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# NEW APPROACHES FOR PREVENTION AND TREATMENT OF INFLUENZA VIRUS INFECTION BY AN ENHANCEMENT OF MUCOSAL IMMUNIZATION AND SUPPRESSION OF PROTEASE EXPRESSION

Hiroshi Kido, Dai Mizuno, Quang Trong Le, Junji Chida, Hiroshi Yamada, Yuushi Okumura and Mihiro Yano

**Abstract** Human influenza virus causes an annual epidemic infection. After influenza virus infection in the airway, infection sometimes spreads with severe neurologic complication and multiple organ failure, resulting in high mortality. In the process of viral spread from the lungs to other organs, significant up-regulated trpsin was observed in various organs. Up-regulated trypsin effectively converted precursor of the viral envelope hemagglutinin (HA) into HA1 and HA2 subunits. The proteolytic activation of HA is a prerequisite for virus multiplication. Administration of the inhibitors of trypsin as new approaches for the treatment of influenza virus infection effectively suppressed viral multiplication. Almost vaccines for influenza virus infection are administered i.m. or s.c., which induce IgG-mediated protection in the systemic immune compartment, but this immunization offers insufficient protection on the mucosal surface at the initial site of viral multiplication. To improve protective mucosal immunity, intranasal vaccination has been studied. The powerful mucosal adjuvants reported are toxin based, such as cholera toxin and *Escherichia coli* heat-labile toxin, but these enterotoxins cause severe side effects. We recently found natural mucosal adjuvant, pulmonary surfactant, in the lungs. Intranasal administration of influenza vaccine combined with Surfacten, a modified pulmonary surfactant free of antigenic c-type lectins, induced high protective mucosal immunity in the airway and systemic immune responses in the blood, the efficacy of both protective immunities by Surfacten being equivalent to those by cholera toxin. In this symposium, we reported the effects of Surfacten on mucosal and systemic immunities.

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**Key words:** influenza virus; mucosal immunization; pulmonary surfactant; ectopic trypsin; influenza-associated encephalopathy

## Cellular trypsin-type processing proteases for influenza virus envelope protein as principal determinants for virus entry

The post-translational proteolytic cleavage of precursors of fusion glycoprotein of the enveloped influenza A virus (IAV) is a prerequisite for viral fusion activity and virus entry into cells[1-4]. However, IAV, a member of *Orthomyxovirus*, as well as Sendai virus and Newcastle disease virus, members of *Paramyxovirus*, do not have any processing protease (s) for their envelope glycoprotein precursors in their genomes. A cellular proteolytic modification is therefore indispensable for viral infection and multiplication within the infected host, and is a major determinant of IAV pathogenicity, rather than the virus receptor sialic acid, which is widely distributed in various cell membranes. There are two systems for proteolytic activation of the IAV envelope fusion protein precursor, hemagglutinin ($HA_0$). One is a ubiquitous intracellular processing system for the virulent strains of avian IAV HA with multiple basic residues in the consensus cleavage motif, Lys/Arg-X-Lys/Arg-Arg, in the cis or medial cisternae of the Golgi complex, or in the trans Golgi network of host cells[6]. Consequently, the virulent avian IAV usually causes systemic infection rapidly followed by death of the

Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 77-8503, Japan

Phone: +81-88-633-7423; Fax: +81-88-633-7425;
E-mail address: kido@ier.tokushima-u.ac.jp

**Table 1** Comparison of the viral activating enzymes

| Enzyme | Species | M.W. SDS-PAGE | M.W. non-reducing condition (composition) | Optimal substrate | Inhibitor specificity | localization | Reference |
|---|---|---|---|---|---|---|---|
| Tryptase Clara | rat | 30 kDa | 180 kDa, (hexamer) | QAR | Aprotinin, leupeptin, antipain, Kunitz-type soybean trypsin inhibitor | Bronchiolar epithelial Clara cells | Kido *et al.*, 1992 |
| Mini-plasmin | rat | 28 kDa + 12 kDa | 38 kDa (heterodimer) | QAR | Aprotinin, Kunitz-type soybean trypsin inhibitor, Bowman-Birk soybean trypsin inhibitor, leupeptin | folded epithelial cells in relatively thick superior bronchiolar divisions | Murakami *et al.*, 2001 |
| Ectopic anionic trypsin I | rat | 22 kDa | 31 kDa (monomer) | EGR QGR | aprotinin, soybean trypsin inhibitor, leupeptin | stromal cells in peri-bronchiolar region | Towatari *et al.*, 2002 |
| Mast cell tryptase | pig | 32-35 kDa | 120 kDa (tetramer) | QAR | Antipain, leupeptin | mast cell | Chen *et al.*, 2000 |
| Tryptase TC30 | pig | 30 kDa | 30 kDa, (monomer) | SIQSR | Aprotinin, benzamidine, leupeptin | ND | Sato *et al.*, 2003 |

M.W.: molecular weight, ND: Not determined

bird. The other is an extracellular proteolytic activation system limited to the respiratory tract or the enteric duct for the human IAV and avirulent avian IAV strains, which has a single basic amino acid arginine in the consensus $HA_0$ cleavage motif, Gln (Glu)-X-Arg[3,4,6,7]. The latter is true for most of the epidemic human IAV known to date, which are pneumotropic, except for a few neurotropic strains, such as that of the 1918-1919 pandemic[8,9] or IAV WSN/33 (H1N1) strain[10].

We have searched the $HA_0$ processing proteases of human IAV in the airways of animal and human and found various trypsin-type, single arginine processing serine proteases, such as tryptase Clara[11], mini-plasmin[12], ectopic anionic trypsin[13], porcine mast cell tryptase[14] and tryptase TC30[15] (Table 1).

These proteases show different distribution in the airway and may play a major role in the spread of IAV from the upper respiratory airways to the lung parenchyma. Why do trypsin-type HA processing proteases capable of potentiating IAV infections exist in the airways? We found that these cellular proteases exhibit different distribution in the airway as well as different potency of proteolytic potentiation of various strains of the viruses[12]. Among the proteases we have examined, trypsin efficiently activated the infectivity of all strains. Mutational evolution of IAV $HA_0$ to adapt to these host trypsin-type processing proteases in the airway allows efficient multiplication of the virus in vivo and ultimately becomes epidemic.

## Ectopic trypsin I highly up-regulated by influenza virus infection in the brain induces tissue damage and encephalopathy

Most of the epidemic human IAV are pneumotropic but a few neurotropic strains, such as the 1918-1919 pandemic or IAV WSN/33 (H1N1) strain[10,16] can multiply both in the airway and in the brain. In addition, a rare, but often fatal encephalopathy from infection by a pneumotropic IAV has been reported in children with Reye's syndrome and influenza-associated encephalopathy[17,18]. However, the processing enzyme (s) in the brain has not been reported so far. Recently, we identified an ectopic trypsin I for the IAV $HA_0$ processing proteases in rat brain[19] (Figure 1).

The localization of brain trypsin I determined by immunohistochemistry and in situ hybridization revealed that this enzyme clusters in the hippocampal pyramidal and dentate gyrus

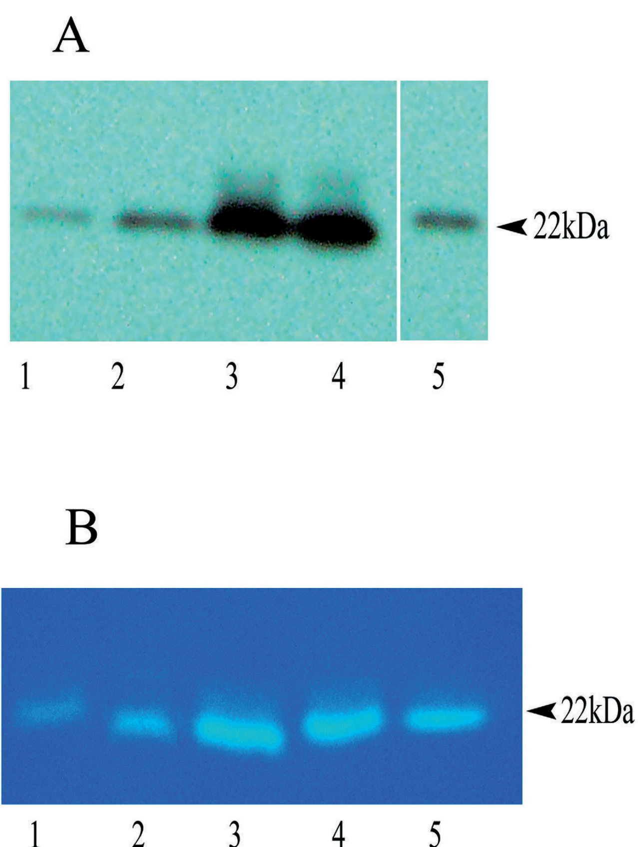


**Figure 1** Brain trypsin I expression is up-regulated after IAV infection. Brain trypsin I expression before and after IAV infection was analyzed by West immunoblotting (A) and fluorescent zymography (B). Brain extracts (50 μg) before virus infection (lane 1) and after virus infection on day 2 (lane 2), day 4 (lane 3) and day 6 (lane 4) were analyzed. Rat pancreatic trypsin (10 ng) as a standard protein was shown in lane 5.

neuronal cells and also distributes in endothelial cells of the brain capillaries, particularly in the allocortex (Figure 2).

The enzyme distribution pattern supports the accumulation of neurovirulent IAV WSN (H1N1) antigen in neuronal cells of the hippocampus[16] and that of pneumotropic IAV Aichi/2/68(H3N2) antigen in the brain capillaries[20]. Up-regulated trypsin in the brain capillaries degraded tight junction components of blood-brain barrier, such as occludin and ZO-1, and also laminin, resulting in encephalopathy with severe brain edema. To prevent the trypsin-mediated degradation cascade and brain edema, development of the drugs for inhibition of trypsin activity in the brain and for suppression of trypsin transcription is necessary.

## Endogenous inhibitory compounds for HA processing proteases

Under physiological conditions, the activity of $HA_0$ processing proteases is strictly regulated by endogenous inhibitors in the airway, such as secretory leukoprotease inhibitor (SLPI) in the upper respiratory tract[21] and pulmonary surfactant as an adsorbent molecule for

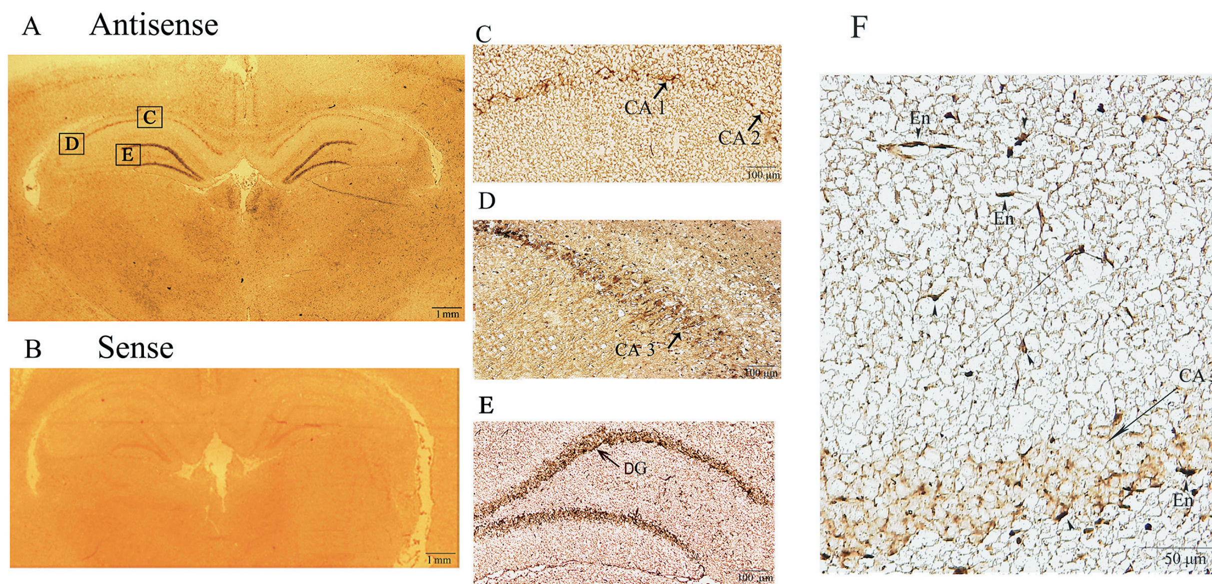


**Figure 2** In situ hybridization of trypsin I in rat brain. Low-power photomicrographs of rat brain section, hybridized with rat trypsin I antisense probe (A) and sense probe (B). Bars in A and B present 1mm. High power photographs of A show that the positive staining is localized in pyramidal neurons of the cornuammonis (CA) 1-2 (C) and CA-3 (D), and in the granular cells of the dentate gyrus (DG) (E). Capillary endothelial cells (En) were also heavily stained (D and F). Bars in C, D and E present 100 μm and bar in F presents 50 μm.

tryptase Clara in the lower respiratory tract [22,23]. However, the levels of these inhibitory compounds are lower than those of trypsin-type proteases under conventional airway conditions.

We identified SLPI as an endogenous inhibitor of tryptase Clara in the upper division of the airway, with a Ki value of $9.7\times10^{-8}$ M and which thus acts as a defensive compound against IAV infection[21]. SLPI is secreted from non-ciliated secretory airway epithelial cells, such as Clara and goblet cells[24,25], and is found in bronchoalveolar lavage fluid (BALF) and nasal and salivary secretions[26], as well as in the walls of the alveoli[27]. Intranasal administration of recombinant SLPI (rSLPI) at a dose of 6μg 15 times every 8 h after infection of rats with $1\times10^{4}$ plaque forming unit (PFU), a semi-lethal dose, of mouse-adapted IAV Asia/1/57 (H2N2) virus, the lung viral titer was markedly reduced to less than 10% of that without rSLPI treatment. The mean (±SD) SLPI levels in normal BALF and nasal fluids were 11.0±1.2 nM[28] and 208.9 ±44.5 nM[29], respectively, which are apparently insufficient for protection against viral infection. These results suggest that intranasal administration of SLPI can efficiently prevent IAV infection.

The other inhibitory compound against IAV infection in the lower division of the airway is pulmonary surfactant, a lipoprotein complex, which coats the alveolar epithelium to lower the surface tension at the air-liquid interface and increase phagocytosis by mononuclear cells and alveolar macrophages. Pulmonary surfactant efficiently adsorbs tryptase Clara and inhibits the activity with a Ki value of 0.13μM, although it does not inhibit anionic trypsin I in the lungs[22]. Intranasal administration of pulmonary surfactant every 6-8 h shortly after infection, multiplication in the lungs of Sendai virus which causes pneumonia in the similar way to influenza virus was suppressed to approximately 10% of

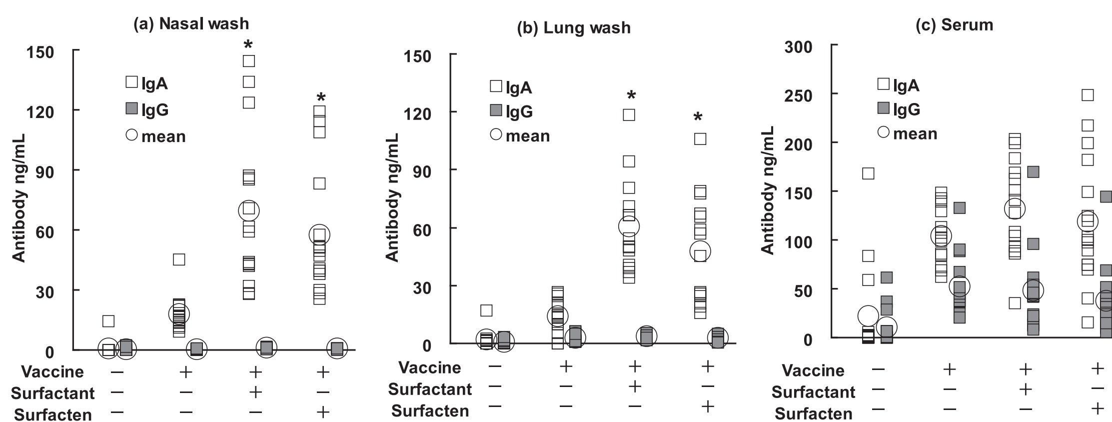


**Figure 3** Effects of intranasal inoculation of pulmonary surfactant, or Surfacten combined with HA vaccine, on the production of antigen-specific IgA and IgG. Anti-influenza virus IgA (open square) and IgG (shaded square) responses in the nasal wash (a), lung wash (b), and serum (c), 2 weeks after boost intranasal inoculation of 0.2 $\mu$g HA vaccine with or without 0.2 $\mu$g of pulmonary surfactant or Surfacten. Each circle represents the mean antibody concentration in each group of 12-15 mice. * indicates a significant ($p<0.01$) increase in the antibody concentrations in comparison with those measured in mice inoculated HA vaccine alone.

control rats treated with the vehicle[23]. These endogenous inhibitory compounds for virus multiplication in the airway play a role as host-defense factors against IAV infection.

## Modified pulmonary surfactant is a potent adjuvant that stimulates mucosal immunity for prevention

In the studies on pulmonary surfactant administration for inhibition of HA processing protease, we found an increase in the secretory IgA levels in BALF. Based on these evidences, we have tested the adjuvanticity of pulmonary surfactant as a natural adjuvant. The intranasal administration of HA vaccine with a pulmonary surfactant or a modified pulmonary surfactant free of antigenic c-type lectins, Surfacten, as a mucosal adjuvant, induced the highest protective mucosal immunity in the airway (Figure 3).

The intranasal immunization of mice with HA vaccine (0.2$\mu$g) -pulmonary surfactant or Surfacten (0.2$\mu$g) selectively induced the neutralizing anti-HA IgA but not IgG, and conferred nearly maximal protection in the airway, without inducing a systemic response[30]. The intranasal administration of HA vaccine alone induced a limited amount of mucosal IgA against IAV. While the subcutaneous administration of HA vaccine prominently induced serum IgG and IgA, Surfacten and CT-B* did not enhance their induction, and the concentrations of antibodies leaking into the airways were insufficient to prevent viral multiplication[30]. The intranasal administration of HA-Surfacten stimulated the expression of MHC class II, CD40 and CD86 molecules in the CD11c-positive cells isolated from the nasal mucosa, but not cells from the lungs or spleens[30]. Our observations suggest that Surfacten, by mimicking the natural surfactant, is an effective mucosal adjuvant in the process of airway immunization for IAV.

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