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Magnitude of influenza virus replication and cell damage is associated with interleukin-6 production in primary cultures of human tracheal epithelium

Mutsuo Yamaya[a,*], Lusamba K. Nadine[a], Chiharu Ota[a], Hiroshi Kubo[a], Tomonori Makiguchi[b], Ryoichi Nagatomi[c], Hidekazu Nishimura[d]

[a] Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[b] Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[c] Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
[d] Virus Research Center, Clinical Research Division, Sendai National Hospital, Sendai 983-8520, Japan

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ABSTRACT

Primary cultures of human tracheal epithelium were infected with influenza viruses to examine the relationships between the magnitude of viral replication and infection-induced cell damage and cytokine production in airway epithelial cells. Infection with four strains of the type A influenza virus increased the detached cell number and lactate dehydrogenase (LDH) levels in the supernatants. The detached cell number and LDH levels were related to the viral titers and interleukin (IL)-6 levels and the nuclear factor kappa B (NF-κB) p65 activation. Treatment of the cells with an anti-IL-6 receptor antibody and an NF-κB inhibitor, caffeic acid phenethyl ester, reduced the detached cell number, viral titers and the LDH levels and improved cell viability after infection with the pandemic influenza virus [A/Sendai-H/N0633/2009 (H1N1) pdm09]. A caspase-3 inhibitor, benzyloxycarbonyl-DEVD-fluoromethyl ketone, reduced the detached cell number and viral titers. Influenza viral infection-induced cell damage may be partly related to the magnitude of viral replication, NF-κB-p65-mediated IL-6 production and caspase-3 activation.

1. Introduction

The high virulence of the influenza virus causes severe illness and increases the fatality rate in patients. Precise information regarding the pathogenic magnitude of the influenza viruses is needed for the effective treatment of influenza viral infection and for the prevention of confusion and fear during a pandemic infection. The pathogenic magnitude of the influenza virus has been reported to be associated with the non-structural (NS) gene segment of the influenza A (H5N1) virus (Cheung et al., 2002), contributing to the increase in inflammatory cytokine production. Furthermore, several studies have reported mechanisms of the high pathogenicity, which include the elevation in pulmonary concentrations of inflammatory cytokines, including interleukin (IL)-1, IL-6 and interferon (IFN)-γ, the decrease in anti-inflammatory cytokine production and the elevation in viral replication (de Jong et al., 2006; Lipatov et al., 2005). Influenza viral infection induces viral replication, cytokine production and cell damage in the airway epithelium, which is the first target of the infection. However, the mechanisms and relationship between viral replication, cytokine production and cell damage in the human airway epithelium have not been well studied.

Influenza viral infection-induced production of inflammatory cytokines, including IL-6 and tumor necrosis factor (TNF)-α, and proteases may cause damage to airway and alveolar epithelial cells and vascular endothelial cells (Mauad et al., 2010; Ruwanpura et al., 2011; Wang et al., 2010) and may subsequently exacerbate bronchial asthma and chronic obstructive pulmonary disease, and develop acute respiratory distress syndrome (Nicholson et al., 1993; Perez-Padilla et al., 2009; Rohde et al., 2003). IL-6 and TNF-α are associated with cell death and the activation of caspases in swine macrophages after pandemic A/H1N1 viral infection (Gao et al., 2012). However, the mechanisms for cytokine production-induced airway cell damage resulting from influenza viral infection have not been well studied.

* Corresponding author. Tel.: +81 22 717 7184; fax: +81 22 717 7576.
E-mail address: myamaya@med.tohoku.ac.jp (M. Yamaya).

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Primary cultures of human tracheal epithelial cells may demonstrate the real pathogenic influence of influenza viral infection on the damage, viral replication and inflammatory cytokine production in the airways because human tracheal epithelial cells cultured using the method that we reported previously (Yamaya et al., 1992) retain the functions of the original tissue. In the present study, we infected primary cultures of human tracheal epithelial cells with four strains of influenza virus and measured the magnitude of viral replication, cell damage and cytokine production. We also examined the relationships and mechanisms among viral replication, cell damage and cytokine production in the infection with the pandemic influenza virus [A/Sendai-H/N0633/2009 (H1N1) pdm09].

2. Materials and methods

2.1. Human tracheal epithelial cell culture

The isolation and culture of the human tracheal surface epithelial cells were performed as described previously (Yamaya et al., 2010) 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. The tracheas used for the cell cultures were obtained from 23 patients after death (age, 72 ± 11 yr; 10 female and 13 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 (Numazaki et al., 1987; Yamaya et al., 2010). The cells were then plated in 96-well plates and cultured.

2.3. Viral stocks

Stocks of influenza viruses were generated by infecting human tracheal epithelial cells with four strains of influenza virus as follows: the pandemic A/H1 2009 virus [A/H1N1 pdm 2009, A/Sendai-H/N0633/2009 (H1N1) pdm09] and three strains of the human influenza virus [A/H1N1 Sendai, A/Sendai-H/108/2009/(H1N1); A/H3N2 New York, A/H3N2/New York/55/2004; A/H3N2 Aichi, A/Aichi/2/68 (H3N2)] (Yamaya et al., 2010). The cells were cultured in 24-well plates in 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. To obtain the influenza virus solution, the supernatants were collected.

To prepare the A/H1N1 pdm 2009 virus and the A/H1N1 Sendai virus, which was isolated during the 2008–2009 season, nasal swabs were collected from patients and suspended in MEM medium (Numazaki et al., 1987). The A/H3N2 New York virus and the A/H3N2 Aichi virus, which were passaged 5–7 times in MDCK cells, were also used for the generation of viral stocks.

2.4. Detection and titration of viruses

The detection and titration of influenza viruses in the culture supernatant were performed using the endpoint method (Condit, 2006) by infecting replicate MDCK cells in plastic 96-well plates with 10-fold dilutions of virus-containing supernatants, as previously described (Yamaya et al., 2010). The presence of the typical cytopathic effects of the influenza virus was then examined. The $TCID_{50}$ (TCID, tissue culture infective dose) was calculated using previously described methods (Condit, 2006), and the viral titers in the supernatants were expressed as $TCID_{50}$ units/ml (Yamaya et al., 2010).

2.5. Viral infection of the cells

The infection of human tracheal epithelial cells with influenza was performed using previously described methods (Yamaya et al., 2010). A stock solution of influenza virus was added to the cells in 24-well plates (400 μl in each well, 1.0×10^3 $TCID_{50}$ units/ml, 0.8×10^{-3} $TCID_{50}$ units/cell of the multiplicity of infection). After a 1-h incubation period, the viral solution was removed, and the cells were cultured in 1 ml of fresh medium at 37 °C in 5% CO_2-95% air.

2.6. Collection of the supernatants

The supernatant (300 μl) was collected 1 day (24 h) and 3 days (72 h) after infection, and the same volume (300 μl) of fresh medium was added. The entire supernatant volume (1 ml) was collected 5 days (120 h) after infection. Furthermore, when the influenza virus titers 7 days (168 h) after infection were measured, the supernatant (300 μl) was collected 1 day (24 h), 3 days (72 h) and 5 days (120 h) after infection, and the same volume (300 μl) of fresh medium was added. The entire supernatant volume (1 ml) was collected 7 days (168 h) after infection.

2.7. Measurement of airway epithelial cell damage

To examine the tracheal epithelial cell damage after influenza infection, the number of floating cells in the supernatants, which were detached from the cell sheets adhered on the culture vessels of 24-well plates, and the number and viability of the adhered cells were measured by trypan blue exclusion. Lactate dehydrogenase (LDH) concentrations in the supernatants were also measured. These parameters demonstrate apoptosis and necrosis (Catalani et al., 2013; Cechetti et al., 2007; Yan et al., 2013).

2.8. Quantification of influenza virus RNA

Viral RNA in the cells was measured to confirm differences in the magnitude of viral replication among the species. A two-step real-time quantitative reverse transcription (RT)-PCR was performed using the TaqMan® Gene Expression Master Mix (Applied Biosystems, Bedford, CA, USA) as described previously (Yamaya et al., 2010). The primers and TaqMan probe for the viruses were designed as previously reported (Lorusso et al., 2010; Yamaya et al., 2010). The expression of viral RNA was normalized to the constitutive expression of β-actin mRNA (Suzuki et al., 2002).

2.9. NF-kappa B assay

The presence of p50 and p65 subunits in the nuclear extracts was assayed using a TransFactor Family Colorimetric Kit-NF-κB (BD Bioscience/CLONTECH) as previously described (Yamaya et al., 2011).

2.10. Measurement of cytokine production

We measured IL-6, TNF-α and IFN-γ levels in the supernatants. The measurement of IL-6 and INF-γ was performed using specific enzyme-linked immunosorbent assays (ELISA), and the measurement of TNF-α was performed using chemiluminescent enzyme immunoassay.

2.11. Statistical analysis

The results are expressed as the mean ± SEM. The statistical analysis was performed using a two-way repeated measures