Combined effect of anti-high-mobility group box-1 monoclonal antibody and peramivir against influenza A virus-induced pneumonia in mice

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ABSTRACT

Human pandemic H1N1 2009 influenza virus causes significant morbidity and mortality with severe acute lung injury (ALI) due to excessive inflammatory reaction, even with neuraminidase inhibitor use. The anti-inflammatory effect of anti-high-mobility group box-1 (HMGB1) monoclonal antibody (mAb) against influenza pneumonia has been reported. In this study, we evaluated the combined effect of anti-HMGB1 mAb and peramivir against pneumonia induced by influenza A (H1N1) virus in mice. Nine-week-old male C57BL/6 mice were inoculated with H1N1 and treated with intramuscularly administered peramivir at 2 and 3 days post-infection (dpi). The anti-HMGB1 mAb or a control mAb was administered at 2, 3, and 4 dpi. Survival rates were assessed, and lung lavage and pathological analyses were conducted at 5 and 7 dpi. The combination of peramivir with the anti-HMGB1 mAb significantly improved survival rate whereas the anti-HMGB1 mAb alone did not affect virus proliferation in the lungs. This combination therapy also significantly ameliorated histopathological changes, neutrophil infiltration, and macrophage aggregation by inhibiting HMGB1, inflammatory cytokines, and oxidative stress. Fluorescence immunostaining showed that the anti-HMGB1 mAb inhibited HMGB1 translocation from type I alveolar epithelial cells. In summary, combining anti-HMGB1 with conventional anti-influenza therapy might be useful against severe influenza virus infection.

KEY WORDS: H1N1; acute lung injury; cytokine; oxidative stress; virus propagation

INTRODUCTION

Influenza causes significant morbidity and mortality globally every year. While most cases appear to be self-limiting and uncomplicated, a considerable number of patients develop respiratory complications that can lead to influenza-related hospitalization and death.[1-3] Antiviral drugs, including neuraminidase inhibitors (NAIs), are usually effective in preventing infection or ameliorating disease severity;[4] however, the effectiveness of NAI was insufficient in many cases of the pandemic H1N1 2009 influenza.[1,2] Thus, there is a pressing need to identify a novel therapeutic target of influenza pathogenesis and progression. These severe cases are typically caused by the cytokine storm, excessive inflammatory infiltration, and virus-induced tissue destruction,[4-6] that is, excessive cytokines and chemokines are the key factors.[7] Therefore, treatment with anti-inflammatory agents along with antiviral agents might be a promising strategy against severe influenza.[8]

The inflammatory role of high-mobility group box-1 (HMGB1) has been recently investigated in influenza infection.[8] HMGB1 is a highly conserved non-histone chromosomal protein and originally identified as a DNA-binding protein. HMGB1 sustains nucleosome structure and regulates gene transcription.[9] It is either actively released by activated immune cells or passively released from damaged/necrotic cells. The extracellular HMGB1 can act as a

proinflammatory cytokine-like mediator. HMGB1 has been reported to be involved in the pathogenesis of a wide range of diseases, including sepsis, traumatic brain injury, and several viral infections.[9,10] The effect of anti-HMGB1 monoclonal antibody (mAb) treatment in a murine model of influenza-induced pneumonia[11] and brain edema[12] has been reported. In the pneumonia model, anti-HMGB1 mAb improved the survival rate significantly and ameliorated lung damage without an anti-influenza drug.[11]

The US Centers for Disease Control and Prevention and the World Health Organization recommend NAI use for the treatment of seasonal and pandemic influenza,[13,14] and treatment with NAI is the main strategy for managing influenza infection.[2] In severe influenza cases that cannot be treated with NAI alone, combination treatment with an anti-HMGB1 mAb represents an interesting treatment option. In the present study, a severe influenza murine model was developed and the combined effect of an anti-HMGB1 mAb and peramivir[15] (intravenous NAI) treatment was examined. Furthermore, the role of the anti-HMGB1 mAb in the translocation of HMGB1 from type I alveolar epithelial cells (AECI) was investigated.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Animal Use Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (No. OKU-2015424), following the guidelines of the National Institutes of Health.

Establishment of NAI-insufficient influenza pneumonia mouse model

Nine-week-old male C57BL/6 mice were obtained from Charles River Laboratories (Yokohama, Japan) and were housed in a specific pathogen-free animal facility controlled at 25 °C with a 12-h light/dark cycle. They were fed a regular pellet diet (Oriental MF; Oriental Yeast, Ltd., Tokyo, Japan) and given *ad libitum* access to drinking water.

The influenza virus strain A/Puerto Rico/8/34 (H1N1) used in this study is a highly virulent, mouse-adapted virus, which was prepared as described previously.[11] An A/Puerto Rico/8/34 (H1N1) infection is treatable with NAI.[16]

The 50% mouse lethal dose (MLD_{50}) was determined as 100 plaque-forming units (pfu). After being anesthetized with intraperitoneal ketamine (50 mg/kg) and pentobarbital (30 mg/kg), the mice were infected intranasally with 1,500 pfu (15 MLD_{50}) of H1N1 in 25 μL of sterile

phosphate-buffered saline (PBS). Then, the mice were allowed to recover. We defined the day of the virus infection as 0 days post-infection (dpi). Peramivir (RAPIACTA®; 10 mg/kg) (SHIONOGI & CO., LTD.) was administered intramuscularly at 2 and 3 dpi in peramivir-treated groups.

After infection with the virus, the mice were randomly divided into four groups: 1, class-matched control mAb only group; 2, anti-HMGB1 mAb only group; 3, peramivir and class-matched control mAb-treated group; 4, peramivir and anti-HMGB1 mAb-treated group. An anti-HMGB1 mAb (#10-22, IgG2a subclass; 7.5 mg/kg) or a class-matched control Ab (anti-keyhole limpet hemocyanin) was given intravenously through the caudal vein at 2, 3 and 4 dpi after the virus infection in accordance with the treatment schedule for each group. These mAbs were produced as described previously.[17] The dose of anti-HMGB1 mAb was considered sufficient.

Survival rate analysis

The survival rates of all mice, including those administered the anti-HMGB1 mAb or control mAb without peramivir, were evaluated until 28 dpi. Our influenza pneumonia model showed severe lung injury; thus, we could not collect favorable samples from the groups without peramivir treatment (Groups 1 and 2 described above). Therefore, the following experiments were performed

among the groups with peramivir treatment (Groups 3 and 4 described above) to assess the additional impact of anti-HMGB1 mAb treatment.

Pathological analysis

After H1N1 inoculation, pathological analyses were conducted at 5 and 7 dpi. Animals were subjected to euthanasia before collecting their blood and bronchoalveolar lavage fluid (BALF) for measuring cytokines/chemokines and hydroperoxides.

The severity of the lung injury was scored as described previously.[18] Briefly, the following four readily identifiable pathological processes were graded semi-quantitatively on a scale of 0 to 4: the onset of bronchiolitis, alveolar and interstitial edema, margination and infiltration of inflammatory cells, and hemorrhage. A score of 0 represented a normal lung; scores 1 to 4 represented mild (1), moderate (2), severe (3), and very severe (4) changes. The calculation was performed by adding the individual grades (mean value for four processes) for each category. Lung histological analysis was performed by two of the authors (K.H. and N.N.) in a blinded manner.

Bronchoalveolar lavage was performed as reported earlier.[19] Briefly, the right lung was lavaged with 1 mL of cold PBS after the ligation of the left main bronchus. The collected BALF was spun down at 2,000 rpm for 10 min at 4 °C, and its supernatant was kept at −80 °C for the

measurement of cytokines. A total neutrophil count in the BALF was performed using a 200-μL pellet aliquot. Cell differentiation was evaluated using Diff-Quick staining (Sysmex, Kobe, Hyogo, Japan) for at least 200 neutrophils on a smear prepared from the pellet. The total number of neutrophils and macrophages in BALF was measured and expressed per animal.

Immunohistochemistry

Immunohistochemical staining was conducted as previously described[17] using antibodies against podoplanin (Sino Biological, Inc., Daxing Qu, Beijing, China), pan-cytokeratin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), ATP-binding cassette sub-family A member (Abcam, Cambridge, UK), TLR4 (Abcam, Cambridge, UK) and HMGB1 (Abcam and R&D Systems Inc.).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the middle part of the left lung using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An aliquot of 1 μg of RNA per reaction was prepared using the NanoDrop™ One (Thermo Fisher Scientific, Madison, USA) and reverse-transcribed to cDNA according to the instructions of the RETROscript kit (Applied Biosystems, Foster City, CA, USA). The 7500 real-time qPCR system was used to

determine virus copy numbers. The PCR primers were designed as described previously.[18,19]

Measurement of toll-like receptor 4 (TLR4), cytokines, and hydroperoxides

TLR4 levels were quantified using commercial enzyme-linked immunosorbent assay kits (USCN Life Science, Inc., Wuhan, China) following the manufacturer's instructions. Granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-α (TNFα), chemokine (C–X–C motif) ligand 1 (CXCL1), monocyte chemotactic protein 1 (MCP1), chemokine (C–C motif) ligand 3 (CCL3), chemokine (C–X–C motif) ligand 10 (CXCL10), and regulated on activation, normal T cell expressed and secreted (RANTES) were detected using a mouse cytokine/chemokine magnetic bead panel (Millipore, Billerica, MA, USA) and a Luminex 100 system (Millipore). Interleukin-6 (IL-6) was quantified using commercial enzyme-linked immunosorbent assay kits (R&D Systems Inc.) according to the manufacturer's instructions.

The hydroperoxide level (whole oxidant capacity of plasma against *N,N*-diethyl-*p*-phenylenediamine in an acidic buffer) in plasma was measured using a free radical elective evaluator (FREE CARRIO DUO; Diacron, Grosseto, Italy). It has been established that one measurement unit (CARR U) corresponds to 0.08 mg/dL hydrogen peroxide.[20]

Statistics

Data are presented as the mean ± standard error of the mean. All data were compared using the Mann–Whitney *U*-test or log-rank test where appropriate. The statistical analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Anti-HMGB1 mAb improves survival by inhibiting TLR4 in the lung

Most mice without peramivir treatment died at 7 and 8 dpi (Fig. 1A). 90.9% of the mice that were treated with a combination of the anti-HMGB1 mAb and peramivir survived, whereas only 45.5% of the control mice (control mAb + peramivir) survived. Because the body weight of the mice was the lowest at 7 dpi, 5 and 7 dpi were chosen as time points for subsequent analysis.

No significant difference of the viral copies in the lung at either 5 or 7 dpi was detected between the anti-HMGB1 mAb-treated and control mAb-treated mice (Fig. 1B). The levels of TLR4 in the BALF at 5 dpi were lower in the anti-HMGB1 mAb-treated mice than those in the control mice (Fig. 1C).

Anti-HMGB1 mAb alleviates lung damage

The histopathological patterns observed in the anti-HMGB1 mAb-treated group differed from those in the control group (Fig. 2). The control mice showed the infiltration of inflammatory cells into the alveoli and the interstitium, diffuse edema, and hemorrhage. These histopathological changes were attenuated in the anti-HMGB1 mAb-treated mice, which led to lower lung injury scores than those in the control mice at 5 and 7 dpi.

Anti-HMGB1 mAb inhibits the infiltration of neutrophils and macrophages to the lung

The anti-HMGB1 mAb-treated mice had significantly lower counts of neutrophils and macrophages in the BALF than the control mice at 5 dpi, whereas these counts were not significantly different from control values at 7 dpi (Fig. 3). These changes could also be observed on immunostained images.

Anti-HMGB1 mAb inhibits the translocation of HMGB1 from AECI

To investigate the origin of HMGB1 in the BALF, lung cells, including AECI and AECII, and bronchial epithelial cells were immunostained. The translocation of HMGB1 from the nucleus to the cytosol was observed in AECI (white arrowhead in Fig. 4B) whereas anti-HMGB1 mAb treatment suppressed the HMGB1 release (white arrows in Fig. 4C). No apparent HMGB1 release was observed from AECII or bronchial epithelial cells (data not shown).

Anti-HMGB1 mAb controls cytokine/chemokine responses and systemic oxidative stress

The cytokine/chemokine measurement results showed that the levels of TNFα, IL-6, G-CSF, CXCL1, MCP1, CCL3, and RANTES in the BALF at 5 dpi were significantly lower in the

anti-HMGB1 mAb-treated group than those in the control group, whereas these levels did not significantly differ between the same groups at 7 dpi (Fig. 5A). The CXCL10 level in the BALF at 5 dpi was lower in the anti-HMGB1 mAb-treated group than that in the control group, but the difference was not statistically significant ($p=0.07$).

The hydroperoxide levels in the plasma at 5 dpi (Fig. 5B) were also significantly lower in the anti-HMGB1 mAb-treated group than those in the control group, whereas these hydroperoxide levels did not significantly differ between the two groups at 7 dpi.

DISCUSSION

An exaggerated innate immune response has been recognized as a major complication in severe influenza cases, accompanied with massive neutrophil infiltration to the lung due to elevated levels of inflammatory cytokines and chemokines.[4,21] Both anti-inflammatory therapy and antiviral treatment are important for severe influenza. Therefore, we aimed to evaluate the additional effect of an anti-HMGB1 mAb therapy in the mouse model of NAI-insufficient influenza. The combination therapy of an anti-HMGB1 mAb and peramivir improved the survival rate of mice with influenza-induced pneumonia without any effect against the propagation of influenza virus. Hence, the improvement in the survival rate might be due to the anti-inflammatory effect of the anti-HMGB1 mAb but not due to the inhibition of virus propagation.

TLR4 is one of the major HMGB1 receptors.[22] In this study, anti-HMGB1 mAb suppressed the TLR4 level in the BALF, associated with the infiltration of inflammatory cells and the elevation of TNFα levels. Shirey et al. have shown that the therapeutic antagonism of TLR4 signaling protects against influenza-induced acute lung injury (ALI).[23] Downregulation of the HMGB1–TLR4–myeloid differentiation primary response 88 (MYD88)–nuclear factor-κB–TNFα

pathway might be the downstream mechanism.[24] Based on the inhibitory effects of the anti-HMGB1 mAb on the levels of cytokines and HMGB1, it can be reasonably assumed that the benefit of anti-HMGB1 mAb treatment in influenza virus-infected mice is due to its ability to inhibit HMGB1-induced TLR4 signaling and a subsequent TLR4-dependent cytokine storm. To investigate the origin of TLR4, we performed immunohistochemical staining of TLR4 of AECI and AECII (data not shown); however, the TLR4 expression could not be visualized in this study. TLR4 expression was reported to be elevated at the ALI caused by LPS or Alarmin S100A8 especially in AECII *in vitro*;[25,26] however, there is no report that shows the expression of TLR4 in AECI and AECII with ALI *in vivo*. Further experiments would be necessary to explain the source of TLR4 in ALI.

In this study, anti-HMGB1 mAb and peramivir treatment inhibited the production of cytokines/chemokines, including TNFα, IL-6, G-CSF, CXCL1, MCP1, CCL3, CXCL10, and RANTES. Reportedly, all these cytokines/chemokines are associated with the pathology of influenza.[4,27] The levels of TNFα and IL-6 are linked to host morbidity and mortality in influenza patients.[28,29] G-CSF was reported as a risk factor of mortality in the 2009 influenza pandemic caused by the 2009pdm influenza virus.[30] CXCL1 functions as a chemoattractant for neutrophils

to sites of inflammation.[31] There was a sustained activation of MCP1 and TNFα in the plasma of severe H1N1 pneumonia patients as compared to those in the plasma of patients with mild infections.[32] CCL3 contributes to the activation of macrophages.[33] CXCL10 plays an important role in T cell attraction and activation.[34] RANTES is induced in bronchial epithelial cells during influenza infection.[35] Inhibition of these proinflammatory cytokines is predicted to result in less infiltration of inflammatory cells to the lungs and alleviation of lung injury.

There are several anti-inflammatory agents that have been evaluated for efficacy against severe influenza using murine models, such as the commercially available TNFα inhibitor etanercept,[28] corticosteroids,[36] COX2 inhibitors,[37] a TLR4 antagonist (Eritoran),[23] and anti-HMGB1 mAb.[11] Considering that there is no reason to treat influenza without NAI in daily clinical settings, we are interested in evaluating the adjunctive effects of these agents with NAI therapy against severe influenza. However, there have been limited studies evaluating the combination therapy. Shirey et al. showed the combined effect of Eritoran and oseltamivir against influenza. While Eritoran monotherapy provided a significant degree of protection from lethality, the combined Eritoran and oseltamivir treatment showed a significant improvement of survival when the therapy initiation was delayed.[38] Zheng et al. showed the combined effect of a COX-2

inhibitor and zanamivir against H5N1. COX-2 inhibitors were not as effective as monotherapy in H5N1 influenza-infected mice but they were effective when used in combination with zanamivir.[37] Walsh et al. showed the combined effect of a sphingosine analog, AAL-R, and oseltamivir against H1N1 2009. Treatment with AAL-R and oseltamivir significantly improved survival rate by limiting pulmonary injury.[39] These studies in addition to our study suggest that combination with antiviral and anti-inflammation therapies is a promising strategy against severe influenza. However, these anti-inflammatory therapies act in influenza infections by exerting immunosuppression. Moreover, our study showed that anti-HMGB1 mAb did not inhibit virus replication. Thus, when we apply these anti-inflammatory therapies with conventional therapy in the future, close follow up of patients and careful monitoring of viral load would be necessary.

In this study, we also found that anti-HMGB1 mAb and peramivir therapy ameliorated the level of hydroperoxides. This finding indicates that this combination therapy provides a broad suppression of local cytokines/chemokines and systemic oxidative stress. We have previously reported that the redox-active protein thioredoxin-1 attenuated H1N1-induced pneumonia in mice through its antioxidative properties.[19] An important role of reactive oxygen and nitrogen species as mediators in influenza virus-induced lung injury has been substantiated by previous studies;

superoxide dismutase, N-monomethyl-L-arginine, or allopurinol suppressed lung injury and improved the survival rate of the infected mice.[40,41] Further research using a multidisciplinary approach for influenza-induced pneumonia studies in appropriate animal models is therefore warranted.

Interestingly, administration of the anti-HMGB1 mAb inhibited HMGB1 translocation from AECI. Yao et al. reported the translocation of HMGB1 from the nucleus to the cytoplasm in lung epithelial cells and its subsequent release into the extracellular lung space in a mouse model of cigarette smoke-induced pulmonary inflammation, which suggested the involvement of a TLR4-mediated signaling cascade.[42] Izushi et al. reported HMGB1 release from the nucleus to the cytosol in AECI in a lipopolysaccharide-induced ALI model.[43] Hirincus et al. reported that the H1N1 (PR8) is widely dispersed in the alveoli including in AECI and AECII.[44] However, considering that more than 98% of the internal surface area in the rodent pulmonary alveolus is covered by AECI,[43] it is reasonable to assume that most of the inflammation-related events are associated with the AECI.

While we have shown the combined effect of NAI and anti-HMGB1 mAb in an

NAI-insufficient severe influenza model, NAI-resistant influenza is another issue.[45] Further research is needed to evaluate the effect of anti-HMGB1 mAb against NAI-resistant influenza.

CONCLUSIONS

Intravenous administration of an anti-HMGB1 mAb against severe influenza pneumonia in a murine model significantly improved the survival rate and reduced histopathological lung injury by inhibiting HMGB1 translocation from AECI, leading to the inhibition of TLR4 signaling, cytokine/chemokine responses, and oxidative stress. This study provides compelling evidence for the effectiveness of anti-HMGB1 therapy in combination with an anti-influenza drug for severe influenza pneumonia.

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DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Fig. 1. Effects of peramivir and anti-high-mobility group box 1 (anti-HMGB1) monoclonal antibody (mAb) treatment on survival, viral load, and toll-like receptor 4 (TLR4) level after H1N1 inoculation.

(A) Survival rate: one mouse (9.1%) in the peramivir + anti-HMGB1 mAb-treated group (n = 11) died, and six mice (54.5%) in the peramivir + control-IgG-treated group (n = 11) died. $*p < 0.05$ by the log-rank (Mantel–Cox) test. No mice survived without peramivir. (B) Viral load in the lung: there were no significant differences in viral load at 5 or 7 dpi. Data represent the mean ± SEM of 11 mice. $*p < 0.05$ by the Mann–Whitney *U*-test. (C) The anti-HMGB1 mAb-treated mice had lower levels of TLR4 in the bronchoalveolar lavage fluid (BALF) than control mice at 5 dpi. The dotted line describes the normal basal level. Data show the mean ± SEM of 11 mice.

Fig. 2. Effects of peramivir and anti-high-mobility group box 1 (HMGB1) monoclonal antibody (mAb) treatment on lung histology after H1N1 inoculation.

(A) Hematoxylin and eosin staining of lung tissue samples at 5 and 7 dpi. These are representative of four independent experiments. Peramivir + control-IgG group lung tissue showed diffuse edema and hemorrhage in alveoli and the interstitium. Destruction of the alveolar structure was detected.

These histopathological changes were attenuated in the peramivir + anti-HMGB1 mAb group. Scale bar = 100 μm. (B) Lung injury scores: data show the mean ± SEM of four independent experiments; *$p < 0.05$ by the Mann–Whitney *U*-test

Fig. 3. Effects of peramivir and anti-high-mobility group box 1 (HMGB1) monoclonal antibody (mAb) treatment on neutrophil and macrophage infiltration to the lung after H1N1 inoculation.

(A,B) Microscopic images of lung tissue samples stained with anti-HMGB1 (red), anti-nucleus (blue), and anti-neutrophil (green) in three groups. White arrows in (A) and (B) indicate infiltration of neutrophils. (C) Neutrophil numbers in the bronchoalveolar lavage fluid (BALF). The dotted line describes the normal basal level. Data show the mean ± SEM of 10–11 mice; *$p <$ 0.05 by the Mann–Whitney *U*-test. (D,E) Microscopic images of lung tissue samples stained with anti-HMGB1 (red), anti-nucleus (blue), and anti-macrophage (green) in three groups. White arrows in (D) and (E) show infiltration of macrophages. (F) Macrophage numbers in the BALF. The dotted line describes the normal basal level. Data show the mean ± SEM of 10–11 mice; *$p <$ 0.05 by the Mann–Whitney *U*-test. Scale bar = 50 μm.

Fig. 4. Effects of peramivir and anti-high-mobility group box 1 (HMGB1) monoclonal antibody

(mAb) treatment on HMGB1 translocation from alveolar epithelial cell I (AECI)

(A–C) Microscopic images of lung tissue samples stained with anti-HMGB1 (red), anti-nucleus (blue), and anti-podoplanin (green) in three groups. White arrows in (A) and (C) indicate the intranuclear localization of HMGB1. The white arrowhead in (B) indicates the disappearance of HMGB1 in the nucleus. The white squares and their magnified pictures indicate the typical patterns of HMGB1 distribution in each group. Scale bar = 20 μm.

Fig. 5. Effects of peramivir and anti-high-mobility group box 1 (anti-HMGB1) monoclonal antibody (mAb) treatment on cytokines and chemokines in the bronchoalveolar lavage fluid (BALF) and hydroperoxides in plasma after H1N1 inoculation.

(A) Effects of peramivir and anti-HMGB1 mAb treatment on cytokines and chemokines in the BALF after H1N1 inoculation. Data show the mean ± SEM of 6–8 mice. The dotted line describes the normal basal level. TNFα, IL-6 and MCP1 were not detectable in the normal mice; $*p < 0.05$, $**p < 0.01$ by the Mann–Whitney *U*-test. (B) Effects of peramivir and anti-HMGB1 mAb treatment on hydroperoxides in the plasma after H1N1 inoculation. Data describe the mean ± SEM of 10–11 mice. The dotted line shows the normal basal level; $**p < 0.01$ by the Mann–Whitney *U*-test.