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Pulmonary surfactant is a potential endogenous inhibitor of proteolytic activation of Sendai virus and influenza A virus

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The pathogenicities of influenza viruses and paramyxoviruses have been proposed to be primarily determined by a host cell protease(s) that activates viral infectivity by proteolytic cleavage of the envelope glycoproteins. We recently isolated a trypsin-type endoprotease, named tryptase Clara, from rat bronchial and bronchiolar epithelial Clara cells, which is secreted into the airway lumen and activates Sendai virus and influenza A virus proteolytically. We report here that surfactant in the bronchial fluid inhibited tryptase Clara specifically, having a K_i value of 0.13 μM, and inhibited the proteolytic activations by tryptase Clara in vitro and in organ cultures of rat lung. Intranasal infection of rats with Sendai virus was shown to stimulate secretion of tryptase Clara without changing the amount of surfactant in the bronchial lumen, resulting in a preferable condition for proteolytic viral activation and multiplication.

Pulmonary surfactant; Sendai virus; Influenza A virus; Tryptase Clara

1. INTRODUCTION

Influenza and parainfluenza viruses are important causative agents of respiratory infections. Control of infections has been a major subject of virus research. Post-translational proteolytic cleavage of envelope glycoprotein precursors, such as F0 of Sendai virus (murine parainfluenza virus type 1) and HA of influenza viruses, is essential for the expression of infectivity of these viruses and multiple cycles of viral replication [1–5]. The HA's of mammalian influenza viruses and F_0 of Sendai virus have a single arginine residue in a consensus cleavage motif, Gln (or Glu)-X-Arg, [3,6–8]. These envelope glycoprotein precursors have been reported to be cleaved in vitro by several proteases, such as trypsin in culture systems [2,9] and the clotting factor Xa in the allantoic fluid of chick embryos [10], and they are not activated by the ubiquitous host protease(s) present in various cell types [3,9,11]. Sendai virus and mammalian influenza viruses, however, are specifically pneumotropic in vivo, infecting only bronchial and bronchiolar epithelial cells, suggesting the presence of a specific trypsin-like protease(s) in the respiratory tract that activates the envelope glycoprotein precursors [5,11].

Recently, we isolated a novel trypsin-like protease, named tryptase Clara, from rat lung [13]. This protease is present only in non-ciliated secretory cells (Clara cells) of the bronchial and bronchiolar epithelia of rats, and is secreted into the airway lumen. Purified tryptase Clara converts F_0 of Sendai virus to its subunits, F_1 and F_2 [14], and HA of influenza A/Aichi/2/68(H3N2) to HA_1 and HA_2 [13] and activates the infectivity of these viruses in a dose-dependent manner in vitro. Antibody against tryptase Clara inhibits Sendai virus activation in the lungs of infected rats and suppresses multiple cycles of viral replication and pathological changes in rat lung [14]. Thus tryptase Clara is considered to be a primary host factor involved in the pneumopathogenicity of these viruses.

The physiological role of tryptase Clara is unknown, but the activity of this enzyme, like those of many proteases, may be strictly regulated by an endogenous inhibitor(s). To identify this endogenous inhibitor(s), we searched for an inhibitor of tryptase Clara in airway lavage fluid of rats. Results showed that surfactant, which is secreted mainly by alveolar type II cells, and to a lesser extent by bronchiolar epithelial Clara cells [15], is a specific endogenous inhibitor of tryptase Clara. In this paper, we describe the inhibitory effects of pulmonary surfactant on proteolytic activation of Sendai virus and influenza virus in vitro and in organ cultures of infected rat lung. We also discuss the potential role of pulmonary surfactant in pneumopathogenic viral infections.

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Abbreviations: HA, haemagglutinin; F0, precursor fusion glycoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Boc, *N*-tert-butoxycarbonyl; MCA, 4-methyl-coumaryl-7-amide; PBS, phosphate buffered saline; SPF, specific-pathogen free; CIU, cell-infecting unit; MEM, minimal essential medium.

2. MATERIALS AND METHODS

2.1. *Materials*

Tryptase Clara from rat lung, and pulmonary surfactant and surfactant protein A from rat alveolar lavage fluid, were purified by the methods of Kido et al. [13], and Sakai et al. [16], respectively. L-α-Phosphatidylcholine, L-α-phosphatidyl-L-serine and L-α-phosphatidyl-DL-glycerol were from Sigma. All other reagents were commercial products of the highest grade available.

2.2. *Enzyme and inhibitor assays*

Amidolytic activation of tryptase Clara was assayed using the synthetic peptide, Boc-Gln-Ala-Arg-MCA, as substrate, as described [13]. Trypsin and mast cell tryptase were measured with Boc-Phe-Ser-Arg-MCA as substrate, plasmin with Boc-Val-Leu-Lys-MCA, and factor Xa with Boc-Ile-Glu-Gly-Arg-MCA. For assay of the effects of surfactant and related compounds on the activity of each protease, the enzyme was preincubated with surfactant or surfactant protein A in water or phospholipids in ethanol for 5 min at 25°C and then residual activity was measured by addition of the substrate. The concentration of ethanol in the reaction mixture did not exceed 1%. The inhibition constant (K_i) for surfactant was determined from a Dixon plot.

2.3. *Radioisotope labeling of virus and gel electrophoresis*

Sendai virus was labeled with [^{3}H]glucosamine in LLC-MK2 cells and purified as described previously [11]. Purified inactive Sendai virus suspended in PBS deficient in Ca^{2+} and Mg^{2+}, pH 7.2, was treated with proteases in 100 mM Tris-HCl buffer, pH 7.2, for 10 min at 37°C. Reactions were terminated by adding 100 μg/ml of aprotinin and the viral polypeptides were separated by SDS-PAGE under reducing conditions followed by fluorography [13].

2.4. *Preparation of bronchoalveolar fluid and Western immunoblotting*

Bronchoalveolar lavage was collected as described previously [13]. The lavage fluid was centrifuged at 600 × *g* for 15 min at 4°C to remove cells and concentrated to 0.5 ml by Diaflow ultrafiltration with a YM-30 membrane. The concentrated samples were separated by SDS-PAGE (10–20% gradient), transferred electrophoretically to an Immobilon transfer membrane and immunoblotted with 1:1,000 dilution of an affinity purified anti-tryptase Clara antibody (1.24 mg/ml), or 1:200 dilution of anti-rat pulmonary surfactant protein A (0.5 mg/ml). Bound antibodies were detected by the anti-rabbit alkaline phosphatase method [13]. The resulting bands were scanned with a densitometer (Shimazu CS-9000).

2.5. *In vitro activations of Sendai virus and influenza A virus infectivities*

Non-activated (non-infectious) Sendai virus and influenza A virus were prepared in LLC-MK2 cells and MDCK cells, respectively, as described by Tashiro et al. [14,17]. Virus suspensions in PBS, pH 7.2, were treated with various concentrations of tryptase Clara or trypsin for 10–20 min at 37°C, and reactions were stopped by adding 100 μg per ml of aprotinin. Activation of infectivity was assayed by the immunofluorescent cell-counting method [5].

2.6. *Organ block cultures of rat lung*

The lung block culture method described for mice [18] was applied to rats with minor modifications. SPF, 3-week-old male CD(SD) rats were infected intranasally with 10^4 plaque forming units of active Sendai virus. After 2 h, the rats were sacrificed and their lungs were removed and cut into blocks. Organ blocks were then incubated in MEM at 34°C, and at the indicated times they were homogenized and assayed for infectivity as described previously [5].

3. RESULTS

3.1. *Effects of pulmonary surfactant and related compounds on the activity of tryptase Clara*

In a search for an inhibitor(s) of tryptase Clara in the bronchial lavage fluid of rats, we found that pulmonary surfactant is a potent inhibitor of the protease. The inhibition was non-competitive and the K_i value of the purified surfactant was 0.13 μM. However, phosphatidylcholine, a major component of surfactant, and minor components, such as surfactant protein A, phosphatidylglycerol and phosphatidylserine [19], by themselves

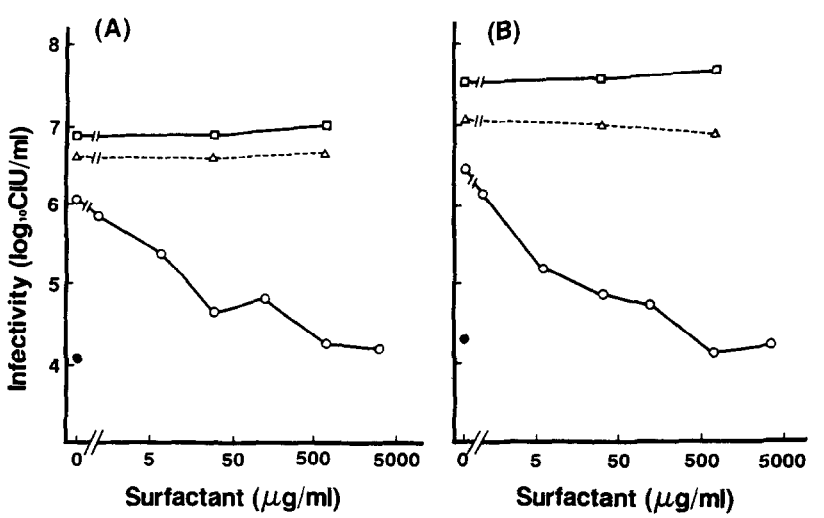

Fig. 1. Inhibitory effects of surfactant on activations of Sendai virus (A), and influenza A/Aichi/2/68(H3N2) (B) by proteases in vitro. Tryptase Clara (50 μg/ml, ○) and trypsin (10 μg/ml, △) were preincubated with surfactant at the indicated concentrations at 0°C for 20 min. Then the mixtures were incubated with inactive Sendai virus grown in LLC-MK2 cells (A, ●) or inactive influenza A virus grown in MDC K cells (B, ●) for 20 min with tryptase Clara, or for 10 min with trypsin at 37°C as described in section 2. The infectivities of the viruses were assayed by the immunofluorescent cell-counting method [5] using LLC-MK2 cells for Sendai virus (A) and MDCK cells for influenza A virus (B). The infectivity of active virus grown in embryonated chicken eggs (□) after treatment with surfactant was also measured. Concentrations of surfactant are expressed as those of phospholipids in the samples. CIU, cell infecting unit [5].

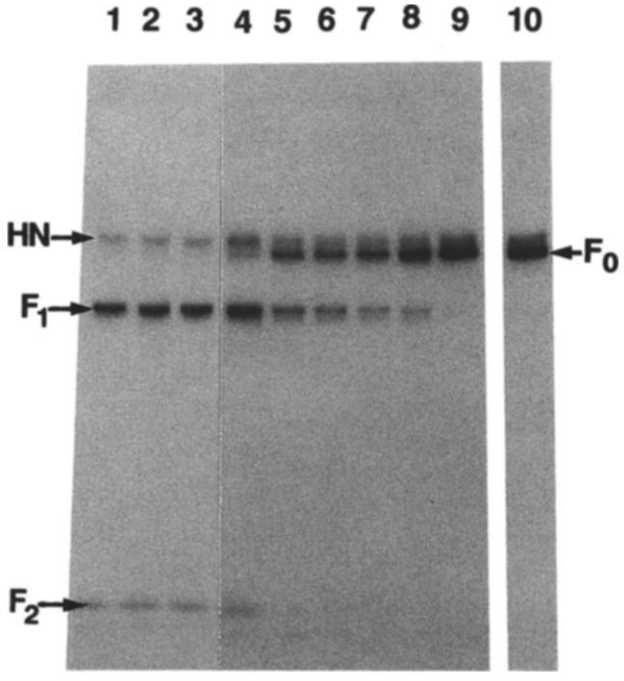

Fig. 2. Effect of surfactant on proteolytic cleavage of F_0 of Sendai virus by tryptase Clara. Trypsin (10 μg/ml, lanes 1–3) and tryptase Clara (50 μg/ml, lanes 4–9) were preincubated without surfactant (lanes 1 and 4) or with 0.26 mg/ml (lanes 2 and 5), 0.52 mg/ml (lane 6), 0.78 mg/ml (lane 7), 1 mg/ml (lanes 3 and 8), 3.0 mg/ml (lane 9) of surfactant in 20 μl of 100 mM Tris-HCl buffer, pH 7.2, at 0°C for 5 min. Then [^{3}H]glucosamine-labeled inactive Sendai virus (lane 10) was treated with the reaction mixtures at 37°C for 10 min as described in section 2. The viral polypeptides were separated by SDS-PAGE under reducing conditions followed by fluorography. Concentrations of surfactant are expressed as those of phospholipids in the samples. HN, haemagglutinin neuraminidase.

had no effect. Surfactant reconstituted with phosphatidylcholine, phosphatidylglycerol, surfactant protein A and phosphatidylserine in a ratio of 67.3:19.3:10.2:3.2 by weight, showed comparable inhibitory activity, with a K_i value of 0.64 μM. These results suggest that the inhibition by surfactant is due to a complex of the phospholipids and surfactant protein A. It should also be noted that the inhibition was specific for tryptase Clara. The other trypsin-type proteases, such as trypsin, factor Xa, plasmin and rat mast cell tryptase [20], were not inhibited at all by surfactant (data not shown).

3.2. *Inhibitory effects of surfactant on activations of Sendai and influenza viruses in vitro*

To determine whether surfactant inhibits the proteolytic activations of Sendai and influenza viruses by tryptase Clara, we examined the infectivities of inactive Sendai and influenza A/Aichi/2/68 (H3N2) viruses after in vitro activation by tryptase Clara or trypsin that had been preincubated with various concentrations of surfactant for 20 min at 0°C. Infectivity was measured by the immunofluorescent cell-counting method [5], in which only the number of activated viruses are counted. Tryptase Clara caused an up to 100-fold increase in the infectivity of both inactive viruses. Surfactant inhibited the viral activation by tryptase Clara concentration dependently, but did not inhibit the activation by trypsin (Fig. 1). In addition, surfactant itself did not affect the infectivities of active viruses grown in the allantoic cavity of chick embryos.

We next examined whether surfactant inhibits proteolytic cleavage of the F_0 protein of Sendai virus by tryptase Clara. For this, the enzyme was preincubated with various concentrations of surfactant for 5 min at 0°C and then tested for proteolytic cleavage of [^{3}H]glucosamine-labeled inactive Sendai virus grown in LLC-MK2 cells. The results of SDS-PAGE analysis are shown in Fig. 2, together with those for trypsin. Surfactant inhibited the cleavage of F_0 to its subunits. F_1 and F_2, by tryptase Clara dose dependently, but not at all the cleavage by trypsin. Proteolytic cleavage of HA of influenza A/Aichi virus by tryptase Clara was also inhibited by pulmonary surfactant (data not shown).

3.3. *Inhibition of Sendai virus activation by surfactant in organ cultures of rat lung*

We examined the effect of surfactant on Sendai virus activation using block cultures of infected rat lung. The results in Fig. 3 also show inhibition of Sendai virus activation by surfactant. In culture medium with surfactant (50 μg/ml), about 90% of the progeny virus remained unactivated, and so viral replication was sup-

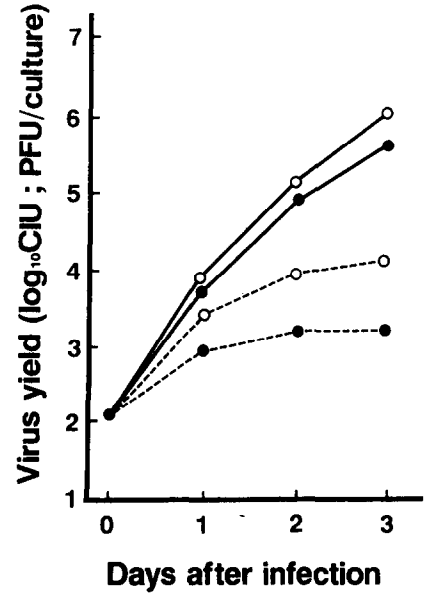

Fig. 3. Inhibitory effect of surfactant on Sendai virus activation in organ cultures of rat lung. SPF, 3-week-old male CD (SD) rats were infected intranasally with 10^4 plaque forming units of active Sendai virus. After 2 h, lung block cultures were prepared as described in section 2 and incubated at 34°C with MEM in the absence (—) orpresence (----) of 50 μg/ml of surfactant. At the indicated times, organ blocks and media were collected and tissue homogenates were assayed for total virus (○) and activated virus (●) yield as described previously [5].

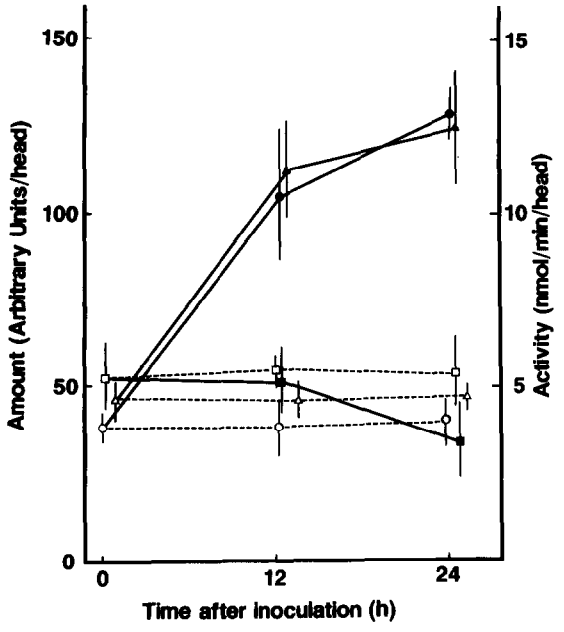

Fig. 4. Alterations in the activity and amount of tryptase Clara and surfactant in bronchial lavage fluid after intranasal infection with Sendai virus. Three-week-old, male SPF rats of the CD (SD) strain were treated intranasally with 2×10^4 plaque forming units of active Sendai virus (—) or saline (----). Then their lungs were lavaged once at the indicated times with 3 ml of sterile saline to obtain 2.5 ml of lavage fluid. Cell-free lavage fluid was concentrated to 0.5 ml and an aliquot (20 μl) of the sample was separated by SDS-PAGE (10–20% gradient), transferred electrophoretically to an Immobilon PVDF membrane and developed with anti-tryptase Clara (●,○) or anti-surfactant protein A antibody (■,□) as described in section 2. The resulting bands were quantified by scanning with a densitometer. The proteolytic activity of tryptase Clara in lavage fluid (▲,△) was calculated from measurements of the activity that was able to be absorbed by anti-tryptase Clara IgG-coupled Sepharose 4B, with Boc-Gln-Ala-Arg-MCA as substrate. Data are means ± S.E.M. (n = 3).

pressed to about 10% of the control value. The inhibitory effects on viral activation and replication were not observed after absorption of surfactant by anti-surfactant protein A antibody (data not shown). These effects of surfactant were quite similar to those of antibody against tryptase Clara on Sendai virus activation [14], supporting our previous postulates that tryptase Clara is a major activating protease of Sendai virus in rat lung, and that viral activation occurs extracellularly in the airway lumen [14].

3.4. *Sendai virus infection stimulates secretion of tryptase Clara but not of surfactant in the airway lumen*

When rats were infected intranasally with Sendai virus, tryptase Clara with a molecular mass of 28.5 kDa, a secretory form in the bronchial lavage fluid [13], was increased significantly in both amount and activity after inoculation for 12–24 h, whereas the amount of surfactant remained almost constant, as shown in Fig. 4. These findings indicate that infection of rats with Sendai virus stimulates secretion of tryptase Clara, but not of surfactant, resulting in enhancement of proteolytic activation of viral infectivity and, in preferable conditions, for viral multiplication in vivo.

4. DISCUSSION

The present study showed that pulmonary surfactant purified from bronchoalveolar lavage fluid of rat inhibits the proteolytic activations of Sendai virus and influenza A virus by tryptase Clara in vitro, and that it also inhibits Sendai virus activation in organ cultures of rat lung. To our knowledge, this is the first report of the inhibitory effect of pulmonary surfactant on proteolytic activation of envelope glycoprotein precursors of viruses. Pulmonary surfactant, a lipoprotein complex, has been reported to coat alveolar epithelium and to lower the surface tension at the air–liquid interface. Recent studies also indicated that pulmonary surfactant and its apoprotein are important in the host defense system in the lung, increasing phagocytosis by mononuclear phagocytes [21] and uptake of liposomes by alveolar macrophages [22]. In addition to these functions, we found that pulmonary surfactant specifically inhibited the proteolytic activity of tryptase Clara in a non-competitive fashion with a relatively low K_i value of 0.13 μM, suggesting that it is a potential endogenous inhibitor of tryptase Clara. Surfactant from bovine lung causes similar inhibition of the activity of tryptase Clara and proteolytic activation of the viruses by tryptase Clara (data not shown). Neither phospholipids nor surfactant protein A inhibited the enzyme activity, but the inhibitory activity was restored by reconstitution of surfactant with these compounds. There is no sequence homology between surfactant protein A and Kunitz-type protease inhibitors of tryptase Clara, such as aprotinin and soybean trypsin inhibitor [13]. Inhibition of enzyme activities by phospholipids and fatty acids have been reported: chymase and α-chymotrypsin are inhibited by fatty acids [23], granulocyte elastase by *cis*-unsaturated fatty acids [24], phospholipase A by phosphatidylcholine [25], and glutamate dehydrogenase by phosphatidylserine [26]. Thus, we speculate that hydrophobic interaction of tryptase Clara with pulmonary surfactant, a complex of phospholipids and surfactant protein A, might alter the conformation of the enzyme, resulting in inhibition of its enzyme activity.

We have recentry shown that that ploteotytic activation of Sendai virus occurs mainly extracellular in the lumen of the respiratory tract [14]. In intact animals, the activity of tryptase Clara may be suppressed at a low level by a physiological concentration of endogenous inhibitor, such as surfactant, in the bronchial lumen, since when inactive Sendai virus is inoculated intranasally into mice, little uncleaved F_0 is converted to an active form in the lung [5]. Although the significance of the effect of surfactant on tryptase Clara in vivo currently remains unclear, after infection by activated Sen-

dai virus the proteolytic activity in the lumen was enhanced by increased secretion of the enzyme from Clara cells without changing the amount of surfactant, resulting in preferable conditions for proteolytic activation and multiplication of progeny virus. An induced imbalance between the amount of tryptase Clara and that of surfactant by activated virus may be responsible for pneumopathogenesis of Sendai virus infection.

Van Daal et al. [27,28] recently reported that intratracheal administration of surfactant is a promising method of therapy in experimental models of adult respiratory distress syndrome associated with Sendai virus pneumonia in rats and influenza A pneumonia in mice. They showed that supplementation of surfactant significantly improved gas exchange by increasing compliance and the lung volume, and also reduced alveolar and bronchiolar edema caused by inflammation. In addition to these effects of surfactant on gas exchange and edema, its effect as an inhibitor of proteolytic activation of viruses described here indicates that it should be therapeutically effective in viral pneumonia.

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