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Up-regulation of ectopic trypsins in the myocardium by influenza A virus infection triggers acute myocarditis

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Aims Influenza A virus (IAV) infection markedly up-regulates ectopic trypsins in various organs, viral envelope glycoprotein processing proteases, which are pre-requisites for virus entry and multiplication. We investigated the pathological roles of trypsin up-regulation in the progression of IAV-induced myocarditis, cytokine induction, and viral replication in the hearts, and also investigated the protective effects of trypsin inhibitor on cardiac dysfunction *in vivo* and selective knockdown of trypsin on IAV-induced cellular damage in cardiomyoblasts.

Methods and results The relationship of the expression among IAV RNA, trypsins, matrix metalloproteinase (MMP)-9, MMP-2, pro-inflammatory cytokines interleukin (IL)-6, IL-1β, and tumour necrosis factor-α was analysed in mice hearts and cardiomyoblasts after IAV infection. The severity of myocarditis was most noticeable during Day 6–9 post-infection, along with peak expression of viral RNA, trypsins, particularly trypsin$_2$, MMPs, and cytokines. Cardiac ATP levels were the lowest at Day 9. Up-regulated trypsins, viral protein, and tissue-injured loci in the myocardium were closely localized. Trypsin inhibitor aprotinin treatment *in vivo* and selective trypsin$_1$- and trypsin$_2$-knockdown, particularly the latter, in H9c2 cardiomyoblasts significantly suppressed viral replication, up-regulation of MMPs, and production of active MMP-9 and cytokines, resulting in marked protection against cellular damage, ATP depletion, and apoptosis. IAV infection-induced cardiac dysfunction monitored by echocardiography was improved significantly by aprotinin treatment.

Conclusions IAV-induced trypsins, particularly trypsin$_2$, in the myocardium trigger acute viral myocarditis through stimulation of IAV replication, proMMP-9 activation, and cytokine induction. These results suggest that up-regulation of trypsins is one of the key host pathological findings in IAV-induced myocarditis.

Keywords Myocarditis • trypsin • Influenza virus • Cytokines • Matrix metalloproteases

1. Introduction

Influenza A virus (IAV) is the most common infectious pathogen in humans, causing significant morbidity and mortality in infants and the elderly.[1,2] Severe influenza causes pneumonia and sometimes cardiac complications, such as myocarditis and acute myocardial infarction.[3,4] In addition, people with underlying chronic cardiovascular diseases are particularly at high risk of IAV-induced myocardial diseases.[5]

IAV initially infects the respiratory mucosal surface and induces various host cellular factors other than anti-viral immunoglobulins, not only in the airway but also in other organs, such as pro-inflammatory cytokines, matrix metalloproteinases (MMPs), and ectopic trypsins, viral envelope haemagglutinin (HA) processing proteases. Among these factors, ectopic trypsins are crucial for viral entry, replication, and spread, because IAV has no HA processing protease in its genes and post-transcriptional HA cleavage by cellular

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proteases is a pre-requisite for membrane fusion activity and virus entry into cells.[6–8] However, the inter-relationship among these factors in the progress of IAV-induced myocarditis in severe influenza remains unclear.

Pro-inflammatory cytokines, tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 mediate cardiac injuries through recruitment of immune cells towards inflammatory loci, and enhance the production of MMPs at the transcriptional level via nuclear factor-kappa B and activator protein 1.[9,10] In addition, these cytokines increase nitric oxide production in the heart.[11] Over production of nitric oxide is followed by myocardial ATP depletion through inhibition of the activity of mitochondrial enzymes complex I and complex II and induction of cardiomyocyte apoptosis by changing the cellular balance between Bak and Bcl-x.[12,13] Induced active MMPs, such as MMP-2 and MMP-9, promote endothelial hyperpermeability and assist inflammatory cell migration through the breakdown of vascular basement membranes and extracellular matrix (ECM).[14] Destruction of ECM further causes cardiomyocyte anoikis/apoptosis,[15] resulting in cardiac dilation and dysfunction. The induced MMP-2 and MMP-9 in turn activate pro-inflammatory cytokines into their active forms.[16,17] Exaggerated expression and activation of MMPs and cytokines result in severe tissue injury.

We previously reported that IAV infection significantly up-regulates ectopic trypsins in the brain endothelial cells and hippocampal neurons,[7] which may potentiate brain vascular hyperpermeability,[18] viral spread, and tissue damage. Since trypsins efficiently convert proMMPs to active MMPs,[19,20] induction of trypsins and proMMPs synergistically degrades vascular basement membranes and ECM. Furthermore, up-regulated trypsins in turn evoke cytokine release via proteinase-activated receptor (PAR)-2.[21] These results suggest close interaction among IAV, proteases, and cytokines in the pathogenesis of severe influenza. Trypsin has three major isoforms; trypsin$_1$ (cationic trypsin, T_1), trypsin$_2$ (anionic trypsin, T_2), and trypsin$_3$ (mesotrypsin, T_3).[22]

The aim of the present study was to clarify the pathological roles of up-regulated trypsins in the progression of myocarditis in severe influenza. The results first defined the pivotal roles of up-regulated trypsins in viral replication, proMMP-9 activation, and cytokine induction in IAV-induced myocarditis. The results also presented the therapeutic effects of trypsin inhibition on IAV-induced myocarditis.

2. Methods

2.1 Influenza myocarditis model

This study conforms 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 the University of Tokushima. Specific pathogen-free 8-week-old male BALB/c mice (SLC, Japan) were infected intranasally with 40 plaque-forming units (PFU) IAV/PR/8/34(H1N1), about one-third of a minimum lethal dose, in 15 μL of saline or saline as a vehicle under ketamine anaesthesia. Mice were euthanized at Day 0, 3, 6, 9, or 12 post-instillation. To analyse the effects of trypsin inhibition, trypsin inhibitor aprotinin (Sigma) was injected intra-peritoneally at 2 mg/kg twice daily immediately after infection for 9 days.

2.2 Haemodynamic measurements

Mouse blood pressure and heart rate were assessed by a blood pressure monitor MK-1030 (Muromachi Kikai). Echocardiograph was performed with a 12 MHz transducer on an Aplio 80 ultrasound machine (Toshiba Medical Systems) under the awake state of mice at Day 9 post-infection. Left ventricular M-mode echocardiogram was obtained from parasternal short-axis view in the left lateral decubitus position. Left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), and per cent fractional shortening (%FS), which was calculated by the formula $[(\mathrm{LVEDD}-\mathrm{LVESD})/\mathrm{LVEDD}]\times 100$, were measured from three consecutive cycles and averaged. Stroke volume (SV) and cardiac output (CO) were also analysed as described in Supplementary material online.

2.3 Tissue preparation

Mice were divided randomly into two groups before tissue preparation. Hearts of 10 mice in the first group were weighed immediately after preparation and then weighed again after drying for 3 days at 70°C to obtain wet/dry weight ratios. Ten mice in the second group were injected intraperitoneally with 200 μL of 2% Evan's blue solution in saline at 3 h before tissue preparation to analyse vascular permeability and injured loci. Isolated heart of each animal was cut into halves; one-half was fixed with 4% paraformaldehyde for histopathological evaluation, and the other half was stored at −80°C for biochemical analyses. All experiments were repeated at least thrice to ensure reproducibility.

2.4 Cell culture, transfection, and infection

Cardiomyoblast H9c2 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/mL penicillin/streptomycin, and 2 mM L-glutamine (growth medium). For gene-silencing experiments, trypsin$_1$-shRNA plasmid (T_1-sh), trypsin$_2$-shRNA plasmid (T_2-sh), control-shRNA plasmid (C-sh), transfection reagents, and medium were purchased from Santa Cruz. Cells in 6-well tissue culture plate (50–70% confluence) were washed with transfection medium, and incubated with 1 μg shRNA plasmid and 3 μL of transfection reagent in 1 mL transfection medium per well for 7 h at 37°C, then supplemented with 1 mL of DMEM containing 20% FCS for 24 h according to the protocol recommended by the manufacturer. Thereafter, the transfection mix was replaced by fresh growth medium. Puromycin-resistant H9c2 cell colonies were selected for 3 weeks after transient transfection for 48 h, and then propagated to establish stable transfectant T_1-sh, T_2-sh, and C-sh cell lines. After washing twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, these cells in serum-free medium supplemented with ITS premix (BD Biosciences) were infected with IAV/PR/8 at multiplicity of infection (m.o.i.) 1 or mock infected for 24 h.

2.5 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with RNeasy Mini kit (Qiagen), and reverse transcribed using oligo primers and universal primers in SuperScript III RT kit (Gibco BRL) for cDNAsynthesis. The primer pairs used to amplify trypsins and IAV non-structure protein 1 (NS1) gene segment, and methods of RT-PCR are described in Supplemental material online. PCR products were analysed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

2.6 Immunoblotting and gelatin zymography

Tissues were homogenized with three volumes of 0.05 M Tris-HCl, (pH 7.6), containing 2% SDS and 0.5 M NaCl. Cultured cells were lysed with RIPA lysis buffer (Santa Cruz). Extracts were concentrated by Amicon Ultra 10K device (Millipore) by centrifugation. Immunoblotting and zymography were performed as described previously.[7] Target proteins were reacted with their specific antibodies against trypsin, MMP-9 and MMP-2 (Santa Cruz), TNF-α (R&D system), IL-6 and IL-1β (Sigma), actin (Chemicon), and IAV (Takara). Immunoreactive bands were visualized by enhanced chemiluminescence detection system (Amersham). For gelatin zymography, the extracts (30 μg protein) were subjected to electrophoresis on 10% gelatin zymogram gels (Invitrogen).

2.7 Immunostaining

Immunohistochemistry of up-regulated trypsin(s) in the myocardium was performed as described previously.[7] Briefly, after blocking endogenous peroxidase activity with 3% H_2O_2 and non-specific binding sites with goat serum, sections were incubated with rabbit anti-trypsin antibody (Santa Cruz). Immuno-deposits were visualized by using the avidin–biotin–peroxidase kit (Vector). For double staining, rabbit anti-trypsin and mouse anti-IAV (Takara) antibodies were used for the initial reaction and immuno-deposits of trypsins and IAV in the hearts were visualized by goat anti-rabbit Alexa Fluor 647 conjugated secondary antibody (Molecular Probes) and goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Sigma), respectively. IAV-infected H9c2 cells were detected with rabbit anti-IAV polyclonal antibodies, followed by goat anti-rabbit Texas red-conjugated secondary antibody (Molecular Probes). Nuclei were stained blue by 4,6-diamidino-2-phenylindole (DAPI) (Dojindo).

2.8 Measurement of mitochondrial membrane potential and apoptosis

Mitochondrial membrane potential ($\Delta\Psi_m$) was analysed by the APO LOGIX^{TM} JC-1 assay kit (Bachem) as described previously.[23] Briefly, cells were incubated with 10 μM JC-1 dye for 15 min at 37°C in darkness and immediately imaged after washing. Apoptosis was detected by the *In situ* cell death detection kit, fluorescein (Roche) according to the protocol offered by the manufacturer.

2.9 ATP level and enzyme-linked immunosorbent assay (ELISA)

ATP in the heart homogenates was extracted with extraction solution in the XL-ATP kit (APRO) according to the protocol offered by the manufacturer. ATP in cultured cells was extracted with 1% trichloroacetic acid after trypsinization for cell count, followed by centrifugation. Resulting supernatants were neutralized with 1 M Tris-acetate buffer, (pH 7.5), as described previously.[23] ATP levels in these extracts were measured by ENLITEM® ATP assay system bioluminescence detection kit (Promega) and normalized relative to the protein concentrations or cell numbers. The levels of IL-6, IL-1β, and TNF-α in the culture medium were measured by ELISA kits (R&D system).

2.10 Statistical analysis

Results are presented as mean ± SD. Significance was calculated by one-way ANOVA. A P value of <0.05 was considered statistically significant.

3. Results

3.1 Increased vascular permeability, tissue oedema, and inflammatory cell infiltration in acute influenza myocarditis

Mice intra-nasally inoculated with 40 PFU of IAV/PR/8(H1N1) exhibited acute myocarditis, as confirmed by histological examination of haematoxylin and eosin-stained sections. Inflammatory infiltrates appeared initially in the subepicardium at Day 3 post-infection, followed by extensive infiltration across the interstitium and perivascular areas deep into the myocardium, accompanied by ECM destruction during Day 6 and 9, and then alleviated at Day 12 (*Figure 1A–C*). Coronary vascular permeability monitored by Evan's blue extravasation and tissue oedema by wet/dry weight ratio started to increase at

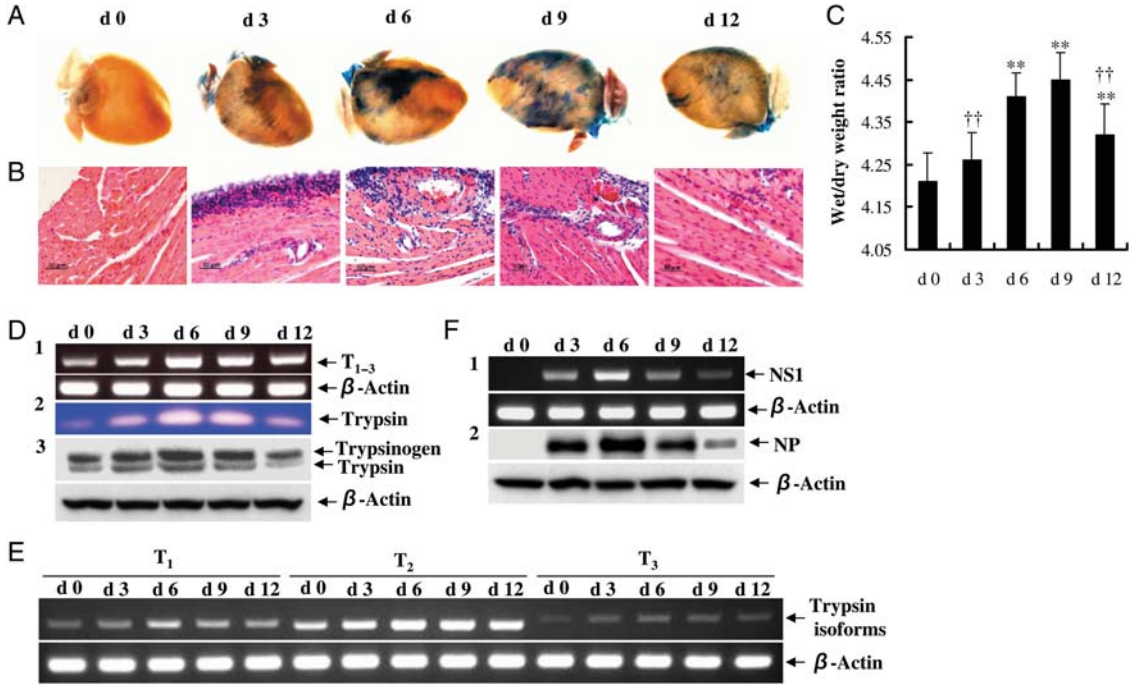

Figure 1 Increased vascular hyperpermeability, tissue oedema, and inflammatory cell infiltration in acute myocarditis, and kinetics of up-regulation of trypsins in myocardium after IAV infection. Vascular hyperpermeability monitored by Evan's blue extravasation during the course of infection from Day 0 (d 0) to 12 (d 12) (*A*). Haematoxylin and eosin staining (*B*). Bar = 50 μm. Heart oedema determined by wet/dry weight ratio (*C*). Data are mean ± SD of 10 mice in each group. $^{**}P < 0.01$ vs. D 0; $^{\dagger\dagger}P < 0.01$ vs. D 9. Detection by RT-PCR of trypsin$_{1-3}$, trypsin isoforms T_1, T_2, and T_3 and IAV NS1 gene in the hearts from Day 0 to 12 post-infection (*D*1, *E*, and *F*1, respectively). Detection by zymography of trypsin activity (D2), by western immunoblotting of trypsinogen and trypsin (D3) and viral nucleoprotein (NP) (F2). Each result is a representative of three experiments.

Day 3 after infection, reaching a peak during Day 6 and 9, and then decreased significantly at Day 12.

3.2 Up-regulation of trypsins by IAV infection in myocardium and close distribution of trypsins with viral antigen and inflammatory injury

Infection of IAV/PR/8(H1N1) began to up-regulate trypsins (T_{1-3}) in the hearts at Day 3 post-infection, reached a peak at Day 6, remained at high levels until Day 9, and then started to decrease at Day 12 (*Figure 1D*). The majorities of up-regulated T_{1-3} were trypsinogens, as determined by western immunoblotting, and were partially converted to active trypsins with a peak at Day 6 monitored by zymography. Among the three trypsin isoforms in murine hearts without infection at Day 0, T_2 was the predominant isoform and the level of T_1 was less than that of T_2, while T_3 was barely detected. After infection, T_2 was predominantly induced with a peak during Day 6 and 9 (*Figure 1E*). The viral levels in the hearts monitored by the NS1 gene and nucleoprotein (NP) showed that it also peaked at Day 6 and started to decrease at Day 9 (*Figure 1F*). Immunohistochemical analysis revealed that the up-regulated trypsins were distributed in the myocardium of infected loci and inflammatory infiltrates in the peripheral infected loci, which were most obvious at Day 6 after infection (*Figure 2A*). Fluorescent analyses of trypsins and viral protein as well as Evan's blue dye, a marker of hyperpermeability and tissue injury, showed their overlapped localization, suggesting their close correlation at infected foci (*Figure 2B*).

3.3 Up-regulation of MMPs and cytokines with cardiac fuel depletion after IAV infection

Limited amounts of trypsin(s) effectively converts various proMMPs, particularly proMMP-9, into active MMPs[19] and the latter in turn convert pro-inflammatory cytokines to active cytokines,[16,17] resulting in cardiomyocyte injury and ECM degradation.[16] The relationship among trypsin up-regulation, MMP activation, and cytokine induction was analysed in IAV-induced myocarditis. Along with an increase in trypsin levels, up-regulated proMMP-9 was readily converted to active MMP-9 (actMMP-9) from Day 3 post-infection, whereas up-regulated proMMP-2 was not (*Figure 2C*). The actMMP-9 levels increased to a peak value during Day 6 and 9, followed by a decrease in the levels at Day 12. Similar time courses of induction of the cytokines IL-6, IL-1β, and TNF-α, which are activated by MMPs,[16,17] were

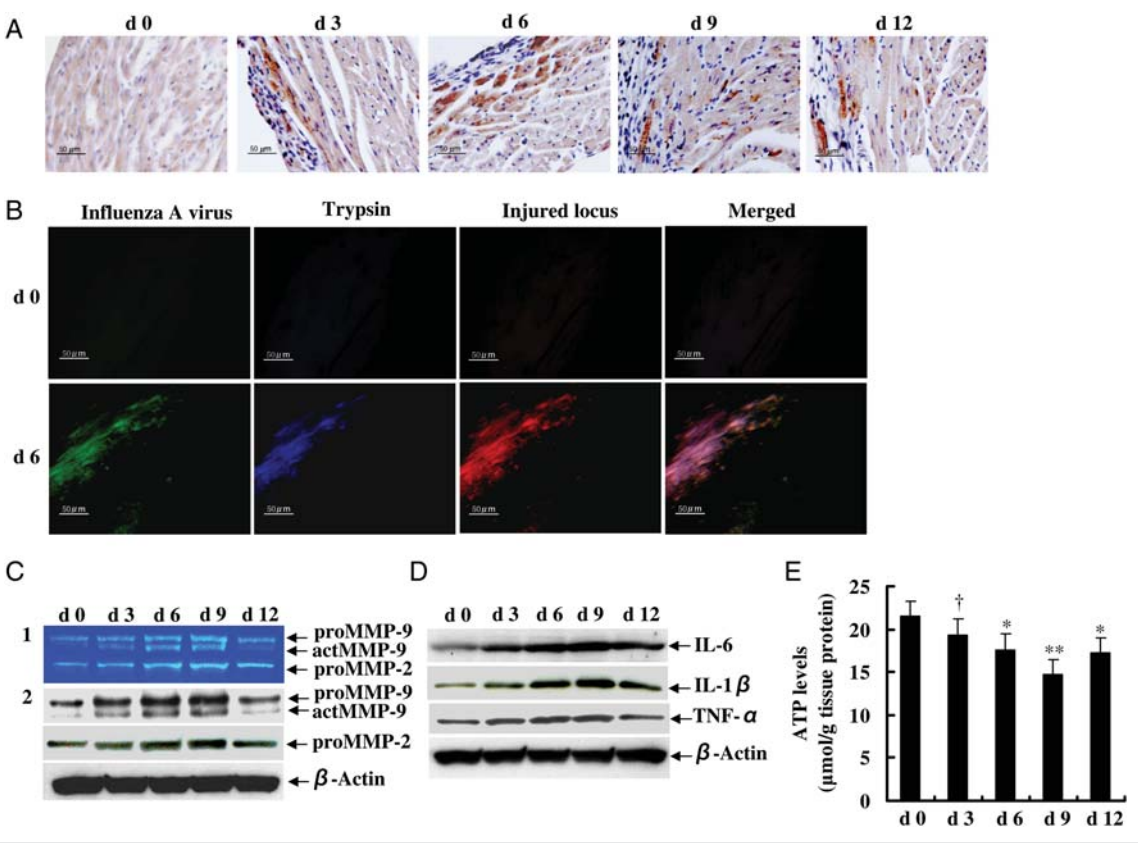

Figure 2 Close distribution of up-regulated trypsins with viral antigen and inflammatory injury, and kinetics of up-regulation of MMPs and cytokines as well as ATP depletion in the hearts after IAV infection. (*A*) Immunohistochemical detection of up-regulated trypsins in the myocardium from Day 0 (d 0) to 12 (d 12) post-infection. Bar = 50 μm. (*B*) Immunofluorescence detection of viral antigen (green), trypsins (blue), and injured loci monitored by Evan's blue extravasation (red) in the hearts at Day 0 and 6. Bar = 50 μm. Right panel shows merge. (*C*) Kinetics of up-regulation of proMMP-9, proMMP-2, and actMMP-9 in the hearts determined by zymography (*C1*) and western immunoblotting (*C2*) from Day 0 to 12 post-infection. (*D*) Kinetics of up-regulation of IL-6, IL-1β, and TNF-α in the hearts determined by western immunoblotting from Day 0 to 12 post-infection. (*E*) Changes in cardiac ATP levels during IAV infection. Data are mean ± SD of three repeated experiments from 10 mice per each group. $^{*}P<0.05$, $^{**}P<0.01$ vs. Day 0; $^{\dagger}P<0.05$ vs. Day 9.

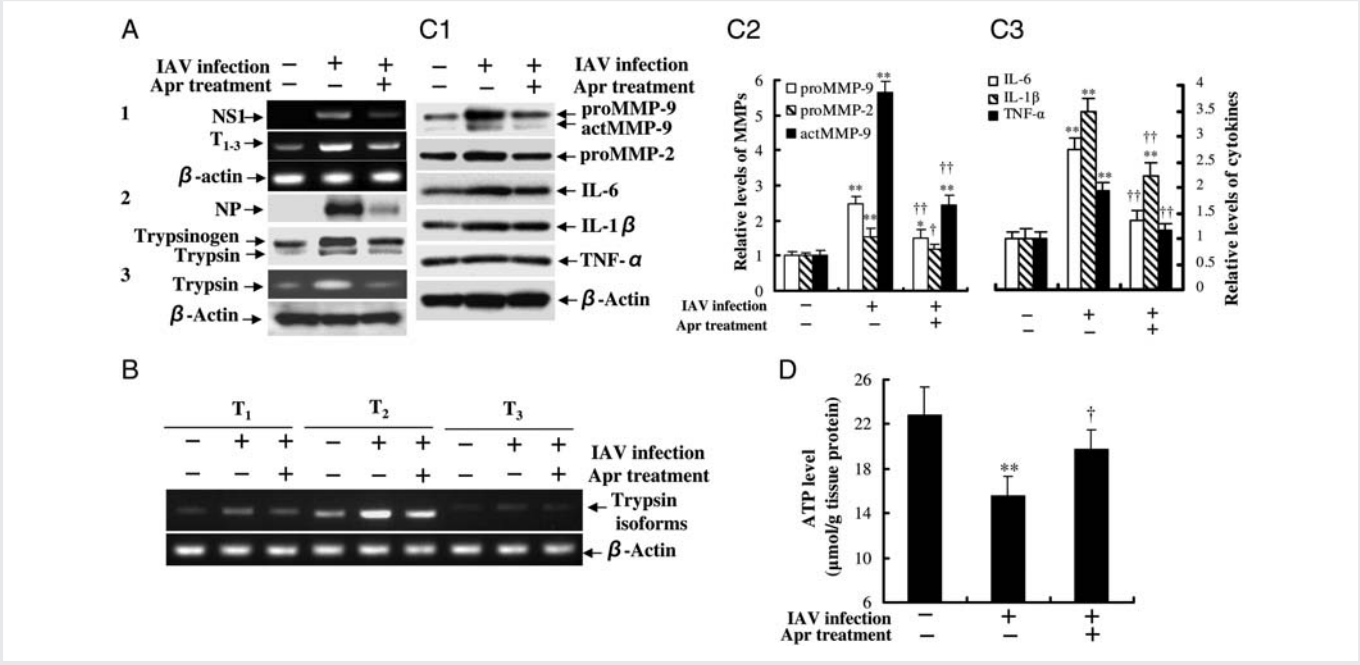

Figure 3 Effects of trypsin inhibitor administration on viral replication and up-regulation of trypsins, MMPs, and cytokines as well as ATP depletion in the hearts. Intraperitoneal administration of aprotinin (Apr) at 2 mg/kg twice a day for 9 days significantly suppressed viral replication and trypsin up-regulation analysed by RT-PCR (*A*1 and *B*), by western immunoblotting (*A*2), and zymography (*A*3), up-regulation of proMMP-9, actMMP-9, proMMP-2, IL-6, IL-1β, and TNF-α analysed by western immunoblotting (*C*1) and ATP depletion (*D*) at Day 9 post-infection. β-Actin as an internal control. Relative levels of MMPs and cytokines after IAV infection vs. mock infection (*C*2 and *C*3). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock infection. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. IAV infection without Apr treatment.

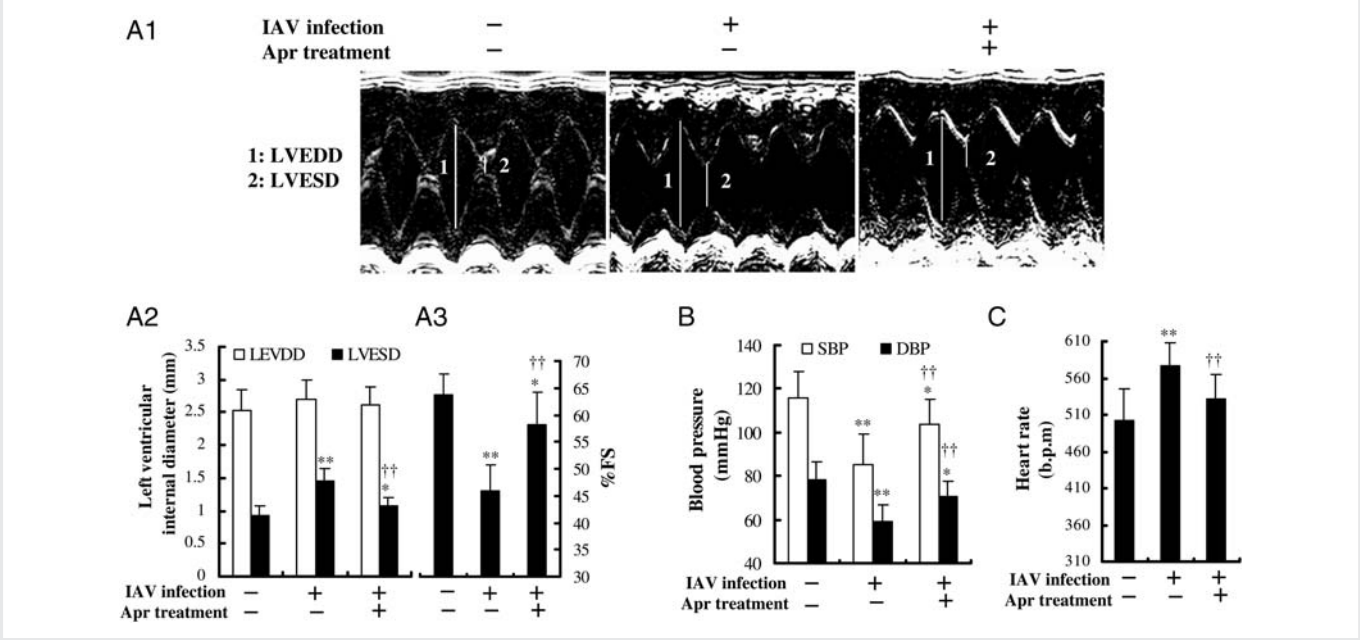

Figure 4 Suppression of cardiac function by IAV infection and its restoration by trypsin inhibitor. Representative M-mode echocardiogram images of mice infected with mock and IAV with or without intraperitoneal administration of aptrotinin (Apr) at 2 mg/kg twice a day for 9 days (*A*1). Measurements of LVEDD and LVESD at Day 9 post-infection (*A*2) and %FS (*A*3). Comparison of blood pressure (*B*) and heart rate (*C*) at Day 9 post-infection among mock and IAV-infected mice treated with or without Apr. SBP, systolic blood pressure; DBP, diastolic blood pressure. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock infection; $^{\dagger\dagger}P < 0.01$ vs. IAV infection without Apr treatment.

observed (*Figure 2D*). Cardiac viability was monitored by ATP levels in the hearts (*Figure 2E*). ATP levels were decreased after viral infection with a valley at Day 9 and then started to recover at Day 12. Increase in the viral load for infection up-regulated trypsin(s) and depleted ATP in the hearts in a dose-dependent manner at Day 6 post-infection (see Supplementary material online, *Figure S1*).

3.4 Administration of trypsin inhibitor suppressed viral replication and up-regulation of trypsins, MMPs, and cytokines as well as ATP depletion, and significantly improved cardiac function

Administration of trypsin inhibitor aprotinin immediately after IAV infection at 2 mg/kg twice a day for 9 days markedly suppressed IAV replication monitored by NS1 gene and NP (*Figure 3A*). Up-regulation by IAV infection of trypsin isoforms and T_{1-3} monitored by RT-PCR, up-regulation of trypsinogens, trypsins, proMMPs, actMMP-9 as well as the cytokines IL-6, IL-1β, and TNF-α, analysed by western immunoblotting, and trypsin activity monitored by zymography were significantly inhibited by aprotinin administration at Day 9 post-infection (*Figure 3A–C*). Depleted cardiac ATP levels were improved by aprotinin treatment (*Figure 3D*). Suppression of cardiac function by IAV infection monitored by echocardiographic evaluation, blood pressure, and heart rate at Day 9 post-infection was evident (*Figure 4*). Left ventricular function monitored by LVESD values and %FS as well as SV and CO (see Supplementary material online, *Table S1*) was significantly suppressed after infection, although LVEDD values were in a similar range between mock and IAV-infected mice. Low CO by IAV infection resulted in low blood pressure and compensatory tachycardia. The cardiac dysfunction was improved significantly by aprotinin with the increase in the values of %FS, SV, CO, and blood pressure as well as the decrease in the values of LVESD and heart rate.

3.5 IAV infection up-regulated trypsins and MMPs in cultured cardiomyoblasts with ATP depletion

IAV replication monitored by NS1 gene and NP was also observed in H9c2 cardiomyoblasts after infection for 24 h at m.o.i. 1 (see Supplementary material online, *Figure S2A–B*). Up-regulation of trypsinogens,

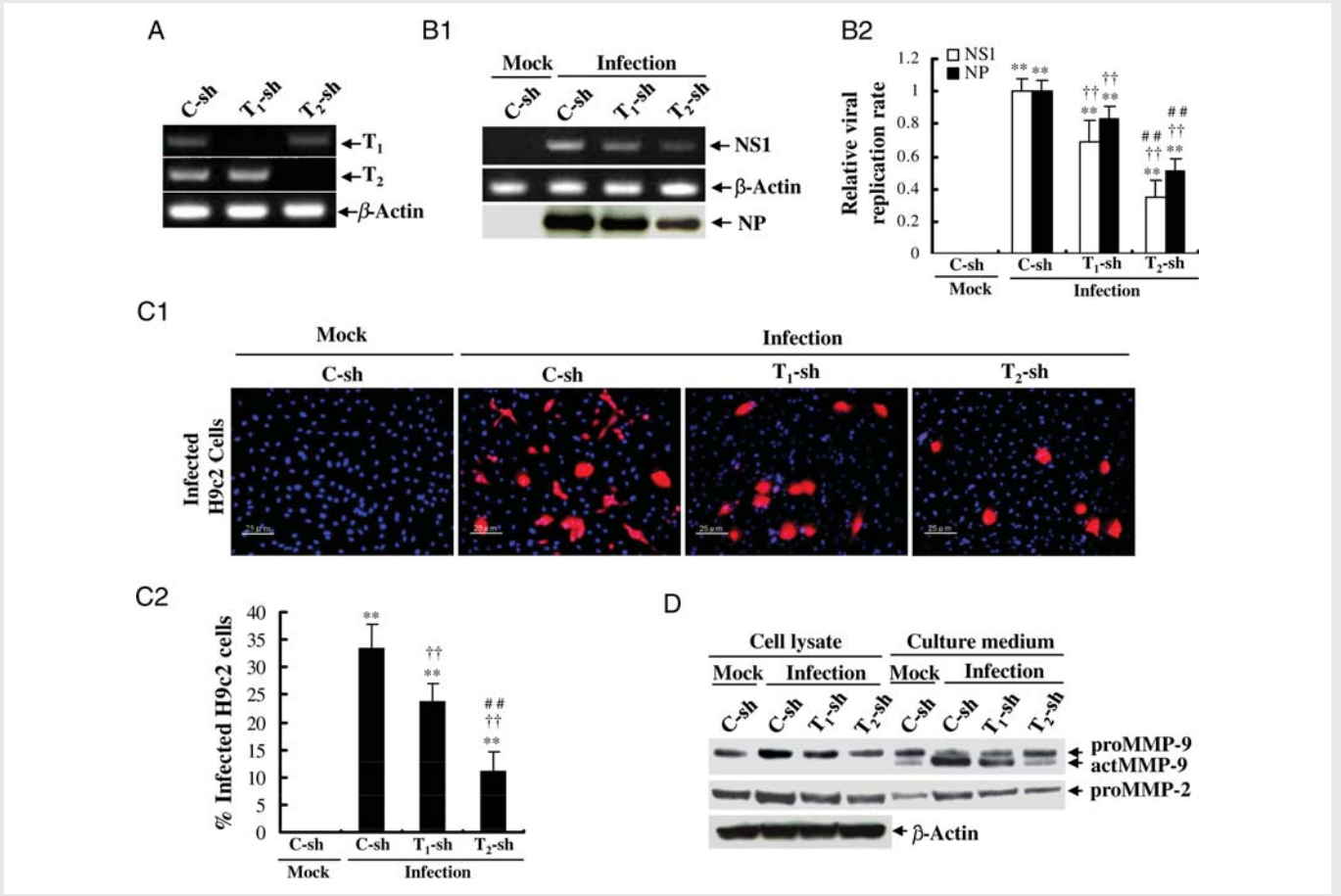

Figure 5 Effects of trypsin knockdown on IAV replication, MMPs up-regulation, and actMMP-9 production in H9c2 cardiomyoblasts. Silence efficiency of T_1 and T_2 genes was determined by RT-PCR in T_1-sh, T_2-sh, and C-sh cell lines (*A*). Effects of T_1- and T_2-knockdown on viral replication monitored by NS1 gene in cell lysate by RT-PCR, NP in culture media by western Immunoblotting (*B1*), and relative values of NS1 and NP vs. infection control (C-sh) (B2). Immunofluorescent detection of viral antigen (red) in H9c2 cells (*C1*) at 24 h post-infection. Nuclei were stained by DAPI. Bar = 25 μm. Percentage of infected cells (*C2*). Data are mean ± SD of three independent experiments. Effects of T_1- and T_2-knockdown on the expression of proMMP-9, proMMP-2, and actMMP-9 in cell lysate and culture medium of T_1-sh, T_2-sh, and C-sh cells infected with IAV or mock monitored by western immunoblotting (*D*). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock infection; $^{\dagger\dagger}P < 0.01$ vs. infection control; $^{\#\#}P < 0.01$ vs. T_1-knockdown.

trypsins, and proMMPs by IAV infection was confirmed in H9c2 cells (see Supplementary material online, *Figure S3*). Among the trypsin isoforms, T_2 was dominant while T_3 was detected in a trace amount, in a similar manner in the hearts. IAV up-regulated precursor forms of proteases, such as trypsinogens, proMMP-9, and proMMP-2, in the cell lysate, while IAV-induced mature forms of proteases, such as trypsins and actMMP-9, in the culture medium. Treatment of the cells with 1.5 μM aprotinin effectively inhibited IAV replication and up-regulation of proteases, particularly the production of actMMP-9. These results indicate that secreted trypsins readily promote IAV replication and convert proMMP-9 to actMMP-9 in extracellular milieu. Increase in the viral load up-regulated trypsins in the culture medium and depleted ATP in H9c2 cells in a dose-dependent manner (see Supplementary material online, *Figure S4*). In neonatal heart cells, accumulated viral antigens (red) were detected after IAV infection not only in cardiomyocytes identified by cardiac myosin (green) but also in non-myocytes (see Supplementary material online, *Figure S5*).

3.6 Trypsin knockdown suppressed viral replication and production of active MMP-9 and cytokines

Since host cellular trypsins trigger IAV entry and viral replication in target cells,[6–8] we analysed the effects of silencing major trypsin genes, T_1 and T_2, on viral replication and production of actMMP-9 and cytokines in stable transfectant T_1-sh, T_2-sh, and C-sh H9c2 cell lines. Expressions of T_1 and T_2 genes determined by RT-PCR were selectively and significantly suppressed in T_1-sh and T_2-sh cells, respectively, in comparison with the levels in control (C-sh) cells (*Figure 5A*). Viral replication monitored by the NS1 gene and the levels of NP showed significant suppression of IAV replication in T_1- and T_2-knockdown cells, the suppression efficiency being significantly higher in T_2-knockdown than in T_1-knockdown (*Figure 5B1–2*). Significant reduction in the number of immunofluorescence-labelled viral antigen in the infected cells (red) was detected under T_1- and T_2-knockdown conditions, particularly T_2-knockdown condition, compared with those in C-sh cells (*Figure 5C1–2*).

Up-regulation of both proMMP-9 and proMMP-2 was detected in C-sh cells after infection in a similar manner *in vivo*, and the up-regulation was significantly suppressed by T_1- and T_2-knockdown, the suppression efficiency in T_2-knockdown being higher than in T_1-knockdown (*Figure 5D* and Supplementary material online, *Figure S6A*). Significant suppression of actMMP-9 production monitored by the ratio of actMMP-9/proMMP-9 was observed in the culture medium of T_1- and T_2-knockdown cells, the efficiency in T_2-knockdown being higher than in T_1-knockdown (*Figure 5D* and Supplementary material online, *Figure S6B*).

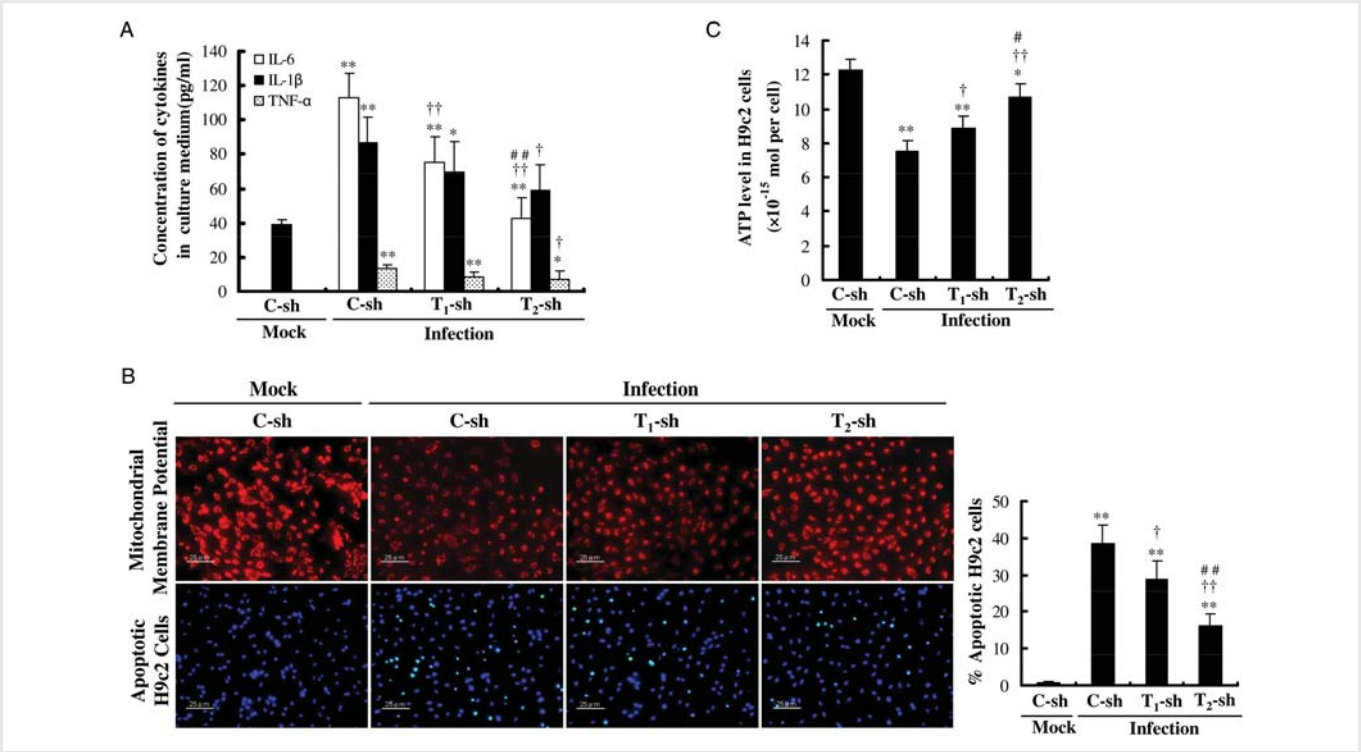

Figure 6 IAV infection-induced cytokine up-regulation, mitochondrial membrane depolarization, ATP depletion and apoptosis, and abrogation by T_1- and T_2-knockdown in H9c2 cardiomyoblasts. Effects of T_1- and T_2-knockdown on IL-6, IL-1β, and TNF-α levels in culture medium were analysed by ELISA at 24 h post-infection (*A*). $\Delta\Psi_m$ of C-sh, T_1-sh, and T_2-sh cells infected with mock or IAV were analysed after IAV infection for 24 h (*B*). Top: $\Delta\Psi_m$ was monitored by treatment for 15 min with JC-1 (red). Bottom: apoptotic cells detected by TUNEL staining (light blue). Nuclei were stained by DAPI. Bar = 25 μm. Right: percentage of apoptotic cells in total cells. ATP levels under the same experimental conditions in B (*C*). Data are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock infection; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. infection control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. T_1-knockdown.

3.7 Effects of trypsin knockdown on cytokine up-regulation, mitochondrial membrane potential, ATP levels, and apoptosis in cardiomyoblasts

Induction of pro-inflammatory cytokines by IAV infection in the culture medium, such as IL-6, IL-1β, and TNF-α, were suppressed under T_1- and T_2-knockdown conditions, particularly IL-6 in T_2-knockdown (*Figure 6A*). We further analysed the effects of trypsin knockdown on $\triangle\Psi_m$, intracellular ATP levels[23,24] and apoptosis after IAV infection for 24 h. Active mitochondria with high $\triangle\Psi_m$ appeared red by cationic lipophilic probe JC-1[23] as shown in the cells with mock infection (*Figure 6B*, C-sh, mock). Viral infection markedly reduced the red fluorescence indicating mitochondrial depolarization and reduction of $\triangle\Psi_m$. T_1- and T_2-knockdown, particularly T_2-knockdown, improved the infection-induced mitochondrial depolarization (*Figure 6B*, T_2-sh, infection). The numbers of apoptotic cells analysed by TUNEL staining were significantly reduced under T_1- and T_2-knockdown conditions, particularly T_2-knockdown condition. ATP levels in H9c2 cells were reduced to about 61% after the viral infection, and this reduction was abrogated by trypsin-knockdown, particularly T_2-knockdown (*Figure 6C*).

4. Discussion

The present study reported several new observations: (i) IAV infection up-regulated ectopic trypsins, particularly T_2 among the three isoforms, MMPs, and cytokines, and also induced ATP depletion in the heart *in vivo* and also cardiomyoblasts *in vitro*. (ii) Administration of trypsin inhibitor aprotinin suppressed viral replication, up-regulation of trypsins, MMPs, and cytokines as well as ATP depletion, and significantly improved cardiac function. (iii) Trypsin-knockdown, particularly T_2-knockdown, in cardiomyoblasts effectively suppressed IAV replication and ATP depletion accompanied by the protection against mitochondrial membrane de-polarization and apoptosis.

Influenza is a recognized cause of myocarditis and acute myocardial infarction, which can lead to significant impairment of cardiac function and mortality.[4,25] However, the pathogenesis of myocarditis has not been fully clarified. We elucidated here the crucial roles of inducible and ectopic host cellular trypsins in the pathogenesis of IAV infection-induced acute myocarditis and found that aprotinin administration and selective trypsin-knockdown effectively suppress up-regulation of cellular pathogenic factors induced by IAV infection, such as trypsins, MMPs, and cytokines, resulting in the improved cardiac function.

Binding sites of nuclear factor-kappa B and activator protein 1 are found in the promoter regions of T_1 and T_2 and these transcriptional factors up-regulate trypsin expression (Talukder SR *et al.*, manuscript in preparation) in a similar manner to MMP-9 up-regulation.[9,10] These data suggest that IL-6, IL-1β, and TNF-α, which induce these transcriptional factors, may play important roles in up-regulation of both trypsins and MMP-9. Trypsins are secreted as trypsinogens and readily converted to trypsins through auto-activation or by widely distributed endogenous activators.[26,27] Trypsins proteolytically trigger various cellular events such as activation of proMMPs[18,19] and pro-urokinase.[28] Trypsins also promote IAV entry and replication,[6,8] intracellular signal transduction, and release of cytokines through PAR-2.[21,29] Close distribution of influenza virus antigens, up-regulated trypsins, inflammatory infiltrates in injured heart loci indicates a mutual close-relationship among these pathogenic factors.

MMP-2 and MMP-9 are type IV collagenases and MMP-9 also digests type I and III collagens while MMP-2 does not.[30] IAV infection up-regulated both proMMP-2 and proMMP-9 in the myocardium and cardiomyoblasts, with only marked proMMP-9 activation, but not proMMP-2, probably because proMMP-9, but not proMMP-2, is readily activated by induced trypsins.[31]

Although both T_1- and T_2-knockdown protected cardiomyoblasts against damage during IAV infection, the protective effects of T_2--knockdown were more efficient than those of T_1-knockdown. The difference might be explained by the following reasons: (i) Abundance of T_2 at nearly double the amount of T_1 in cardiac tissue and cardiomyoblasts. (ii) T_2 activates proMMP-9 efficiently even at a molar ratio of 1:1000, the lowest concentrations reported so far for any proMMP-9 activators.[32] (iii) T_2 also degrades type I collagen and serves as an efficient activator of a pro-urokinase cascade and pro-collagenases MMP-1,-8, and -13,[33] and thus may cause an even more serious tissue destruction.

To date, there are no effective therapies for IAV infection-induced myocarditis apart from anti-viral drugs and symptomatic treatments. Previous studies indicated that inhibition of cytokine production and MMP-9 activity has protective effects on myocarditis.[34,35] However, recent studies showed that MMP-9-deficient mice display reduced recruitment of leucocytes to the infected sites, resulting in impaired host defence and enhanced dissemination of the infection.[36,37] Our present data suggest that administration of the trypsin inhibitor aprotinin and selective trypsin knockdown suppress IAV multiplication by intercepting the interrelationship among IAV replication, up-regulation of trypsins, MMPs, and cytokines, and also suppress ATP depletion and apoptosis in the myocardium and cardiomyoblasts. In addition, aprotinin administration improved heart function, monitored by LVESD, %FS, SV, CO, blood pressure, and heart rate. We are currently investigating the therapeutic effects of various trypsin inhibitors other than aprotinin on IAV infection-induced acute myocarditis *in vivo*. In conclusion, the presented data demonstrated that up-regulated trypsin, particularly T_2, is one of the key pathogenic host factors in IAV infection-induced myocarditis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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