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The serine protease inhibitor camostat inhibits influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells

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ABSTRACT

Background: Serine proteases act through the proteolytic cleavage of the hemagglutinin (HA) of influenza viruses for the entry of influenza virus into cells, resulting in infection. However, the inhibitory effects of serine protease inhibitors on influenza virus infection of human airway epithelial cells, and on their production of inflammatory cytokines are unclear.
Methods: Primary cultures of human tracheal epithelial cells were treated with four types of serine protease inhibitors, including camostat, and infected with A/Sendai-H/108/2009/(H1N1) pdm09 or A/New York/55/2004(H3N2).
Results: Camostat reduced the amounts of influenza viruses in the supernatants and viral RNA in the cells. It reduced the cleavage of an influenza virus precursor protein, HA0, into the subunit HA1. Camostat also reduced the concentrations of the cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α in the supernatants. Gabexate and aprotinin reduced the viral titers and RNA levels in the cells, and aprotinin reduced the concentrations of TNF-α in the supernatants. The proteases transmembrane protease serine S1 member (TMPRSS) 2 and HAT (human trypsin-like protease: TMPRSS11D), which are known to cleave HA0 and to activate the virus, were detected at the cell membrane and in the cytoplasm. mRNA encoding TMPRSS2, TMPRSS4 and TMPRSS11D was detectable in the cells, and the expression levels were not affected by camostat.

Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; ANOVA, analysis of variance; CLEIA, chemiluminescent enzyme immunoassay; COPD, chronic obstructive pulmonary disease; DF-12, mixture of Dulbecco's modified Eagle's medium-Ham's F-12 medium; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; F-12, Ham's F-12 medium; HA, hemagglutinin; HAT, human trypsin-like protease; IFN, interferon; IL, interleukin; KIU, Kallikrein Inhibitor Unit; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MDCK, Madin Darby Canine Kidney; MEM, Eagle's minimum essential medium; MOI, multiplicity of infection; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline with Tween® 20; PVDF, polyvinylidene difluoride; RT, room temperature; SARS-CoV, severe acute respiratory syndrome coronavirus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCID, tissue culture infective dose; TMPRSS, transmembrane protease serine S1 member; TNF, tumor necrosis factor; USG, Ultroser G.

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Conclusions: These findings suggest that human airway epithelial cells express these serine proteases and that serine protease inhibitors, especially camostat, may reduce influenza viral replication and the resultant production of inflammatory cytokines possibly through inhibition of activities of these proteases.

1. Introduction

Infection with pandemic or seasonal influenza virus causes a rapid onset of various symptoms, including fever, sore throat, cough and general fatigue [1,2]. Influenza virus infection can be complicated by pneumonia, brain edema and exacerbations of chronic pulmonary diseases [2–4].

Influenza vaccination reduces the mortality rate in elderly people with chronic obstructive pulmonary disease (COPD) [5], and vaccination against influenza is also recommended to prevent the severe side effects of influenza and exacerbations of bronchial asthma [6,7]. Clinically used anti-influenza drugs, which include neuraminidase inhibitors such as oseltamivir and zanamivir, are beneficial for uncomplicated pandemic and seasonal human influenza infection [8,9].

However, several patients with pandemic influenza virus infection have died of pneumonia and multi-organ system failure despite intensive drug treatments, including neuraminidase inhibitors and steroids [2]. Patients infected with highly pathogenic influenza viruses experience increased viral replication and subsequent hypercytokinemia [10,11]. Oseltamivir-resistant influenza A (A/H1N1) virus infection has also been reported, and this type of seasonal influenza has caused severe disease in immunocompromised patients [12]. Therefore, the further development of drugs with anti-influenza and anti-inflammatory effects is needed.

Homma and Ohuchi showed that Sendai virus was activated when trypsin cleaved the viral surface glycoprotein, fusion protein (F), from the inactive precursor (F0) to F1 and F2 heterodimers [13,14]. The cleaved protein fused the viral envelop to the host cell membrane, inducing the viral gene to enter the host cell. Subsequent reports demonstrated that trypsin and other host proteases, by cleaving the surface glycoproteins important for membrane fusion, contribute to the spread of infection and the pathogenicity of many types of viruses, including paramyxoviruses and influenza virus [15,16]. Serine proteases, such as trypsin, transmembrane protease serine S1 member (TMPRSS) 2 and human trypsin-like protease (HAT; also known as TMPRSS11D) are important examples of host proteases responsible for the proteolytic cleavage of the influenza virus hemagglutinin (HA), which is essential for viral gene entry into the cell and the start of viral replication [17,18].

The serine protease inhibitor aprotinin and similar agents, such as leupeptin and camostat, suppress virus HA cleavage and reduce the replication of influenza viruses with a single arginine in the HA cleavage site [19]. The effects of protease inhibitors, including aprotinin, gabexate and camostat, have been studied in Madin Darby Canine Kidney (MDCK) cells and in mice after influenza virus infection [19–21]. Zhirnov et al. showed that viral replication in human adenoid epithelial cells was also inhibited by aprotinin [22]. Another serine protease inhibitor, sivelestat, has been used to treat patients with acute respiratory distress syndrome [23]. However, the inhibitory effects of serine protease inhibitors in clinical use on influenza viral replication and the production of inflammatory mediators in human tracheal and bronchial epithelial cells, the first target of the infection, have not been studied.

In this study, primary cultures of human tracheal epithelial (HTE) cells, which retain the functions of the original tissue [24], were infected with the 2009 pandemic influenza virus, or a seasonal influenza virus, and the effects of serine protease inhibitors on viral replication and cytokine release from the cells were examined.

2. Material and methods

2.1. Human tracheal epithelial cell culture

Human tracheal surface epithelial cells (HTE cells) were isolated and cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (DF-12) medium containing 2% Ultroser G (USG) serum substitute as described previously [24,25]. Tracheas for cell cultures were obtained from 25 patients after death (age, 64 ± 3 yr; 10 female and 15 male). This study was approved by the Tohoku University Ethics Committee.

2.2. Culture of Madin Darby Canine Kidney cells

Madin Darby Canine Kidney (MDCK) cells were cultured in T_{25} flasks in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum [25]. The cells were then plated in 96-well plates and cultured.

2.3. Viral stocks

Stocks of influenza viruses were generated by infecting HTE cells with the pandemic A/H1N1 pdm 2009 virus [A/Sendai-H/N0633/2009 (H1N1) pdm09] or the seasonal A/H3N2 virus [A/New York/55/2004 (H3N2)] [25,26]. The cells were cultured in 24-well plates in a mixture of 0.9 mL of DF-12 medium and 100 μL of MEM containing virus for 1 h. The culture supernatants containing virus were then removed, and the cells were cultured in DF-12 medium containing 2% USG at 37 °C in 5% CO_2-95% air. The supernatants were collected to recover the influenza virus.

To prepare the influenza A/H1N1 pdm 2009 virus, nasal swabs were collected from patients and suspended in MEM medium [26]. The influenza A/H3N2 virus, which was passaged 5–7 times in MDCK cells, was also used to generate viral stocks.

2.4. Detection and titration of viruses

The detection and titration of influenza viruses in the culture supernatants were performed using the endpoint method [27], by infecting replicate MDCK cells in plastic 96-well plates with 10-fold dilutions of virus-containing supernatants, as previously described [25]. The presence of the characteristic cytopathic effects of the influenza virus was then determined. The $TCID_{50}$ (TCID, tissue culture infective dose) was calculated using previously described methods [26], and the viral titers in the supernatants were expressed as $TCID_{50}$ units/mL/24 h [25].