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Original Article

Increase of Apoptosis in a Murine Model for Severe Pneumococcal Pneumonia during Influenza A Virus Infection

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SUMMARY: The mechanisms of severe pneumonia caused by co-infection of bacteria and influenza A virus (IAV) have not been fully elucidated. We examined apoptosis and inflammatory responses in a murine model for pneumococcal pneumonia during IAV infection. Inflammation, respiratory epithelium apoptosis, and inflammatory-cell infiltration increased in a time-dependent manner in the lungs of mice co-infected with Streptococcus pneumoniae and IAV, in comparison with those infected with either S. pneumoniae or IAV. According to appearance of terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling positive cells, caspase-3 and -8 were activated 24 h after S. pneumoniae infection, and caspase-3 activation decreased after 48 h, whereas inflammatory cytokine levels continued to increase in co-infected mice. In contrast, in mice infected with either IAV or S. pneumoniae, apoptosis and activation of factors related to caspase-3 peaked at 48 h. Furthermore, Fas-associated death domain was significantly expressed in the lungs of co-infected mice 24 h after S. pneumoniae infection. These data suggest that early onset of apoptosis and its related factors play important roles in fulminant pneumonia resulting from bacterial pneumonia complicated by co-infection with influenza virus.

INTRODUCTION

Influenza A virus (IAV) is responsible for influenza pandemics. Although IAV frequently causes severe pneumonia that rapidly progresses to acute respiratory distress syndrome, a considerable proportion of mortality is attributed to secondary bacterial infections (1,2).

Studies on the 1918 influenza pandemic identified interactions between IAV and bacterial pneumonia (1,3). The pathophysiological effects of IAV modify the effects of co-infecting pathogens. Proposed mechanisms of these interactions include destruction of the physical barriers against tissue invasion, decreased mucociliary clearance, enhanced receptor expression, and increased adherence to epithelial cells (1,3). We previously identified a significant induction of several mediators, including Toll-like receptors and cytokines/chemokines (4), and DNA microarrays and two-dimensional gel electrophoresis (2DE) have revealed an up-regulation of platelet activating factor-related compounds and neutrophil proteases, respectively, in mice co-infected with IAV and Streptococcus pneumoniae (4–6). We have also shown that both the severity of pneumonia and levels of high mobility group box-1 (HMGB-1) protein are significantly increased in patients co-infected with IAV and S. pneumoniae (7,8). These findings suggest that immune system dysregulation is critically associated with the severity of pneumonia associated with IAV and S. pneumoniae co-infection.

The mechanisms by which IAV proteins impair host defenses during bacterial infection have not been fully elucidated; however, it is known that IAV induces apoptosis of airway epithelial cells in vivo and in vitro, thus damaging the lungs (9–11). In addition, Engelich et al. have demonstrated that neutrophils exhibited decreased survival when incubated with both IAV and S. pneumoniae, compared to that observed when incubated with either IAV or S. pneumoniae in vitro; this effect was mediated in part by accelerated neutrophil apoptosis (12). These results suggest that IAV-induced neutrophil dysfunction is an important factor predisposing animals to bacterial infection.

Here, we investigated the correlation between apoptosis and disease severity in a murine model for pneumococcal pneumonia with IAV infection.

MATERIALS AND METHODS

Viral and bacterial infection and sampling: We prepared a mouse-adapted influenza virus A strain (A/PR8/34; H1N1) and S. pneumoniae (penicillin-susceptible S. pneumoniae serotype 19F, ATCC 49619) as previously described (4,5). Male 6-week-old CBA/J mice purchased from Charles River Laboratories (Hino, Shiga, Japan) were intranasally inoculated with 5 × 10^6 plaque-forming units (PFU) of the influenza virus, followed by 5 × 10^6 colony-forming units (CFU) of S. pneumoniae 48 h later. Mock-infected mice were intranasally inoculated with 50 μL of saline at the same time points. Lungs harvested at various time points thereafter were homogenized in saline (2 mL) and separated by centrifugation at 13,000 × g for 5 min at 4°C.
Supernatants were pooled, aliquoted, and stored at −80°C for later analysis. We anaesthetized 5–6 mice with pentobarbital (50 µg/kg body weight) for each experiment as previously described (14). All animal experiments were approved by the University Committee on Use and Care of Animals at Nagasaki University.

**Histopathological analysis and TUNEL staining:** Lung tissue sections were paraffin-embedded and stained with hematoxylin and eosin (HE) using standard procedures. Paraffin-embedded sections were assayed by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) using Apoptosis *in situ* Detection Kits (Wako, Osaka, Japan).

**Immunohistochemistry for FADD:** We used immunohistochemistry (IHC) to detect Fas-associated death domain (FADD) protein using VECTASTAIN ABC kits (Vector Laboratories, Burlingame, Calif., USA).

**Lung sections were digested with proteinase, and endogenous peroxidase was removed. Non-specific binding was blocked with diluted normal serum and then the sections were incubated with anti-FADD antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), followed by diluted biotinylated secondary antibody. The sections were incubated with VECTASTAIN ABC reagent, and the products were visualized by diaminobenzidine staining.

**ELISA of mouse lungs:** Concentrations of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, macrophage inflammation protein (MIP)-2, granulocyte macrophage colony-stimulating factor (GM-CSF), and keratinocyte-derived chemokine (KC) in lung homogenates were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn., USA).

**Measurement of caspase-3 activity:** Caspase-3 activity was measured using a colorimetric assay (R&D Systems). The chromophore p-nitroanilide (pNA) was spectrophotometrically detected after cleavage from its substrate, N-acetyl-Asp-Glu-Val-Asp (Ac-DEVD)-pNA, by caspase-3. Data were expressed as fold increase compared with control values.

**Western blotting:** Western blotting was performed as previously described (4,5) using primary antibodies, anti-caspase-8 (1:1,000; Cell Signaling Technology, Beverly, Mass., USA) and anti-caspase-3 (1:1,000; Cell Signaling Technology) and the loading control anti-β-actin (1:2,000, Abcam, Cambridge, UK).

**Statistical analysis:** Differences between experimental groups were statistically evaluated using analysis of variance, followed by Fisher’s protected least significant difference test. The data were analyzed using Statview Ver. 5 software (SAS Institute, Cary, N.C., USA). The data are expressed as means ± SD, and *P* values of <0.05 were considered statistically significant.

**RESULTS**

**Pneumonia in co-infected mouse lungs:** Histopathological changes were examined in lung sections stained with HE.

Mild-to-moderate bronchopneumonia progressed over time in the lungs of mice infected with either IAV (Fig. 1A, B, C, and D) or *S. pneumoniae* (Fig. 1E and F). In contrast, acute and progressive pneumonia in the lungs of co-infected mice was accompanied by massive, dense, and diffuse inflammatory-cell infiltration (Fig. 1G) that resulted in focal destruction 48 h after *S. pneumoniae* infection (Fig. 1H).

**Lung inflammatory response in co-infected mice:** We measured levels of TNF-α, IL-6, KC, MIP-2, GM-CSF, and IL-10 (Fig. 2) in mouse lungs over time using ELISA.

Levels of IL-10 were slightly increased after 48 h, and levels of TNF-α, IL-6, KC, MIP-2, and GM-CSF gradually increased until 96 h after infection with IAV.
alone. Levels of these cytokines and chemokines were increased 48 h after infection with *S. pneumoniae* alone. In the lungs of co-infected mice, the levels of these cytokines and chemokines were significantly elevated in a time dependent manner, compared to those in mock- or singly-infected mice. Although these levels were already higher in co-infected mice 24 h after *S. pneumoniae* challenge (72 h after IAV infection), they further increased 48 h after *S. pneumoniae* infection, unlike in mice infected with either agent.

**TUNEL analysis:** Because apoptosis is important for eliminating damaged or infected cells and for down-regulating inflammation (13), we performed TUNEL staining to assess the relationship between apoptosis and the severity of influenza virus-related pneumonia after *S. pneumoniae* infection.

The number of TUNEL-positive cells gradually and time-dependently increased in mice infected with either influenza virus (Fig. 3A, B, G, and H) or *S. pneumoniae* (Fig. 3C, D, I, and J) and peaked 48 h after bacterial challenge. In contrast, TUNEL-positive cells acutely increased 24 h after *S. pneumoniae* infection in co-infected mice (Fig. 3E and K), and the lungs of these mice were obviously damaged. The number of TUNEL-positive cells then slightly decreased 48 h after *S. pneumoniae* infection (Fig. 3F and L). A few respiratory epithelial cells and many inflammatory cells, such as alveolar macrophages and some neutrophils, were TUNEL-positive.

These data suggested that apoptosis was induced significantly earlier in the lungs of co-infected mice compared to the lungs of mice infected with either virus or bacteria.

**Caspase expression and activation:** Caspase-8 is a key enzyme that has been intensively studied as an initiator, and caspase-3 plays a central role in the regulation of apoptosis (15,16). We therefore evaluated caspase-3 activity and the expression of caspase-3 and -8 in mouse lungs.

Caspase-3 activity was induced after 24 h and rapidly decreased 48 h after *S. pneumoniae* infection in co-infected mice, whereas such activity gradually increased and peaked at 48 h in the lungs of mice infected with either IAV or *S. pneumoniae* (Fig. 4).

The cleaved forms of caspase-3 and -8 showed tendency to up-regulation and peaked 24 h after *S. pneumoniae* infection in lungs of co-infected mice; however, statistical significance was not achieved when compared to either 0 h or 48 h after *S. pneumoniae* infection (Fig. 5). These data indicate an earlier onset and an increased number of TUNEL-positive cells in the lungs of co-infected mice.

Levels of caspase-3, -8, and their cleaved forms also showed tendency to higher expression in the lungs of co-infected mice compared to the lungs of mice that were infected with either IAV or *S. pneumoniae* 24 h after *S. pneumoniae* inoculation (72 h after IAV); however, statistical significance was not found (Fig. 6).

**Expression of FADD protein:** We used IHC to detect FADD protein, which is the main adaptor protein and essential for transmitting apoptotic signals through death receptors (17). The number of cells positive for FADD protein was significantly increased in the lungs of co-infected mice (Fig. 7D and H) compared to the lungs of mice infected either IAV or *S. pneumoniae* 24 h after *S. pneumoniae* infection (Fig. 7B, C, F, and G).
Fig. 3. TUNEL staining of lung sections at × 200 (A–F) and × 400 magnification (G–L). Mice infected with influenza virus 72 h (A and G) and 96 h (B and H), with S. pneumoniae 72 h (C and I) and 96 h (D and J), and co-infected mice 72 h (E and K) and 96 h (F and L) after influenza virus inoculation. Cells with brown nuclei are undergoing apoptosis.

Fig. 7. Immunohistochemical staining to detect FADD in mice infected with IAV and/or S. pneumoniae. Mice were mock-infected (saline, A and E), or infected with IAV (B and F), or S. pneumoniae (C and G), or both (D and H) and then lungs were collected 72 h after IAV inoculation. Magnification ×200 (upper panels) and ×400 (lower panels). Cytoplasm and nuclei in cells from lungs are intensely stained with anti-FADD antibody.

Many stained alveolar macrophages in the lungs of co-infected mice correlated with inflammation and apoptosis; in contrast, alveolar epithelial cells were intensely stained in the lungs of mock-infected mice (Fig. 7A and E), but the decreased number of FADD-positive cells in the lungs of co-infected mice, which were negatively correlated with inflammation and apoptosis.

DISCUSSION

Apoptosis is a highly regulated, morphologically and biochemically defined form of cell death (17) that eliminates damaged or infected cells (14). Infection with IAV results in the induction of apoptosis both in vitro and in vivo (10,18,19), and IAV recruits several host pro-apoptotic signaling cascades in infected cells (20). However, it remains unclear whether these responses are advantageous for viral replication or for host cell defenses (15,20). In addition, apoptosis occurs during pneumococcal pneumonia and it is an established fea-
Fig. 5. Time course of caspase-3 and -8 expression. Caspase-3 and -8 expression in mice co-infected with influenza virus and *S. pneumoniae* was determined at 0, 48, 72, and 96 h by Western blotting (A), and intensity of cleaved caspase-8 (B) and -3 (C) bands was analyzed. Lung extracts were probed with antibody against caspase-8 (57 kDa, full length; 18/45 kDa, cleaved fragments) and caspase-3 (35 kDa, full length; 17/19 kDa, large catalytic subunit) in immunoblots. Internal control is β-actin. *P < 0.05. Each experiment was repeated 4–6 times.

Fig. 6. Caspase-3 and -8 expression in mice infected with influenza virus and/or *S. pneumoniae* 24 h after bacterial challenge. Expression was determined by Western blotting (A), and intensity of cleaved caspase-8 (B) and -3 (C) bands was analyzed. *P < 0.05. Each experiment was repeated 4–6 times.
tue of *S. pneumoniae* infection (13,21–23).

In the present study, infection with IAV or *S. pneumoniae* induced the caspase-dependent apoptotic pathway and caused a slow increased in the number of TUNEL-positive cells, which mainly comprised alveolar macrophages. In contrast, secondary pneumococcal infection induced an earlier and significantly more severe response in mice 24 h after bacterial challenge. Furthermore, the expression of caspase-3, -8, and their cleaved forms as well as the activity of caspase-3 peaked 24 h and then decreased 48 h after *S. pneumoniae* infection in co-infected mice.

The expression of FADD protein, which leads to caspase-8 and -3 activation through extrinsic receptors, was also significantly induced by bacterial inoculation 24 h after co-infection, compared with IAV or *S. pneumoniae* infection alone. These data suggested that earlier and more vigorous activation of apoptosis and its related molecules induces fulminant and more severe pneumonia in co-infected lungs, compared with either viral or bacterial infection.

Besides its central role in regulating death signals, FADD can positively or negatively regulate the NF-κB signaling pathway (16). One type of FADD-mediated NF-κB activation paradoxically involves stimulation through death receptors. Furthermore, TNF-R1 and Fas signaling can induce either apoptosis or several non-apoptotic processes, such as cell growth and proliferation.

Although FADD might contribute to both TNF-R1- and Fas-induced NF-κB activation (24), the Fas-FADD-mediated apoptosis of vascular smooth muscle cells (SMC) under physiological conditions also leads to the transcription of genes encoding chemokines, including IL-1α, IL-8, and monocyte chemoattractant protein-1 (MCP-1), which account for the recruitment of macrophages within the blood vessels (25). Macrophages normally contribute to the removal of cell bodies and remodeling of the extracellular matrix. However, excessive Fas-FADD-induced SMC apoptosis results in a massive inflammatory response. Therefore, the balance between FADD-mediated cell death and FADD-mediated NF-κB activation could be important for converting Fas-apoptotic signals into an inflammatory NF-κB-mediated response (16).

We could not observe stained SMC, but we observed that stained alveolar macrophages in the lungs of co-infected mice correlated with inflammation and apoptosis. These findings suggest that excessive inflammation was induced in the lungs of co-infected mice, which in turn contributed to their severe lung damage.

In contrast, alveolar epithelial cells were intensely stained in the lungs of mock-infected mice, whereas the decreased number of FADD-positive epithelial cells in the lungs of co-infected mice negatively correlated with inflammation and apoptosis. Although alveolar epithelial cells were damaged and disappeared from the severely inflamed lungs of co-infected mice, the discrepancy in FADD expression between alveolar macrophages and epithelial cells might be critical for the mechanisms of severe influenza-related pneumonia. Further investigation is required to examine this hypothesis.

Although induced directly by viral and bacterial infections, the earlier and more striking apoptosis of lung cells including airway epithelial cells and infiltrating macrophages/neutrophils in co-infected mice during the evolution of infection can also be driven by other factors. One likely candidate is TNF-α, which promotes apoptotic pathways, and its expression is enhanced in co-infected mice. Alternative candidate pathways include interactions with Fas-Fas ligands, which are major mediators of alveolar epithelial cells apoptosis in acute lung damage and severe influenza pneumonia. We did not find increased levels of Fas-Fas ligands as an increase in TUNEL-positive cells and caspase activity in co-infected or singly-infected lungs (data not shown).

We speculate that the apoptosis of lung structural cells contributes to the enhanced severity of lung damage in co-infected mice.

The immune response to infection requires a balance between inflammatory cell proliferation and elimination (26). However, we found that levels of inflammatory cytokines/chemokines continued to increase, whereas caspase-3 activity had already declined 48 h after pneumococcal infection in co-infected mice. We previously reported that number of inflammatory cells increase in the bronchoalveolar lavage fluid and that neutrophil-related proteases are significantly upregulated 48 h after *S. pneumoniae* infection in co-infected mice (4,5). Although we did not detect TUNEL-positive neutrophils in this study, an imbalance between apoptotic signals and proliferative factors associated with alveolar macrophages might disrupt immune responses and reflect excessive inflammation, such as a "cytokine storm," during co-infection with IAV and *S. pneumoniae*.

The cytokine storm results from excessive cytokine/chemokine production, which paradoxically damages tissue during severe inflammatory infections, such as sepsis and influenza pneumonia (27–29). Therefore, the innate immunity system is not only the first line of host defense against infection, but can also be a "double-edged sword" (30). We also previously described a possible role for excessive inflammation in the severity of influenza pneumonia in IRAK-M knockout mice (31). Further studies are required to understand the function and mechanisms of cytokine/chemokine imbalance, apoptosis, and caspase-related molecules and to determine whether therapeutic interventions against cytokines/chemokines and/or apoptosis would be effective in the treatment of severe pneumococcal pneumonia during IAV infection.

In summary, we analyzed the apoptotic response to simultaneous infection with IAV and *S. pneumoniae* in a mouse model. High levels of caspase-3 and -8 were expressed and activated, and the number of TUNEL-positive cells and levels of FADD protein expression were significantly increased during the early inflammatory response. The early onset of apoptosis might be causally related to the more severe pneumonia during co-infection.

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**Conflict of interest** None to declare.
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