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/αichi/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.

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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-
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/Chick/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 H2O2. 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 H2O2 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.
Assessment of lung injury

Protein estimation in the BALF supernatant was performed by the Lowry method using the RC DC Protein assay (Bio-Rad, Hercules, CA). We measured MMP-2 and MMP-9 protein levels in BALF by Western blotting, and their activities by gelatinase zymography. The levels of T1-α and thrombomodulin released into the BALF were also determined by Western blotting (Himmelreich et al., 1994; McElroy and Kasper, 2004). An equal volume of each BALF sample was loaded for electrophoresis to ensure that equivalent amounts of BALF were evaluated. For gelatinase zymography, each BALF supernatant (20 μl) was mixed with protein sample buffer comprising 250 mM Tris–HCl pH 6.8, 40% glycerol, 8% SDS, and 0.01% bromophenol blue, and then loaded on 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gels were rinsed with 2.5% Triton X-100 for 30 min, followed by rinsing twice with water to remove Triton X-100. The gels were then incubated at 37 °C for 16 h in developing buffer containing 50 mM Tris–HCl pH 7.7, 200 mM NaCl, 5 mM CaCl2 and 0.2% Brij-35. The gels were stained with 0.5% Coomassie brilliant blue R-250 for 2 h, and destained with 10% methanol, 5% acetic acid in water. Gelatinase activity was depicted as clear zones within a blue-stained background, resulting from digestion of the gelatin present in the gels (Snoek-van Beurden and Von den Hoff, 2005). The activities of MMP-2 and MMP-9 in the culture supernatants of H3N2-infected LA-4 cells were also ascertained as described above.

Table 1
Sequences of primers used for semi-quantitative and quantitative real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>Influenza PA2</td>
<td>CACTATGTTTCAAACACAGGA</td>
<td>TGGGACATGTGATCCAAATAG</td>
</tr>
<tr>
<td>MMP-2</td>
<td>AGCTGTGGACACCTTGCCGAAA</td>
<td>GCTGCTATCTGGGATTGCCAC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>GACTGGGACCACGCTGACTT</td>
<td>TACATGGCCCTTGCCGGCAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>ACTGGGACACATT GGAA</td>
<td>TTCTAGCTGTTGGTGAAG</td>
</tr>
</tbody>
</table>

Classical and real-time quantitative RT-PCR (qRT-PCR) assay for relative mRNA expression

Total RNAs were isolated from the frozen lung tissues using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany), and reverse-transcribed into cDNA with the MMLV Reverse Transcription system (Promega, Madison, WI) using random primers. Classical RT-PCR was carried out using influenza viral PA2 gene primers, and the amplified products subjected to agarose gel electrophoresis. The qRT-PCR assay was performed by the SYBR Green-based technique in a LightCycler system (Roche, Indianapolis, IN) using specific primers targeting MMP-2, MMP-9, and β-actin as the control housekeeping gene (Table 1). The reactions were subjected to an initial denaturation of 95 °C for 10 min, followed by 40 cycles each of 95 °C for 10 s, 55–60 °C for 5 s and 72 °C for 30 s. The Ct values as well as melting curves were derived using the LightCycler software to calculate the relative expression levels (Leong et al., 2005).

Statistical analyses

Results obtained were expressed as mean ± standard deviation (SD). Student’s t-test was employed to perform statistical analyses and comparisons of samples. Values of p<0.05 were considered statistically significant. In our study, Student’s t-test was sufficient since only pair-wise comparisons were made between two groups each time.

Results

Influenza virus infection induces expression and activities of MMP-2 and MMP-9 both in vivo and in vitro

Immunohistochemical detection of influenza virus antigen confirmed viral presence in the bronchiolar epithelium and alveolar regions, with relatively stronger and more diffuse staining on 3 dpi compared to 6 dpi. No virus staining was observed in uninfected control mice (data not shown). H3N2 virus infection induced expression of MMP-2 and MMP-9 in murine lungs in vivo and alveolar epithelial cells in vitro. Protein levels of MMP-2 and MMP-9 in mouse BALF samples were significantly increased at 3 and 6 dpi (Fig. 1A and C). Gelatinase zymography of infected BALF samples at 6 dpi revealed significantly pronounced gelatinolytic activity of MMP-2 and MMP-9 (data not shown). At 6 dpi, there was good correlation with real-time RT-PCR data, when MMP-2 mRNA expression in lung tissues of infected mice (n=10) increased by 9.5-fold versus its uninfected control (n=6), while MMP-9 mRNA expression was even more upregulated in the infected group (n=10) at 13.5-fold higher than its control (n=6), with statistical significance of p<0.05. However, on 3 dpi, no statistically significant elevation of MMP-2 and MMP-9 mRNA expression was detected by real-time RT-PCR (data not shown).

Although neutrophils and macrophages constitute the major source for gelatinases in the lung, influenza virus also stimulated expression of MMPs in mouse lung epithelial cells. H3N2 virus infection of LA-4 cells induced expression of both MMP-2 and MMP-9 in the culture supernatant (Fig. 1B and D). At 48 h after infection of LA-4 cells, gelatinase zymography also revealed enhanced gelatinolytic activity of MMP-2 and MMP-9 by 1.9-fold (p<0.01) and 4.5-fold (p<0.05) respectively to their controls. Virus replication in LA-4 cells was confirmed by cytopathic effect, and by virus titers that increased significantly at 12, 24 and 48 h post-infection, i.e. 7.8 × 104, 4 × 105 and 6.5 × 103 pfu/ml, respectively. Classical RT-PCR with influenza viral PA2 gene primers verified viral replication, with amplicons of increasing intensity with time (data not shown).

DOX treatment reduces expression and activities of MMP-2 and MMP-9 in H3N2-infected lungs

Given that the mice were challenged with a sublethal virus dose and that the animals eventually recovered, the effects of DOX treatment would be more relevant to recovery than mortality. Hence, day 6 post-infection was selected as the experimental endpoint guided by our preliminary studies at this time-point that revealed significant pulmonary damage, with weight loss of 25% that required euthanasia according to IACUC regulations (data not shown). Single daily doses of DOX were sufficient in our study, since this drug is known to be dehydrating and have gastrointestinal side-effects. Animal experiments were performed in two separate batches, and the data from both batches were found to be reproducible and pooled.

Gelatinase zymography and Western blot analyses of the BALF samples were performed to evaluate the effects of DOX treatment on MMP-2 and MMP-9 activities. Significantly decreased expression (Fig. 2A and C) and activities (Fig. 2B and D) of both MMP-2 and MMP-9 were observed after DOX treatment of H3N2-infected mice. The reductions in MMP protein levels and enzymatic activities were comparable in both groups treated with 20 mg/kg and 60 mg/kg DOX. Treatment with DOX alone did not exhibit any changes in the basal MMP activities, and were comparable with control BALF samples.

DOX treatment decreases leukocyte recruitment and MPO levels in H3N2-infected lungs

To determine the cellular infiltrates in the lungs after infection and DOX treatment, BALF-associated cells were subjected to Giemsa
**Fig. 1.** In vivo and in vitro induction of MMP-2 and MMP-9 during influenza virus infection. Western blot analyses depicting MMP-2 and MMP-9 protein expression in control and infected mouse lung samples and LA-4 cell culture supernatants. (A) Blots of lung homogenates of uninfected control mice (CON: n = 6), and mice infected with mouse-adapted influenza A/Aichi/2/68 H3N2 virus (INF: n = 10) on days 3 and 6 post-infection are shown. Recovery of BALF was more than 90% from each animal. Each BALF sample (20 μl) from control and infected animals were loaded for Western blot experiments. (B) Blots representing LA-4 cell culture supernatants from uninfected control (CON: n = 8) and influenza-infected (INF: n = 8) samples are shown at 0, 12, 24, and 48 h time-points. Equal volumes of each culture supernatant were loaded for Western blotting. (C) Densitometric analyses of MMP-2 and MMP-9 bands of BALF samples depicted in (A). Data are represented as percentages of controls ± SD. (D) Densitometric analyses of MMP-2 bands of cell culture supernatants depicted in (B). A statistically significant 2-fold increase in MMP-9 band intensity (p < 0.05) was only noted at 48 h (data not shown).

**Fig. 2.** Effects of DOX treatment on expression and activities of MMP-2 and MMP-9 in mouse lungs. BALB/c female mice were infected with influenza H3N2 virus, and treated with 20 mg/kg or 60 mg/kg DOX. Equal volumes of BALF samples collected at day 6 post-infection were loaded onto the corresponding gels. (A) Western blot analyses for MMP-2 and MMP-9 levels in BALF. The groups shown are uninfected control mice (CON: n = 4); infected mice (INF: n = 8); infected mice treated with 20 mg/kg DOX (n = 8); and infected mice treated with 60 mg/kg DOX (n = 8). (B) Gelatinase zymography gels for the detection of MMP-2 and MMP-9 activities in BALF. The groups shown are uninfected control mice (CON: n = 4); uninfected mice treated with 20 mg/kg DOX (n = 4); uninfected mice treated with 60 mg/kg DOX (n = 4); infected mice (INF: n = 8); infected mice treated with 20 mg/kg DOX (n = 8); and infected mice treated with 60 mg/kg DOX (n = 8). Densitometric analyses of (C) Western blot bands, and (D) gelatinase zymography bands (inverted to black against light background), each expressed as the absolute intensity of the band. Mean values ± SD are shown for the animals in each group.
staining. Elevated numbers of total leukocytes including macrophages, neutrophils, and lymphocytes were observed following H3N2 virus infection. Untreated control mice exhibited low numbers of total inflammatory cells per 10 μl each of their BALF specimens (2.4 × 10^4), compared with the infected control mice (7.6 × 10^4). However, treatment of infected animals with DOX significantly reduced the leukocytic infiltrations (Fig. 3A), implying inhibition of inflammation.

In one batch of experiments, MPO activities of the untreated infected group on 3 dpi (n=10) were 72 units/mg versus 40 units/mg for its control (n=6), whereas on 6 dpi (n=10) these increased to 110 units/mg versus 53 units/mg for its control (n=6), with the differences being significant (p<0.05). This was compatible with the neutrophil infiltration into the lungs of infected mice. Notably, MPO activities were significantly diminished in infected animals treated with DOX (Fig. 3B).

**DOX treatment diminishes lung injury**

AlI was evaluated by protein leakage and detection of membrane proteins of alveolar epithelium and endothelium in BALF. Protein concentration in the BALF of H3N2-infected animals was elevated ~6-fold more than uninfected control mice, suggesting massive protein leakage due to damaged pulmonary lining. However, in infected mice subjected to DOX treatment, BALF protein concentrations were reduced significantly (Fig. 4A). Amelioration of lung injury was further supported by the decreased protein levels of both T1-α and thrombomodulin in the BALF of infected animals treated with DOX (20 mg/kg), indicating diminished damage of the alveolar-capillary barrier (Fig. 4B and C). Interestingly, the 60 mg/kg of DOX treatment group did not exhibit T1-α and thrombomodulin reduction, despite the significantly improved lung pathology. Uninfected control samples showed the presence of only traces of these membrane proteins in the BALF.

**DOX treatment ameliorates H3N2-induced pathologic lesions in lungs**

Previously, we demonstrated that our mouse-adapted virus induces severe inflammation and causes AI (Narasaraju et al., 2009). In this study, we evaluated DOX treatment on histopathologic changes. DOX treatment alleviated the lung injury compared to the untreated infected group. The uninfected control group did not show any changes in the lung architecture (Fig. 5A, panel i), and was compatible with treatment with DOX alone (Fig. 5A, panels ii, iii). In contrast, severe bronchiolitis with massive inflammatory infiltration and diffuse alveolar damage were observed in infected animals. Extensive inflammation was accompanied by pulmonary edema, and accumulation of cellular debris was more prominent (Fig. 5A, panel iv). Interestingly, there was significant reduction in inflammation and damage to bronchioles after DOX treatment. Administration of both DOX treatments decreased the extent of inflammation, alveolitis and pulmonary edema (Fig. 5A, panels v, vi).

At 6 dpi, the lung histopathology scores with both DOX treatments were lower at ~3, compared to the untreated infected group with a score of ~3.5 (Fig. 5B), whereas the score for the untreated infected...
Complications of ARDS with alveolar-capillary damage, pulmonary edema, and severe hypoxemia are the commonest causes of death in influenza pneumonia (Bdeir et al., 2010; Fingleton, 2007; Quispe-Laime et al., 2010). We have recently demonstrated that neutrophils recruited into the lungs contribute to ALI and ARDS in influenza pneumonia. In this study, we showed that DOX treatment ameliorates ALI and pathologic complications that are aggravated during influenza pneumonia. DOX treatment reduced the degree of lung inflammation, and reduced MMP-2 and MMP-9 enzyme activities. However, DOX treatment did not alter the lung viral titers and body weights of infected animals. These results suggest that DOX may be used for reducing ALI in influenza pneumonia.

We and other investigators have found that gelatinase enzymes are induced during influenza virus infection (Dessing et al., 2009; Gualano et al., 2008; Narasaraju et al., 2011; Wang et al., 2010). The accumulated MMP-2 and MMP-9 can aggravate pulmonary damage by degrading the basement membranes of the alveoli. The induction of MMP-9 by TNFα can activate the mitogen-activated kinase (MAPK) pathway during influenza virus infection. Interestingly, MMP-9 is also induced in the brain and heart, which may contribute to the multi-organ failure observed in certain cases of severe influenza pneumonia (Wang et al., 2010). Previously, we also found that our mouse-adapted virulent H3N2 virus causes inflammation, and damage of other major organs, including the brain and heart (Narasaraju et al., 2009). Proteolytic activity of gelatinases is also associated with ALI/ARDS in several other diseases (Corbel et al., 2000; O’Connor and FitzGerald, 1994). The increased concentration of MMP-9 may be induced by elevated levels of activated neutrophils in the influenza virus-infected respiratory tract since MMP-9 is secreted by neutrophils, and its level is significantly correlated with the increased number of neutrophils in the lung (Fijgier et al., 2006; Masure et al., 1991; Ricou et al., 1996; Torii et al., 1997). Besides neutrophils, activated macrophages can also synthesize MMP-2 and MMP-9 de novo, and these proteinases play a role in macrophage-dependent ALI (Chakrabarti et al., 2006; Gibbs et al., 1999). Lymphocytes also constitute another potential source of gelatinases (Corbel et al., 2000; St-Pierre et al., 2003). We documented that H3N2 infection upregulated the expression of both MMP-2 and MMP-9 in LA-4 alveolar epithelial cells. Interestingly, influenza infection of Vero monkey kidney cells showed induction of
MMP-9, but not MMP-2 (Yeo et al., 1999). Thus, induction of MMPs in epithelial cells may facilitate virus invasion into deeper tissues and dissemination to other organs causing systemic infections.

Induction of MMP activities by virulent influenza suggests that inhibition of these proteases may offer protective effects by reducing immunopathology. Intensive research on MMP inhibitors has been conducted on malignant, cardiac and pulmonary diseases including ARDS (Elkington and Friedland, 2006; Fingleton, 2007; Foda et al., 1999; Overall and Lopez-Otin, 2002). Interestingly, pyrrolidine dithiocarbamate or N-acetyl-L-cysteine inhibits TNFα-induced MAPK-NFκB and AP1-dependent signaling, thus decreasing IκBα phosphorylation and inhibiting NFκB activation, resulting in suppression of MMP-9 activity and tissue injury (Wang et al., 2010). DOX is a tetracycline-related antibiotic that also acts as a non-specific MMP inhibitor. DOX is known to preferentially inhibit MMP-2, MMP-9 and MMP-8 activities, and is a much weaker inhibitor of MMP-1 (Cena et al., 2010). In this study, prior DOX treatment of mice infected with virulent H3N2 virus suppressed MMP-2 and MMP-9 gelatinases (at both protein levels and activities) in BALF samples. It was previously reported that DOX reduces MMP-9 expression in asthma and pulmonary fibrosis models (Fujita et al., 2006; Lee et al., 2004), while DOX treatment decreases gelatinase activities in patients and rat models with abdominal aortic aneurysm (Curci et al., 2000; Petrinec et al., 1996).

Our data demonstrated that prior DOX treatment significantly inhibited inflammation in the lungs after H3N2 infection. DOX treatment significantly improved lung pathology, and decreased protein leakage and alveolar-capillary damage. These results are promising since early tissue damage during influenza infection is associated with the outcome of disease. Furthermore, the positive effects of inhibition of MMPs on ALI support the contribution of these MMPs in the pathogenesis of severe influenza. These results favor the potential application of DOX treatment of influenza pneumonia to control lung damage. Despite diminished histopathologic lesions and scores observed in animals treated with the higher DOX dose of 60 mg/kg, relatively high amounts of membrane proteins of alveolar epithelium and endothelium were detected in the BALF samples. The viral titers in the DOX-treated group were higher than the untreated infected group, suggesting that DOX has no effect on viral replication. The elevated virus titers in the group treated with the higher dose of DOX may be attributed to the significant reduction of inflammatory cellular infiltration which is essential for restricting viral replication during the early stages of infection (Narasaraju et al., 2010). In addition, DOX treatment did not alter animal weight loss, which may be explained by the documented gastrointestinal side-effects of DOX, including anorexia, nausea and diarrhea. However, our histopathologic analysis of the gastrointestinal organs (including stomach and intestines) displayed normal tissues without any pathologic lesions (data not shown). Thus, the weight loss in
DOX-treated infected mice was unlikely to be due to toxic effects on host organs, but may be due to their aversion to food and drink. Secondary bacterial infections are common following primary influenza, and usually lead to considerable morbidity and mortality (van der Sluijs et al., 2010). In our study, we considered the possibility that secondary bacterial infection following influenza may have contributed to lung injury, and that DOX may have exerted its antibiotic effect. However, the absence of any bacterial growth in representative lung homogenate samples of all groups suggested that the reduction of influenza-induced lung injury by DOX was independent of its antibacterial properties, but is mediated through its anti-inflammatory and MMP inhibitory mechanisms.

In conclusion, these findings reveal that MMPs play a critical role in instigating pulmonary injury in mice upon virulent influenza H3N2 virus infection. Treatment with DOX inhibits these protease activities to ameliorate lung injury, thus suggesting that DOX may serve as a useful therapeutic agent in containing ALI during influenza pneumonia. It would be interesting to study whether similar results would be obtained with different DOX concentrations and doses, or in older mice that are at least 10 weeks old. Further studies are also warranted to investigate whether DOX treatment in combination with anti-viral agents may offer a more synergistic strategy in the management of influenza pneumonia.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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