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The Neuraminidase Inhibitor Peramivir Ameliorates Myocarditis Induced by Influenza A (H1N1pdm) Virus in a Murine Model

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Abstract

Severe influenza sometimes causes myocarditis. To analyze the effects of peramivir on influenza A (H1N1pdm) virus myocarditis, we investigated survival rates, cardiac function, histological findings and cytokine induction in murine influenza A (H1N1pdm) virus-induced myocarditis. Eight-week-old BALB/c male mice were infected intranasally with influenza A (H1N1pdm) virus, and then divided into 2 groups: control group, which was injected with saline; and peramivir-treatment group, which was treated with peramivir. Histological studies, echocardiograms and quantitative analysis of viral RNA and mRNA for inflammatory cytokines and adhesion molecules were performed. Treatment with peramivir led to a significant improvement in survival ($p<0.01$ vs. control group). Fractional shortening (FS) of peramivir-treatment group (44.7%, $p<0.01$) was significantly higher than that of control group (27.7%) on day 8. Histological examinations revealed localized myocarditis with lymphocyte infiltration, and myocarditis lesions were found in perivascular areas or associated with pericarditis; treatment with peramivir improved these findings. Quantity of influenza virus genome in heart and lung tissues was suppressed in peramivir-treatment group. The expression of cytokine and adhesion molecule mRNA was suppressed in peramivir-treatment group. Peramivir improved influenza A (H1N1pdm) virus-induced myocarditis.

Keywords: Myocarditis; Influenza; Peramivir

Introduction

Acute myocarditis is a potentially lethal disease; etiological agents include *Enteroviruses*, *Adenoviruses*, *Parvoviruses*, *Cytomegalovirus*, *Influenza* virus and others [1-6]. Fulminant myocarditis causes severe hemodynamic dysfunction and requires high-dose catecholamine and mechanical circulatory support [1,6-8]. An influenza pandemic occurred in 2009 [6,9,10]. The causative organism, influenza A (H1N1pdm) virus, has been reported to cause fatal myocarditis as well as pneumonia [11-14]. Based on national surveillance in Japan, we previously reported that 19 fulminant myocarditis patients (adults: 13, children: 6) with influenza A (H1N1pdm) virus were seen in the 2009/2010 season, while only 2 adult patients were seen in the 2010/2011 season [12-14].

The course of cardiac dysfunction and recommended timing of interventions are described in the guidelines for diagnosis and treatment of myocarditis of the Japan Circulation Society (JCS2009). Myocarditis is treated in three ways: (1) intervention to eliminate the cause, (2) intervention to improve hemodynamic compromise, and (3) intervention to address cardiac dysfunction [1,6,8]. In addition, not only viral myocarditis but also other infections by various microbials in various animals are exacerbated by non-steroidal anti-inflammatory drugs (NSAIDs) use [15,16]. Intervention to avoid exacerbating cause such as NSAIDs including salicylates may reduce serious complication including myocarditis.

The neuraminidase inhibitors represent an important advance in the treatment of influenza [17-24]. Treatment with neuraminidase inhibitors is also recommended by the Japanese association of Infection for all patients infected with influenza [25,26]. Sugaya reported Japan may have had the lowest case fatality rate for symptomatic illness (<0.001%, 198/20.7 million) in the H1N1 pandemic because of the universal implementation of early treatment with neuraminidase inhibitor [21]. On the other hand, Hama et al. reported oseltamivir could induce sudden deterioration leading to death with 12 hours of prescription [27]. Effects of neuraminidase inhibitors on mortality seemed to be controversial.

It was also difficult to show that the neuraminidase inhibitors significantly improved the survival rate of patients with fulminant myocarditis associated with influenza [6,7,11-14]. Peramivir is a selective inhibitor of influenza neuraminidase that is effective when administrated intramuscularly and intravenously for the treatment of influenza virus infection in mouse models [17-20,23]. To analyze the effects of peramivir on influenza A (H1N1pdm) virus myocarditis, we investigated survival rates, cardiac function, histological findings and cytokine induction in murine influenza A (H1N1pdm) virus -induced myocarditis.

Materials and Methods

Cell culture and virus

The influenza A (H1N1pdm) virus (IAV) used in this study was obtained from the lung of a patient who died of IAV pneumonia in 2009 in Japan (A/Niigata/09F098/2009). IAV was plaque-purified in

Madin-Darby canine kidney (MDCK) cells, and passaged three times in MDCK cells. The virus fluids were stocked at -80°C until used.

Animal experimentation

Specific pathogen-free 8-week-old male BALB/c mice weighing 16 g-20 g each were obtained from SLC laboratories (Shizuoka, Japan). Mice were permitted an acclimatization period of greater than 48 h prior to virus inoculation, during which time the animals were observed for signs of disease and/or physical abnormalities. Peramivir trihydrate was obtained from shionogi pharmaceuticals (Osaka, Japan).

The 8-week-old male BALB/c mice were infected intra-nasally with 50 plaque-forming units of IAV under anesthesia, and then were divided into 2 groups: control group (group C), which was injected with saline as a vehicle; and peramivir-treatment group (group P), which was treated with peramivir at 50 mg/kg by intramuscular injection daily for 3 days after virus inoculation. There were 20 mice in each group, and we used another 30 mice for echocardiographic study and histological study in control group and peramivir-treatment group. Uninfected mice (n=20) treated with saline were used for the uninfected group (group U).

To assess cardiac function, an echocardiograph was performed with a 12 MHz transducer on an aplio ultrasound machine (Toshiba medical systems, Tochigi, Japan) under controlled anesthesia on post-infection day 8. Left ventricular end-diastolic dimension (LVeDD) and left ventricular end-systolic dimension (LVeSD) and the percent fractional shortening (FS), which was calculated by the formula [(LVeDD-LVeSD/LVeDD) × 100], were measured from five consecutive cycles and averaged.

In each group, 6 mice were killed on days 5 and 8, after anesthesia administration, and the hearts were cross-sectioned across both ventricles after measurement of body weight and heart weight. Heart weight (mg)/body weight (mg) (HW/BW) ratio was calculated. Half of each heart and lung was fixed in 10% (v/v) buffered formalin and used for histology by staining with hematoxylin and eosin. The other half of each heart and lung was frozen in liquid nitrogen and used for subsequent PCR analysis. This study protocol conformed to the guide for the care and use of laboratory animals (NIH Publication No. 85-23, 1996) and was approved by the animal care committee of Osaka medical college.

Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from myocardial tissues and lung with an RNeasy mint kit (Qiagen, Tokyo, Japan), and reverse transcription was performed with random hexamers and reverse transcriptase. Quantitative PCR was performed using a Step One system (Applied Biosystems, Drive Foster, CA, USA). The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative quantity of each target mRNA [IAV genome, brain natriuretic peptide (BNP), interferon-γ (IFN-G), interleukin-6 (IL-6), and vascular cell adhesion molecule-1 (VCAM-1)] was evaluated by comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. We used pairs of primers and TaqMan® probes for step one system purchased from applied biosystems for IFN-G, IL-6, VCAM-1 and BNP. We used primers of neuraminidase segments of influenza virus for quantitative RT-PCR.

Statistical analysis

Results are presented as mean ± SD. One-way analysis of variance (ANOVA) was performed to evaluate differences among the 3 groups. Wilcoxon's two-rank-sum test (Mann–Whitney U test) was used to evaluate differences between 2 groups. Kaplan-Meier survival analysis was applied to assess survival number differences. P values of <0.05 were considered statistically significant.

Results

Mortality and histological findings

Treatment with peramivir resulted in significant improvement in survival ($p<0.01$ vs. control group) (Figure 1). Body weight (17.9 g ± 0.6 g) of control group was significantly smaller than peramivir-treatment group (19.7 g ± 0.6 g) ($p<0.01$), and HW/BW ratio (6.1 ± 0.4) was significant higher than peramivir-treatment group (5.5 ± 0.2) ($p<0.01$) on day 8 (Figure 1). Mice intra-nasally inoculated with IAV exhibited acute pneumonia and myocarditis, as confirmed by histological examination of hematoxylin and eosin-stained sections. Histological findings showed myocyte degeneration with lymphocyte infiltration and interstitial edema associated with pericarditis, and myocarditis lesions were also found in the perivascular area (Figure 2A-2C), and mononuclear cells infiltrated in lung in control group (Figure 2E). However, histological studies showed localized myocarditis with lymphocyte infiltration. Peramivir ameliorated these histopathological findings (Figures 2D and 2F).

Figure 1.

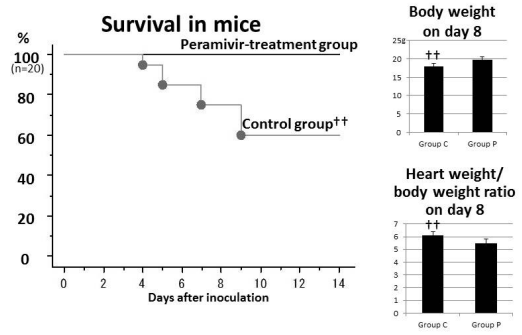

Figure 1: Effects of peramivir on survival, body weight and heart weight/body weight ratio in mice infected with *Influenza A* virus ; we used 20 mice in each group in the survival study.(100%: n=20). ††p<0.01, peramivir-treatment group vs. control group.

Echocardiographic findings

Echocardiograms revealed left ventricular dilatation and left ventricular systolic dysfunction in group V. The LVeDD in peramivir-treatment group (3.1 mm) was significantly smaller than in control group (3.4 mm) on day 8 ($p<0.05$). The FS of uninfected group (51.5%, $p<0.0001$) and peramivir-treatment group (44.6%, $p<0.001$) was

significantly greater than the FS of control group (27.7%) on day 8 (Figure 3).

Figure 2.

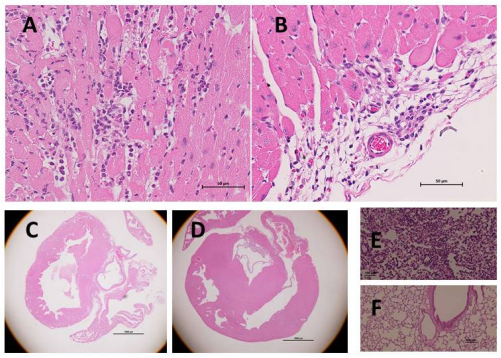

Figure 2: Histological analysis of the effects of peramivir on the development of myocarditis. Cross sections of the heart of mice in **(A, B, C)** the control group and **(D)** the peramivir-treatment group stained with hematoxylin and eosin on study day 5. Histological findings showed myocyte degeneration with lymphocyte infiltration and interstitial edema associated with pericarditis, and myocarditis lesions were also found in the perivascular area in control group. Cross sections of the lung of mice in **(E)** the control group and **(F)** the peramivir-treatment group stained with hematoxylin and eosin on study day 5. Histological findings showed mononuclear cells infiltration in lung in control group. Scale bar: 50 μm in A and B, 1,000 μm in C and D, 100 μm in E and F.

Quantitative real-time polymerase chain reaction analysis

The relative expressions of IAV genome and pro-inflammatory cytokines, BNP and VCAM-1, are summarized in Figure 4. Peramivir treatment significantly decreased quantity of IAV genome in the heart and lung tissues on day 5, compared with control group (Figure 4), and significantly decreased the expression of BNP in the myocardium on day 8, compared with control group. Compared with control group, expression of IFN-G, IL-6, and VCAM-1 mRNA was suppressed in peramivir-treatment group. However there was no significant difference of IL-6 in heart tissue between control group and peramivir-treatment group. There was no significant difference of IFN-G or VCAM-1 in lung tissue between control group and peramivir-treatment group.

Discussion

This study demonstrated the effect of peramivir treatment on influenza viral myocarditis in a mouse model. Treatment with peramivir improved the survival rate, histological findings and echocardiographic findings with suppression of quantity of IAV genome and BNP mRNA expression in this paper.

Figure 3.

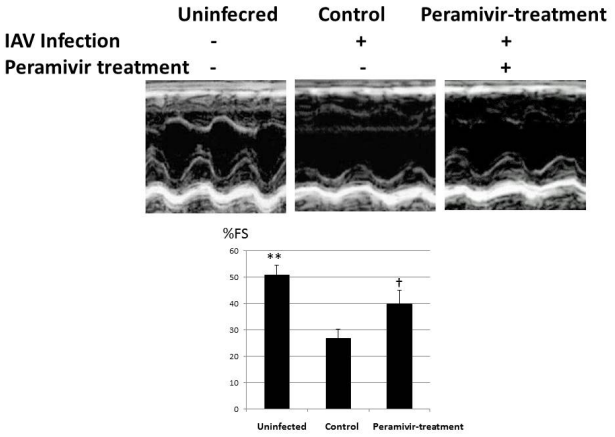

Figure 3: Echocardiograms show the suppression of cardiac function by *Influenza A* virus (IAV) infection and its improvement by peramivir. Representative M-mode echocardiogram images of mice infected with IAV with or without intramuscular administration of peramivir at 50 mg/kg once daily for 3 days. Measurements of left ventricular end-diastolic dimension (LVeDD) and % fractional shortening (FS) were performed on day 8. $^{\dagger}p<0.05$, peramivir-treatment group vs. control group. $^{**}P<0.01$, unincected group vs. control group.

Figure 4.

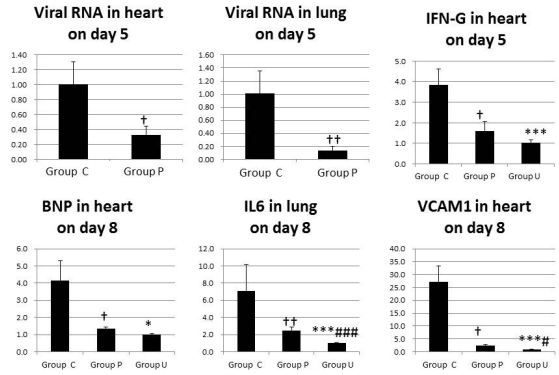

Figure 4: Relative expression of the *Influenza A* virus (IAV) genome in heart or lung tissue by RT-PCR. Relative expression (corrected by GAPDH expression) of target mRNA in heart or lung tissues by RT-PCR. Columns and bars: mean ± SD. $^{\dagger}p<0.05$, peramivir-treatment group (P) vs. control group (C). $^{\dagger\dagger}p<0.01$, peramivir-treatment group (P) vs. control group (C). $^{*}P<0.05$, unincected group (U) vs. control group (C). $^{***}P<0.001$, unincected group (U) vs. control group (C). $^{\#}P<0.05$, unincected group (U) vs. peramivir-treatment group (P). $^{\#\#\#}P<0.001$, unincected group (U) vs. peramivir-treatment group (P). interferon-γ (IFN-G), brain natriuretic peptide (BNP), interleukin-6 (IL-6), and vascular cell adhesion molecule-1 (VCAM-1).

Peramivir is one of a selective inhibitor of neuraminidase of influenza virus [17,19]. It was reported that peramivir increased survival rate of animals infected with lethal dose of influenza virus by the pharmaceutical company of peramivir, and its *in vitro* activity has been demonstrated to be comparable to or better than that of oseltamivir carboxylate and zanamivir [17,28]. Peramivir has been approved in Japan for the treatment of general seasonal influenza in adults, as a single dose of 300 mg intravenously [18,21]. A single dose or multiple daily doses of 600 mg of peramivir are administered intravenously depending on the condition of the patient. We used the 50 mg/kg dose in mice for 3 days, which is approximately a human-equivalent dose of 300 mg [19]. We showed suppression of quantity of IAV genome in this study. However, we did not analyze myocardial virus titer or neutralizing antibody titers, which is one of the limitations of this study [29-31]. Pan et al reported the influenza viral levels in the hearts monitored by the nonstructural protein1 gene and nucleoprotein was in peak on day 6 [32]. However, there is no evidence of viable virus in this paper.

Influenza is a recognized cause of myocarditis, which can lead to significant impairment of cardiac function and mortality [2,3,6]. Myocarditis is not the major cause of death from influenza infection, and is very rare. However the number of influenza myocarditis increased in the H1N1 pandemic, and it was serious problem for cardiologists [12,13]. The pathological effects of influenza viral myocarditis in humans and mice are reportedly milder and more localized than those seen in *coxsackievirus* myocarditis [32-35]. In this study, histological findings showed localized myocarditis with lymphocyte infiltration, and myocarditis lesions were found in the perivascular area, or myocarditis was associated with pericarditis. Peramivir ameliorated these histopathological findings. Kotaka et al. reported that murine influenza myocarditis was histologically mild and brief in duration compared to coxsackievirus B3 myocarditis, and electron microscopic findings of the heart from a murine influenza myocarditis model showed many infiltrating lymphocytes directly attached to the cardiac myocytes [33]. Pan et al. investigated the molecular mechanism of influenza virus-associated myocarditis and revealed the importance of trypsin induction and the increased production of matrix metalloproteinase and proinflammatory cytokines in the pathogenesis of acute myocarditis [32,35]. Along with the direct effect of influenza virus infection, proinflammatory cytokines and endothelial cell dysfunction are thought to contribute to the pathogenesis of severe clinical features, including severe cardiac dysfunction, in patients infected with influenza virus [2,3,6,32,35].It was reported that osertamivir has pleotropic effects [30,36-38]. In human, compared with the partial reduction of viral shedding, pro-inflammatory cytokines are completely suppressed by oseltamivir use [36]. Oseltamivir reduced cellular response in the BALF and pro-inflammatory cytokines without reducing viral load in the lung if administered to mice infected with mild influenza virus [30]. Moore et al reported administration of oseltamivir in animals challenged by respiratory syncytial virus that lacks a neuraminidase gene showed a symptom-relieving effect and inhibition of viral clearance [38]. Evidence of pleotropic effects of peramivir is poor. Tanaka et al reported that a multiple-dose regimen of intravenous peramivir was more efficacious than a single peramivir dose or multiple doses of oseltamivir for improving outcomes in pneumococcal pneumonia following influenza virus infection in mice, and the production of inflammatory cytokines/chemokines was also significantly suppressed by multiple dosing of peramivir compared with oseltamivir. [29] Expression of IFN-G, IL-6, and VCAM-1 mRNA was suppressed by peramivir treatment in this study. Peramivir may have potentiality to suppress pro-inflammatory cytokines.

We previously reported that the frequency of cardiac involvement is likely elevated in influenza A (H1N1pdm) virus infection compared to seasonal influenza infection, according to results of national surveillance in Japan [12-14]. The myocardial toxigenicity of the seasonal influenza virus seems to be rather weak, so we used the pandemic virus obtained from the lung of a patient who died of IAV pneumonia in this study.

In conclusion, peramivir improved influenza A (H1N1pdm) virus-induced murine myocarditis.

Conflict of Interest

All authors declare that there are no conflicts of interest.

Acknowledgement

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