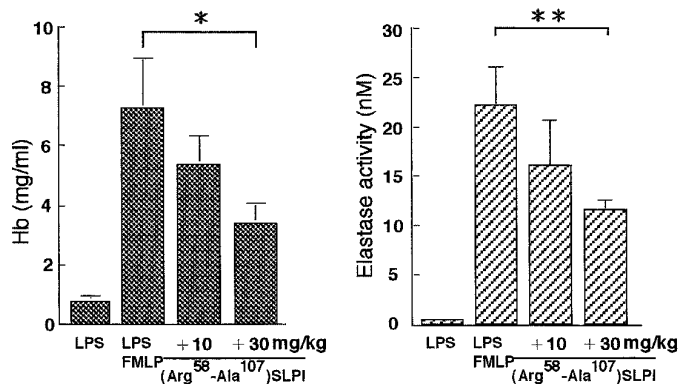


Fig. 4. Effect of i.v. treatment with truncated SLPI, (Arg⁵⁸-Ala¹⁰⁷)SLPI, on the LPS and FMLP-induced acute lung injury in hamsters. Each value is the mean \pm S.E.M. (Arg⁵⁸-Ala¹⁰⁷)SLPI was administered i.v. 2 min before FMLP administration. Truncated SLPI inhibited dose-dependently the lung hemorrhage and attenuated the increase in elastase activity. * $P < .05$ and ** $P < .01$, significantly different from LPS and FMLP-treated group.

cated SLPI attenuated these effects of the additional FMLP treatment of LPS-treated animals (fig. 5).

Identification of elastase-like protease in BAL fluid of LPS and FMLP-induced lung injury model. Elastase-like activity of BAL fluid in the LPS and FMLP-induced injury model was completely inhibited by the addition of DFP, a serine protease inhibitor, but was scarcely reduced by the addition of EDTA, a metallo protease inhibitor (fig. 6).

Effects of several drugs on LPS and FMLP-induced lung injury. Finally, we investigated the effects of several drugs on the lung injury induced by LPS and FMLP. These drugs included truncated SLPIs, a protease inhibitor, an elastase inhibitor, anti-inflammatory agents and radical scavengers. Intratracheal administration of α 1-PI at 20 mg/kg, that of the specific elastase inhibitor CMK at 0.2 mg/kg and that of truncated SLPIs all inhibited lung hemorrhage significantly (table 1). Dexamethasone inhibited lung hemorrhage only slightly, and indomethacin aggravated rather

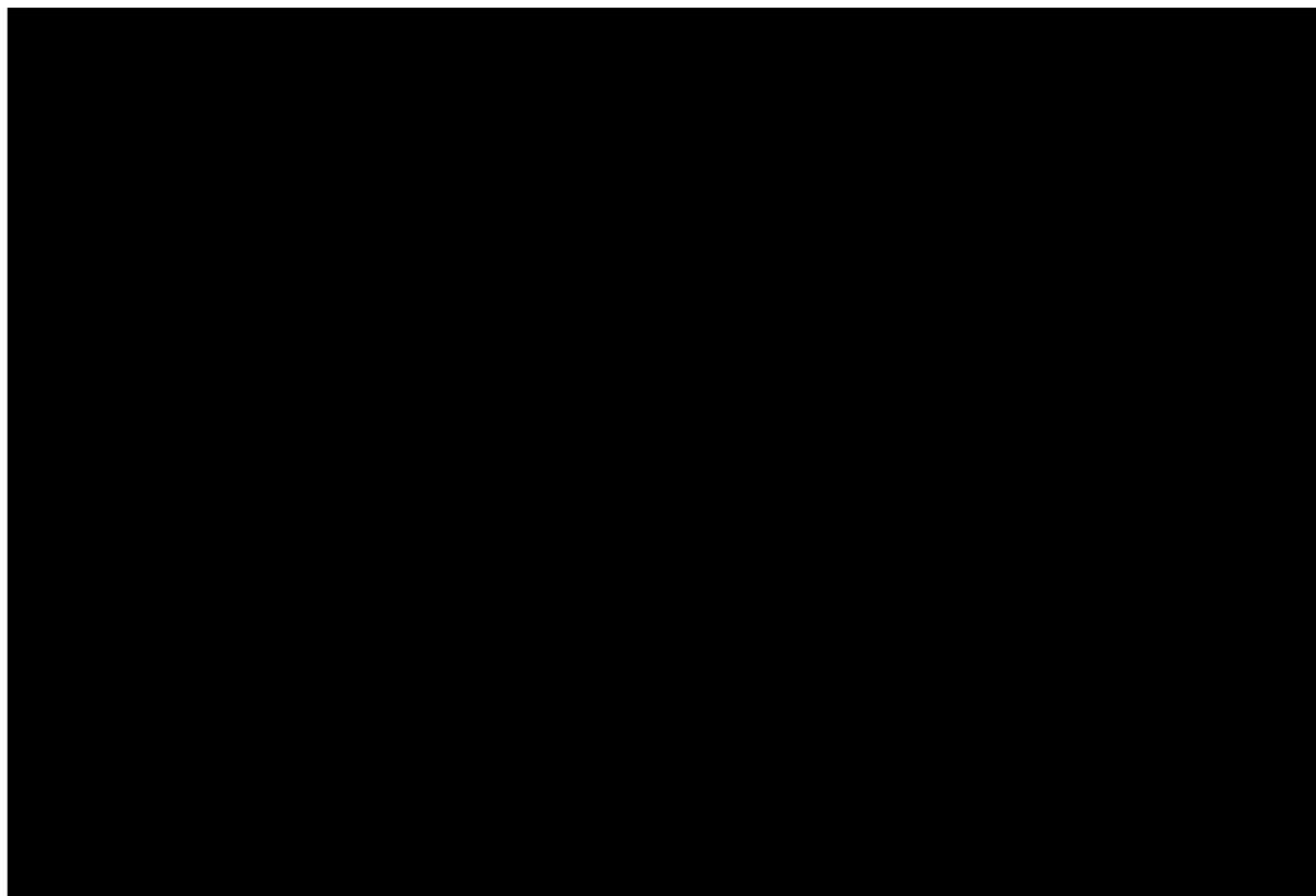


Fig. 5. Light micrographs of the lung field around an airway in hamsters with LPS and FMLP-induced lung injury. A) Intact animal ($\times 61.25$). B) LPS-treated animal ($\times 61.25$). C) LPS and FMLP-treated animal ($\times 61.25$). D) LPS and FMLP-treated animal with truncated SLPI-treatment ($\times 61.25$, 30 mg/kg i.v.).

than inhibited it. The radical scavengers (SOD and catalase) were ineffective under our experimental conditions.

Discussion

A number of elastase inhibitors have been evaluated for their activity to inhibit HSE-induced lung hemorrhage (Fletcher *et al.*, 1990; Herbert *et al.*, 1992; Shah *et al.*, 1992; Durham *et al.*, 1994). However, this model alone appears to be inappropriate for evaluation of elastase inhibitor activity *in vivo*, because it is not surprising that an elastase inhibitor would inhibit elastase-induced lung injury, especially that assessed after intratracheal administration. Thus we undertook this study not only to evaluate the potency of truncated SLPI by the HSE-induced lung injury model but also to develop a specific neutrophil-mediated lung injury model for use in evaluating the potency of truncated SLPI.

The presence of bacterial endotoxin in combination with neutrophil infiltration is known to result in lung injury (Pierce *et al.*, 1977). And it has been suggested that the neutrophil plays a role in the pathogenesis of endotoxin-associated lung injury (Repine *et al.*, 1982). But the mechanism by which endotoxin results in lung dysfunction appears to be complex, because it is known that endotoxin injures lung tissue directly and stimulates monocytes to release tumor necrosis factor (Bachwich *et al.*, 1986), interleukin-1

(Wewers *et al.*, 1984) and numerous lipid mediators (Brown *et al.*, 1988; Rylander and Beijer, 1987). Actually, although intratracheal administration of LPS produces lung injury, we could not find any correlation between the degree of lung injury and the number of neutrophils (data not shown). These findings imply that LPS-induced acute lung injury may be associated not only with neutrophils but also with many other factors.

Accordingly, to make a specific neutrophil-dependent model and to investigate the mechanism by which neutrophil promotes lung injury, we tried intratracheal administration of FMLP, which causes the release of neutrophil proteases and oxidants, 24 hr after LPS administration. We found that additional treatment with FMLP aggravated the LPS-induced lung injury, and we accepted this new model to be one of neutrophil-mediated, exudative and hemorrhagic lung injury based on histopathological observation.

The i.v. and intratracheal administration of truncated SLPI attenuated this lung injury, and the increase in elastase-like activity in LPS and FMLP-treated animals prompted us to consider that neutrophil protease might participate in the lung injury. Thus we focused our interest on elastase-like protease and investigated its possible participation in the injury. The source of elastase-like protease was suggested to be neutrophil from the results of DFP and EDTA treatment (fig. 6). Further, to clarify the participation

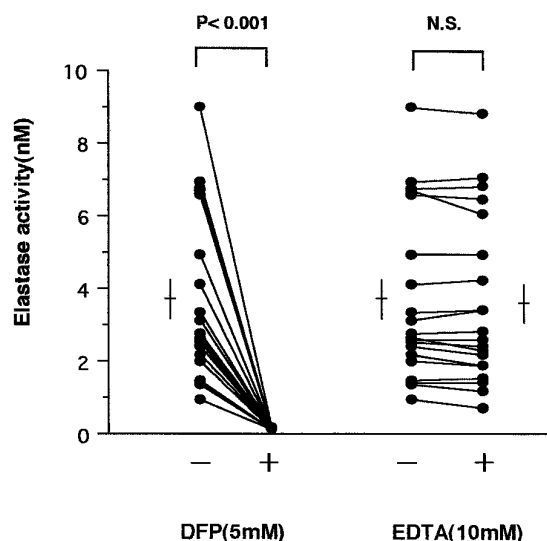


Fig. 6. Identification of elastase-like activity in BAL fluid of hamsters with LPS and FMLP-induced lung injury. Addition of DFP, but not that of EDTA, completely inhibited elastase activity of BAL fluid in the LPS and FMLP-induced injury model.

of neutrophil protease in this injury model, we investigated the effects of α 1-PI, of CMK, a synthetic neutrophil elastase inhibitor, of our recently developed truncated SLPIs and of several anti-inflammatory drugs (table 1). This lung injury was attenuated only by administration of elastase inhibitors such as α 1-PI, CMK and the truncated SLPIs, and not by dexamethasone, indomethacin and radical scavengers. Weiss *et al.* (1986) described the synergy between neutrophil elastase and oxidants in neutrophil-induced tissue injury and indicated that the injury may be mediated in large part by the action of neutrophil elastase. Therefore, our results appear to support their contention. These results suggest that LPS and FMLP-induced lung injury is mediated mainly by neutrophil proteases.

Interestingly, our data show that a nearly 20-fold higher dose of SLPI (0.85 mg/kg *vs.* 47 μ g/kg) was required to inhibit LPS and FMLP-induced lung hemorrhage than to inhibit the HSE-induced injury. In these two models, we used different administration timing. Specifically, in the HSE-induced model we evaluated the efficacy of SLPI as a pretreatment

because it seems reasonable to assume that elastase-induced lung injury would be inhibited by co-administration with its inhibitor. In the LPS/FMLP-induced model, this consideration does not arise. We chose co-administration because we thought the physical irritation of successive intratracheal administrations might injure the respiratory tract of the animals and thus affect the results. However, we do not believe that the different experimental conditions caused the discrepancy in the results. It appears that LPS and FMLP-induced lung injury is mediated by hamster neutrophil proteases. Recently we found a species difference in the inhibitory activity of truncated SLPI toward elastase; *i.e.*, its inhibition of hamster elastase was 30-fold weaker than that of human elastase (data not shown). We think that this difference explains the difference in effective dose between the two models. Gillissen *et al.* (1993) indicated that SLPI augments the glutathione level in lung epithelial lining fluids in sheep. The increase in the glutathione level in such fluids means an increased antioxidant screen on the respiratory epithelial surface. Moreover, truncated SLPI inhibits not only neutrophil elastase but also cathepsin G, chymotrypsin, trypsin, collagenase and chymase (Masuda *et al.*, 1994). Therefore, this inhibitory activity against multiple proteolytic enzymes and oxidants might have added to the protective effects of the truncated SLPI on neutrophil-mediated acute lung injury. Moreover, in a recent study using an immunofluorescence technique, Willems *et al.* (1986) showed SLPI to be localized in association with elastic fibers in airway walls and alveolar septa. This evidence suggests that SLPI may play a role in protecting not only the epithelium but also the connective tissues from the enzymatic injury. We have preliminary data showing that the truncated SLPI administered *i.v.* becomes distributed to the lung tissue quickly. These results imply the efficacy not only of topical but also of systemic treatment with it.

In conclusion, our study has demonstrated that truncated SLPI attenuates the HSE-induced acute lung injury and the neutrophil-mediated acute lung injury induced by the combination of LPS and FMLP administration. Therefore, truncated SLPI appears to be a good candidate for the treatment of destructive lung diseases due to neutrophils, such as chronic bronchitis, emphysema and ARDS.

TABLE 1

Effects of several drugs on LPS/FMLP-induced lung injury

Drugs	Treatment	N	Inhibition of Hemorrhage (%)
Dexamethasone ^a	0.05 mg/kg p.o.	9	0
	1.0 mg/kg p.o.	8	24.3
Indomethacin ^a	0.3 mg/kg p.o.	9	-0.1
	1.0 mg/kg p.o.	9	-23.2
α 1-Protease inhibitor ^b	20 mg/kg intratracheal	8	61.6
	0.2 mg/kg intratracheal	10	55.9
MeoSuc-Ala-Ala-Pro-Val-chloromethylketone ^b (Asn ⁵⁵ -Ala ¹⁰⁷)SLPI ^c	3 mg/kg <i>i.v.</i>	11	32.3
	30 mg/kg <i>i.v.</i>	12	48.0
(Arg ⁵⁸ -Ala ¹⁰⁷)SLPI ^c	10 mg/kg <i>i.v.</i>	12	29.0
	30 mg/kg <i>i.v.</i>	11	59.1
(SOD ^d Catalase	(50000 U/kg s.c. + 6000 U/kg/30 min, infusion 90000 U/kg s.c.)	6	0

^a Administered 1 hr before FMLP treatment.

^b Administered simultaneously with FMLP treatment.

^c Administered 2 min before FMLP treatment.

^d SOD (s.c.) and catalase (s.c.) were administered 2 hr before FMLP treatment and followed by SOD infusion from 10 min after FMLP treatment.

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