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Doxycycline treatment attenuates acute lung injury in mice infected with virulent influenza H3N2 virus: Involvement of matrix metalloproteinases

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ABSTRACT

Acute respiratory distress syndrome, a severe form of acute lung injury (ALI), is a major cause of death during influenza pneumonia. We have provided evidence for the involvement of recruited neutrophils, their toxic enzymes such as myeloperoxidase and matrix metalloproteinases (MMPs), and neutrophil extracellular traps in aggravating alveolar-capillary damage. In this study, we investigated the effects of doxycycline (DOX), an inhibitor of MMPs, on influenza-induced ALI. BALB/c mice were infected with a sublethal dose of mouse-adapted virulent influenza A/Aichi/2/68 (H3N2) virus, and administered daily with 20 mg/kg or 60 mg/kg DOX orally. The effects of DOX on ALI were determined by measuring inflammation, capillary leakage, and MMP activities. Furthermore, levels of T1-α (a membrane protein of alveolar type I epithelium) and thrombomodulin (an endothelial protein) in the bronchoalveolar lavage fluid were evaluated by Western blot analysis. Our results demonstrate significantly decreased inflammation and protein leakage in the lungs after DOX treatment. Levels of MMP-2 and MMP-9 activity, T1-α and thrombomodulin were also diminished in the DOX-treated group. These findings were corroborated by histopathologic analyses, which demonstrated significant reduction in lung damage. Although DOX treatment reduced ALI, there were no effects on virus titers and body weights. Taken together, these results demonstrate that DOX may be useful in ameliorating ALI during influenza pneumonia. Further studies are warranted to determine whether DOX can be used in combination with anti-viral agents to alleviate severe influenza pneumonia.

Introduction

Influenza A viruses pose significant public health problems with frequent outbreaks worldwide (Ivan et al., 2012). The majority of deaths associated with influenza pneumonia are attributed to the sequelae of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) (Yokoyama et al., 2010). We previously demonstrated that depletion of macrophages results in excessive influx of neutrophils during influenza pneumonia, and contributes to ALI/ARDS with severe hypoxemia. In contrast, depletion of neutrophils leads to only mild lung injury. We have also provided evidence for generation of neutrophil extracellular traps (NETs) that can aggravate alveolar-capillary damage (Narasaraju et al., 2011). Human cases and animal models of influenza pneumonia reveal prominent neutrophilic infiltration within the affected areas, thus implicating their role in lung injury (Wang et al., 2008). The recruitment and activation of neutrophils, together with their released toxic products such as matrix metalloproteinases (MMPs), myeloperoxidase (MPO), elastase, and reactive oxygen intermediates (ROI) can contribute to lung injury. Gelatinases (including MMP-2 and MMP-9) are zinc-dependent endopeptidases, degrade major components of the basement membrane such as gelatin and collagen IV, and exert deleterious effects on the epithelium and endothelium in the thin alveolar-capillary barrier (O'Connor and FitzGerald, 1994).

Gelatinases are implicated in many pathologic conditions including ARDS, cancer, and pulmonary fibrosis (Malemud, 2006; O'Connor and FitzGerald, 1994; Rundhaug, 2003). Infection of different cell lines with influenza virus can activate MMPs. Infection of Madin-Darby canine kidney (MDCK) cells with influenza virus increases MMP-2 activity and decreases MMP-9 activity, whereas MMP-9 activity in Vero cells increases with infection (Yeo et al., 1999). The induction of MMP-9 by tumor necrosis factor-α (TNFα) in the brain has been linked with encephalitis due to influenza infection. Cigarette smoke exposure prior to influenza virus infection further enhances the activities of MMP-2 and MMP-9 (Gualano et al., 2008). Although induction of MMPs during influenza is documented, their functional roles and mechanisms remain unknown. Several studies have shown protective effects of MMP inhibitors during ventilation-induced lung injury and ARDS. Chemically-

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modified tetracyclines (CMTs), doxycycline (DOX), and sivelestat sodium have been tested for their ameliorative effects on ALI. Treatment with a combination of sivelestat sodium and an antiviral agent (oseltamivir) indicated promising results in a patient infected with the novel 2009 swine-origin influenza (Quispe-Laime et al., 2010). Via its MMP inhibitory actions, DOX has been reported to be protective in various pulmonary conditions such as toluene diisocyanate-induced asthma, lipopolysaccharide-induced ALI and pulmonary fibrosis (Fujita et al., 2006, 2007; Lee et al., 2004; Liu et al., 2006). The aim of this study was to investigate whether DOX, an inhibitor of MMPs, exerts ameliorative effects in a murine model of ALI induced by a virulent mouse-adapted strain (P10) of human influenza H3N2 virus (Narasaraju et al., 2009).

Materials and methods

Animal infections and DOX treatment

Female 6–8 week old BALB/c mice were housed in micro-isolator cages in an animal BSL-2 laboratory facility. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore. Six groups of animals were anesthetized with a mixture of 7.5 mg/ml ketamine and 0.1 mg/ml medetomidine, and revived with 100 μl of 1.0 mg/ml of Antisedan (atipamezole hydrochloride) solution by intraperitoneal injection. Mice in the infected group each received a sublethal dose of mouse-adapted strain of influenza A/Aichi/2/68 H3N2 virus, i.e. 40 μl comprising 2×10^5 plaque-forming units (pfu) intranasally, while control mice each received an equal volume of uninfected lung homogenate. DOX treatment was administered daily, starting from 3 days prior to virus infection until 6 days post-infection (dpi). DOX (20 mg/kg or 60 mg/kg) was dissolved in water, and administered orally using a metal gavage. The DOX control group received the same amounts of oral DOX alone. Prophylactic and therapeutic administration of the drug was carried out since previous studies showed that DOX administration after the onset of disease proved to be not as effective as prior administration (Fujita et al., 2006; Ryan et al., 2009).

Cell lines and virus infection

Cell lines including MDCK and LA-4 murine lung epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDCK cells were cultured in minimum essential medium with 10% fetal bovine serum, while LA-4 cells were cultured in F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 20% FBS. LA-4 cells in 6-well plates were infected (at a multiplicity of infection or MOI of 2) with mouse-adapted influenza H3N2 virus containing 0.5 μg/ml of TPCK-trypsin. The plates were then incubated at 37 °C for 2 h, and the medium was replaced with serum-free medium. Aliquots of culture supernatants were collected at 0, 12, 24, and 48 h post-infection for virus titer determination.

Determination of virus titer by plaque assay

Virus titers were assayed by infectivity in MDCK cells by the plaque assay. Briefly, lung homogenates or cell-culture supernatants were serially diluted (from 10^{-1} to 10^{-6}), and added to MDCK cell cultures in 96-well plates. After incubation for 1 h, the inocula were removed, and overlaid with 1 ml of 1.2% Avicel RC-591 (FMC BioPolymer, Philadelphia, PA) before incubating at 35 °C for 2 days. The overlay was then removed, the cells were fixed with 20% formaldehyde solution, and stained with 1% crystal violet. The plates were then washed with water, and the number of pfu of virus per unit measure of sample was calculated.

Bacterial detection by plating on blood agar

To exclude the possibility that our test animals were associated with pulmonary bacterial infection, we plated representative lung samples obtained from mice euthanized at 6 dpi onto blood agar. Using sterile loops, lung homogenates from control and infected mice, with or without DOX treatment were streaked on the surface of blood agar, an enrichment medium to detect the growth of common and even fastidious bacteria. The agar plates were incubated at 37 °C for 48 h, and observed for colony formation.

Evaluation of lung histopathology and viral infection

Lungs from each animal in all groups were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Lung sections (4 μm thick) were stained with hematoxylin and eosin (H&E). A semi-quantitative histopathologic scoring system was implemented in a blinded manner by an experienced pulmonary pathologist (J.E. Seet) at 100× and 400× magnification. Owing to the varying amount of lung tissue present on any one slide, an overall score was assigned per case rather than evaluating a specific number of fields. This approach was logical since influenza pneumonia typically affects the lungs diffusely. The following scoring scheme was based on the extent of damage, i.e. 0: normal; 1: bronchiolitis only, not affecting surrounding alveoli; 2: bronchiolitis and peribronchiolar alveolitis; 3: bronchiolitis and alveolitis affecting airspaces away from bronchioles, patchy, <50%; 4: same as 3 but patchy, >50%; 5: same as 4 but diffuse, >50%. For immunohistochemistry, lung sections were incubated with primary rabbit anti-influenza antibody (1:300 dilution), washed with PBS, and incubated with 1:200 dilution of secondary antibody conjugated to Alexa Fluor 555 dye (Molecular Probes, Eugene, OR). After washing, the slides were mounted with DAPI and examined using an Eclipse E600 microscope (Nikon, Tokyo, Japan) at 400× and 1000× magnification (Narasaraju et al., 2009).

Determination of MPO enzymatic activity

MPO activity in the lung homogenate was assayed as described previously (Narasaraju et al., 2010). Briefly, lung homogenate (20 μl) was mixed with MPO assay solution (980 μl). The latter was prepared fresh before use by mixing 107.6 ml of water, 12 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.192 ml of guaiacol, and 0.4 ml of 0.1 M H_2O_2. The generation of tetraguaiacol was measured spectrophotometrically at 470 nm wavelength, and the change in optical density (OD) per min was calculated from the initial rate. The MPO activity was then calculated using the formula (units/ml = OD/min × 45.1), and expressed as units per mg protein. One unit of the enzyme is defined as the amount that consumes 1 μmol of H_2O_2 per min.

Collection of bronchoalveolar lavage fluid (BALF)

Animals were anesthetized, the trachea was exposed, and the lungs were washed twice with 0.5 ml of cold phosphate-buffered saline (PBS). The recovery of the lavage fluid was more than 90%. Larger volumes of PBS for washing were not used because concentrated BALF samples were needed for Western blot analyses. The BALF samples were centrifuged at 1100×*g* for 10 min, and the supernatants were immediately frozen at −80 °C until further use. The cell pellets were resuspended in PBS, and total inflammatory cell counts were measured using a hemocytometer. For differential cell counts, the cells were processed onto microscopic slides using a cytocentrifuge, and subjected to modified Giemsa staining. Cells (500 per animal) were examined at a magnification of 400×, identified by their typical morphology, and counted.