Ectopic trypsin in the myocardium promotes dilated cardiomyopathy after influenza A virus infection

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¹Department of Cardiology, Affiliated Hospital of Nantong University, Institute of Cardiovascular Research, Nantong University, Jiangsu, China; and ²Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan

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Pan H, Sun H, Xue L, Pan M, Wang Y, Kido H, Zhu J. Ectopic trypsin in the myocardium promotes dilated cardiomyopathy after influenza A virus infection. Am J Physiol Heart Circ Physiol 307: H922–H932, 2014. First published July 18, 2014; doi:10.1152/ajpheart.00076.2014.—We have previously reported that ectopic trypsin in the myocardium triggers acute myocarditis after influenza A virus (IAV) infection. As myocarditis is a common precursor to dilated cardiomyopathy (DCM), the aim of the present study was to investigate the influence of trypsin on the progression of DCM after IAV infection. IAV-infected mice treated with saline or trypsin inhibitor were euthanized on days 0, 9, 20, 40 and 60 postinfection. Trypsin expression colocalized with myocardial inflammatory loci and IAV-induced myocarditis peaked on day 9 postinfection and alleviated by day 20 but persisted until day 60 postinfection, even though replication of IAV was not detected from day 20 postinfection. Similar time courses were observed for the activation of pro-matrix metalloproteinase (pro-MMP)-9 and expression of the proinflammatory cytokines IL-6, IL-1β, and TNF-α. Degradation of collagen type I, proliferation of ventricular interstitial collagen, and expression of collagen type I and III mRNA increased significantly during acute and chronic phases; collagen type III mRNA increased more significantly than collagen type I mRNA. Cardiac function progressively deteriorated with progressive left ventricular dilation. The trypsin inhibitor aprotinin suppressed pro-MMP-9 activation and cytokine release, alleviated myocardial inflammation, and restored collagen metabolism during acute and chronic phases of myocarditis. This effectively prevented ventricular dilation and improved cardiac function. These results suggest that ectopic trypsin in the myocardium promoted DCM through chronic activation of pro-MMP-9, persistent induction of cytokines, and mediation of collagen remodeling. Pharmacological inhibition of trypsin activity might be a promising approach for the prevention of viral cardiomyopathy.

trypsin; myocarditis; dilated cardiomyopathy; influenza virus; extracellular matrix remodeling

THE INFLUENZA A VIRUS (IAV) is the most common infectious pathogen in humans. Acute myocarditis is a well-known complication of influenza infection and a common precursor to inflammatory dilated cardiomyopathy (DCM) that can lead to chronic heart failure (1, 49). A long-term followup study (18) in patients with acute myocarditis documented the development of DCM in 21% of patients over a mean followup period of 3 yr. The course of viral myocarditis has three distinct, successive stages: acute viral infection, immune cell infiltration, and cardiac remodeling (29). Although progress has been made in understanding the pathogenesis of DCM after viral infection, the precise mechanisms involved in the transition from viral myocarditis to DCM are not well understood.

During IAV infection, host factors such as proinflammatory cytokines, matrix metalloproteinases (MMPs), and ectopic trypsin are induced (39). Among these factors, ectopic trypsin, serving as a viral envelope hemagglutinin-processing protease, is crucial for viral entry, replication, and spread, because the IAV genome does not have a hemagglutinin-processing protease and hemagglutinin cleavage by cellular proteases at the posttranscriptional level is a prerequisite for membrane fusion activity (16). The expression profile of trypsin is likely a major determinant of IAV tissue tropism and pathogenicity. In addition, trypsin converts pro-MMPs to active MMPs (20, 39, 44) and promotes cytokine release through proteinase-activated receptor (PAR)-2 (35). Active MMPs, such as MMP-2 and MMP-9, degrade vascular basement membranes and the extracellular matrix (ECM), which could promote endothelial hyperpermeability and inflammatory cell migration (53). Degradation of the ECM could initiate anoikis in neighboring healthy tissue (31), leading to cardiac dilation and dysfunction. Furthermore, active MMP-2 and MMP-9 convert proinflammatory cytokines into their active forms (9, 28). Activated cytokines, such as TNF-α, IL-1β, and IL-6 recruit inflammatory cells and increase nitric oxide production in the heart (42) and promote cardiac remodeling via serpin A-3n (5). Overproduction of nitric oxide contributes to the development of DCM by inducing myocardial ATP depletion and cardiomyocyte apoptosis (14, 48).

The myocardial ECM is mainly composed of collagen type I (Col I; 85%) and Col type III (Col III; 11%) (8), which provide architectural support for cardiac myocytes and are important in myocardial function (41). Collagenases like MMP-9 and MMP-2 can remove collagen from struts and tethers, which are critical structures for preventing myocyte slippage (11, 23). Proinflammatory cytokines induce new collagen deposition, which can be misdirected to intercellular septa with a defective content of permanent cross-links (24). These effects can contribute to heart overextension and dilation.

We have previously reported that IAV-induced trypsin expression in the myocardium triggers acute viral myocarditis through stimulation of IAV replication, pro-MMP-9 activation, and cytokine induction (39). The aim of the present study was to clarify the role of trypsin in the progression of DCM after IAV infection. We defined the kinetics of trypsin upregulation during acute and chronic phases of myocarditis and investigated the effects of trypsin upregulation on the persistence of myocardial inflammation, changes in myocardial interstitial collagen components, and DCM development. The results...
suggested that trypsin inhibition might prevent DCM with improved cardiac function after IAV infection.

METHODS

Influenza myocarditis model. This study conformed with Animal Research: Reporting In Vivo Experiments guidelines (17) and was approved by the Animal Care Committee of Nantong University. We randomly assigned 90 specific pathogen-free 8-wk-old male BALB/c mice (Comparative Medicine Center of Yangzhou University, Jiangsu, China) to a mock-infected group or to IAV-infected groups treated with saline or the trypsin inhibitor aprotinin (n = 10 mice/group). Under chloral hydrate anesthesia, mice were injected intranasally with 40 plaque-forming units of IAV/PR/8/34 (H1N1) (VR1469, American Type Culture Collection) in 15 μl saline or saline vehicle and euthanized on days 0, 9, 20, 40, or 60 postinfection. To analyze the effects of trypsin inhibition, aprotinin (Sigma-Aldrich Shanghai Trading, Shanghai, China) was injected intraperitoneally at 4 mg/kg daily until euthanasia.

Histopathological preparation. Transthoracic echocardiography was performed in mice using a VisualSonics vevo 770 ultrasonograph with a 30-MHz transducer (VisualSonics, Toronto, ON, Canada) under light anesthesia from an intramuscular injection of ketamine (30 mg/kg) before euthanasia. Left ventricular (LV) M-mode echocardiograms were obtained from a parasternal short-axis view. LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), LV posterior wall thickness at end diastole (LVPWd), and LV posterior wall thickness at end systole (LVPWs) were recorded. Fractional shortening (FS) was calculated as follows: FS = (LVEDD – LVESD)/LVEDD × 100. LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were measured from the parasternal long-axis view and calculated using Simpson’s formula (4). Stroke volume (SV) and cardiac output (CO) were calculated by the following formulas: SV = LVEDV – LVESV and CO = SV × heart rate, respectively. LV ejection fraction (EF) was calculated as follows: EF = (SV/LVEDV) × 100. All parameters were measured from three consecutive cycles and averaged.

ELISA. Mouse blood was collected from the right ventricle after anesthesia. Plasma levels of Col I cross-linked carboxy-terminal telopeptide (ICTP) were measured with ELISA kits according to the recommendation of the manufacturer (Antibodies-online).

Tissue preparation. Isolated hearts were cut into halves with one portion fixed with 4% buffered paraformaldehyde solution for histopathological evaluation and the other portion stored at −80°C for biochemical analyses. All experiments were repeated at least three times.

Histopathological preparation. Tissues fixed in 4% paraformaldehyde were dehydrated in graded ethanol, embedded in paraffin, and sliced into 5-μm sections. After deparaffinization, sections were subjected to routine hematoxylin and eosin staining or van Gieson staining for the identification of collagen distribution.

Immunostaining. Immunohistochemistry for upregulated trypsin in the myocardium was performed as previously described (22, 39). Endogenous peroxidase activity was quenched with 3% H2O2 in methanol. Non-specific binding sites were blocked with goat serum. Immune depositions in sections were detected with rabbit anti-trypsin antibody (Santa Cruz Biotechnology). Avidin-biotin-peroxidase kits (Vector Labs, Burlingame, CA) were used to visualize trypsin. For immunofluorescent staining, rabbit anti-trypsin (Santa Cruz Biotechnology) and mouse anti-IAV (Takara Bio, Shiga, Japan) were used as primary antibodies. Goat anti-rabbit IgG Texas red-conjugated antibody (Molecular Probes) and goat anti-mouse IgG FITC-conjugated antibody (Sigma-Aldrich) were used as secondary antibodies to visualize trypsin and IAV immunodeposits in hearts.

RT-PCR. Total RNA was extracted with TRIzol Reagent (Sangon Biotech, Shanghai, China), and 2 μg RNA was reverse transcribed with a TIANScript cDNA Synthesis kit (Tiangen Biotech, Beijing, China) for the synthesis of host and IAV cDNA. Trypsin, IAV nonstructural protein (NS1), Col I, and Col III gene segments were amplified with a Taq PCR MasterMix kit (Tiangen Biotech) as previously described (15, 39). RT-PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Immunoblot analysis and gelatin zymograph. Tissues were homogenized with three volumes of 0.05 M Tris-HCl (pH 7.6) containing 2% SDS and 0.5 M NaCl. Immunoblot analysis was performed as previously described (22). Target-specific antibodies were used to detect trypsin, MMP-9, MMP-2, TNF-α, IL-6, IL-1β, and actin (Santa Cruz Biotechnology). Immunoreactive bands were visualized by an enhanced chemiluminescence detection system (Cell Signaling Technology). MMP-2 and MMP-9 activities were analyzed by zymography. Protein extracts were separated by electrophoresis on 10% gelatin zymogram gels (Invitrogen) at 4°C. Gels were incubated in renaturing buffer (Invitrogen) at room temperature for 30 min and in developing buffer (Invitrogen) at 37°C for 4 h. Bands of gelatin degradation were visualized by staining with Coomassie brilliant blue R-250 and destaining as appropriate.

Morphometric quantification of trypsin and collagen. Digital images from immunohistochemistry and van Gieson staining were obtained using a Nikon E800 microscope with a ×10 objective lens (Nikon Instruments, Tokyo, Japan). Trypsin and collagen were quantified from 12 separate fields over 3 stained sections for each heart using Image Proplus software and expressed as percentages of the stained area per total myocardial tissue area.

Statistical analysis. Results are presented as means ± SD from 7–10 mice/group. Significance was calculated by one-way ANOVA. For survival analysis, the Gehan-Breslow-Wilcoxon test was used for the analysis of survival difference. P values of <0.05 were considered statistically significant.

RESULTS

Kinetics of body weight, survival rate, and myocardial inflammation as well as restoration by trypsin inhibitor. We have previously reported that the inflammatory response in the myocardium was most serious on day 9 after viral infection (39). Biventricular dilation consistent with DCM was observed on day 35 postinfection in male BALB/c mice and on day 60 postinfection in male C3H/He mice (38, 46). Therefore, we observed the progression of myocardial inflammation from day 9 to 60 postinfection. The severity of IAV infection was evaluated by weight loss, survival rate, and myocardial pathological changes. Mice intranasally inoculated with 40 plaque-forming units of IAV/PR/8 (H1N1) appeared lethargic and anorexic with significant weight loss in the acute stage (P < 0.01; Fig. 1A). About 20–30% of mice died around day 9 postinfection if not treated with aprotinin. No mice died after day 20 postinfection (Fig. 1B). Surviving mice exhibited acute and chronic myocarditis, as confirmed by histological examination of hematoxylin and eosin-stained sections. Extensive inflammatory infiltration across the interstitium accompanied by focal necrosis and ECM destruction were observed on day 9 in infected mice not treated with the trypsin inhibitor aprotinin. The prevalence of infiltration was alleviated by day 20 postinfection but persisted with sparse, diffuse inflammatory cells in the interstitium on day 60 postinfection (Fig. 1C,1). Aprotinin significantly reduced the loss of body weight, decreased morbidity, and suppressed the inflammatory response, which alleviated cardiac lesions (Fig. 1, A, B, and C,2).
Kinetics of viral replication and activation of trypsinogen with suppression by trypsin inhibitor. The kinetics of viral replication in hearts, as monitored by viral NS1 gene expression, showed that viral RNA peaked on day 9 postinfection and then diminished to undetectable levels by day 20 postinfection (Fig. 2A). Trypsin gene expression as monitored by RT-PCR and expression of trypsinogen plus trypsin and calculation of the trypsin-to-trypsinogen ratio based on Western blot analysis also peaked on day 9 and then slowly declined to minimum levels by day 60 postinfection, although levels were still higher than controls (day 0; Fig. 2, A–D). Immunofluorescent staining showed that trypsin distributed in the myocardium of infected loci during the acute stage. Immunohistochemical staining revealed that trypsin was mainly expressed by myocardial cells, which colocalized with inflammatory infiltrates (Fig. 2, E and G). The level of stained trypsin depositions quantified by area percentage was highest on day 9 postinfection and declined gradually as myocardial inflammation declined during the chronic phase (days 20 to 60 postinfection; Fig. 2F). Aprotinin significantly suppressed viral replication on day 9 postinfection and inhibited upregulation of trypsin expression around inflammatory loci and the trypsin-to-trypsinogen ratio during both acute and chronic phases.

Kinetics of MMPs and cytokine upregulation by IAV and effects of trypsin inhibitor. The relationships among trypsin upregulation, MMP activation, and cytokine induction were analyzed in acute and chronic myocarditis. Along with an increase in trypsin levels, upregulated pro-MMP-9 was effectively converted to active MMP-9, although upregulated pro-MMP-2 was not activated (Fig. 3). MMP-9 activity, total MMP-9 (pro-MMP-9 plus active MMP-9) expression, and the active MMP-9-to-pro-MMP-9 ratio increased to a peak value on day 9 postinfection followed by a slow decrease to day 60 postinfection (Fig. 3). Similar induction time courses were observed for IL-6, IL-1β, and TNF-α (Fig. 4). Aprotinin significantly inhibited MMP-9 activity and suppressed upregulation of MMPs and activation of pro-MMP-9 as well as the induction of cytokines during acute and chronic myocarditis.

Increased Col I degradation in the myocardium was prevented by trypsin inhibitor. Both trypsin and MMP-9 efficiently degrade Col I (33, 37), and ICTP is released during hydrolysis of Col I fibrils. The amount of ICTP in the circulation is proportional to the amount of degraded fibrillar collagen (26). Therefore, ICTP was used as a marker for Col I fibrils. The amount of ICTP in the circulation is proportional to the amount of degraded fibrillar collagen (26). Therefore, ICTP was used as a marker for Col I fibrils. Aprotinin significantly decreased ICTP levels during acute and chronic phases, suggesting a prevention of Col I degradation (Fig. 5A).
Increased collagen deposition in the myocardium reduced by trypsin inhibitor. During cardiac remodeling, loss of collagen from increased collagen degradation leads to replacement by newly synthesized collagen (21). To observe collagen proliferation and deposition after IAV infection, we used van Gieson staining. Heavy accumulation of collagen was seen around the blood vessels and inflammatory loci on day 9 postinfection. From day 20 postinfection, an extensive proliferation of collagen across the ventricular interstitium was observed and was most obvious on day 60 postinfection. The increased collagen deposition was reduced in aprotinin-treated mice during acute and chronic phases (Fig. 5, B and C).

Increased Col I and Col III mRNA in the myocardium was suppressed by trypsin inhibitor. Cardiac ECM remodeling is associated with significant changes in Col I and Col III expression (45). Synthesis of Col I and Col III was assessed as Col I and Col III mRNA using RT-PCR. Both Col I and Col III mRNA significantly increased after IAV infection during acute and chronic phases (Fig. 5D). Myocardial Col I mRNA increased 2.9- to 1.8-fold from days 9 to 60 postinfection (Fig...
5E). Col III mRNA increased 5.0- to 2.4-fold over the same time (Fig. 5F). The differential increase in Col I and Col III led to a decreased Col I-to-Col III ratio in IAV-induced acute and chronic myocarditis (Fig. 5G). Aprotinin significantly suppressed collagen upregulation and restored the Col I-to-Col III mRNA ratio.

**LV dilation and cardiac dysfunction after IAV infection and effects of trypsin inhibitor.** The dynamic changes of cardiac function, LV internal dimension, and LV posterior wall thickness were observed by echocardiography. IAV-infected mice were significantly impaired in LV function, as shown by a significant reduction in FS, EF, SV, and CO and a significant increase in LVESD on day 9 postinfection, although LVEDD, LVPWd, and LVPWs values were similar to levels on day 0. LV function was transiently improved by alleviation of acute myocarditis on day 20 postinfection and then deteriorated with progressive dilation of LV internal dimension and thinning of LV posterior wall thickness to day 60 postinfection. Deterioration of cardiac function and dilation of heart chambers were improved significantly by aprotinin with increased values for FS, EF, SV, and CO, reduced values for LVEDD and LVESD, and restored values for LVPWd and LVPWs during acute and chronic myocarditis (Fig. 6 and Table 1).

**DISCUSSION**

We investigated the pathological mechanisms underlying the development of DCM from IAV-induced myocarditis. First, we found that IAV infection induced a persistent upregulation of ectopic trypsin, which localized in myocardial inflammatory loci, throughout acute and chronic stages. Persistently upregulated trypsin led to continuous activation of proMMP-9 and release of cytokines. Second, myocardial inflammatory infiltration persisted after IAV could not be detected in the myocardium. The severity of myocardial inflammation was consistent with the kinetics of trypsin expression. Third, Col I
degradation, collagen deposition, and Col I and Col III mRNA in the myocardium increased in IAV-induced acute and chronic myocarditis. The differential increase in Col I and Col III mRNA led to a decreased Col I-to-Col III ratio. Fourth, cardiac function was transiently impaired in the acute stage and deteriorated with progressive ventricular dilation during the chronic stage. Finally, trypsin inhibitor aprotinin suppressed pro-MMP-9 activation and cytokine release, alleviated myocardial inflammation, reduced collagen proliferation, and restored collagen metabolism, thus effectively preventing ventricular dilation and DCM for improved cardiac function.

Consistent with our previous study (39), we found that IAV infection significantly upregulated ectopic trypsin, pro-MMPs, and proinflammatory cytokines in acute myocarditis. However, by day 20 postinfection, although replication of IAV could not be detected in the myocardium, upregulation of trypsin, MMPs, and proinflammatory cytokines persisted during the chronic stage. One possible reason for the persistent upregulation of these factors might be the effects of inflammatory cells infiltrating the myocardial interstitium. These cells release IL-1β, IL-6, and TNF-α, which can promote transcription of trypsinogen and pro-MMPs (50, 51). In this model, the interrelationship of trypsin, MMP-9, and cytokines is a cycle that contributes to persistent upregulation of these factors even after IAV is no longer present in the myocardium. Another reason for the persistent upregulation of these factors might be the effects of inflammatory cells infiltrating the myocardial interstitium. These cells release IL-1β, IL-6, and TNF-α, which can promote transcription of trypsin and MMPs genes via NF-κB and AP-1 (39, 51). Trypsin and MMP-9 synergistically promote inflammatory cell migration across the ECM to inflammatory loci (39, 53). These migrating cells further induce upregulation of trypsin and MMPs by releasing cytokines in inflammatory loci, leading to persistent upregulation. The colocalization of upregulated trypsin and inflammatory infiltrates we observed during acute and chronic stages suggests a close relationship among these pathogenic factors that results in chronic myocardial inflammation.

Fig. 3. Upregulation and activation of pro-matrix metalloproteinases (pro-MMPs) in the myocardium and effects of trypsin inhibitor. A: kinetics of activity and upregulation of MMPs in hearts as determined by zymography (1) and Western blot analysis (2) from days 0 to 60 postinfection. β-Actin was the internal control. B–D: densitometry showed significant inhibition of upregulation of pro-MMP9 + active (act)MMP9 (B), pro-MMP-2 (C), and activation of pro-MMP-9 (D) by Apr after IAV infection. Data are averages ± SD of 3 independent experiments from 7–10 mice/group. *P < 0.05 and **P < 0.01 vs. day 0; △P < 0.05 and △△P < 0.01 vs. no Apr treatment at the same time after IAV infection.
Although IAV replication, myocardial inflammation, and expression of MMPs, trypsin, and proinflammatory cytokines were significantly inhibited by day 9 postinfection in mice treated with aprotinin, the pathological changes in the myocardium persisted without recovery until after 40–60 days of continuous treatment with aprotinin. These findings suggested that trypsin might be involved in the pathological process throughout acute and chronic phases of myocarditis after IAV infection.

In the trypsin-MMP-9-cytokine cycle, the regulation of trypsin expression is crucial for cycle maintenance. In the acute stage, trypsin was significantly upregulated by IAV infection and peaked on day 9 postinfection. Induced trypsin activates PAR-2 to evoke cytokine release, which, in turn, upregulates trypsin expression through activation of NF-κB and AP-1 (40, 50). However, PAR-2 is the “sensory” arm of a negative feedback mechanism to downregulate trypsin expression (25). Activated PAR-2 forms a complex with β-arrestin and ERK1/2, which effectively prevents ERK1/2 translocation into the nucleus and after transcription of trypsigen (6, 47). Trypsin expression gradually declined from day 20 postinfection accompanied by the clearance of IAV. The final level of trypsin depended on the balance between its up- and downregulation mechanisms.

In the present study, both pro-MMP-2 and pro-MMP-9 in the myocardium were upregulated during acute and chronic phases. However, only pro-MMP-9 was activated, probably because pro-MMP-2 is resistant to activation by trypsin (19). MMP-9 also cleaves Col I and Col III, whereas MMP-2 does not (36). Trypsin has three major isoforms. In cardiac tissue, trypsin2 is the major isofrom and its abundance is nearly double that of trypsin1, whereas trypsin3 is barely detectable (39). Trypsin2 degrades Col I and is an efficient activator of a plasminogen cascade and procollagenases (33). Consistent with the kinetic expression of trypsin and MMP-9 that we observed in the myocardium, circulating ICTP, which reflects degradation of Col I, increased prominently in early stages and then slightly in late stages. In response to increased collagen degradation, the lost collagen was replaced by newly synthesized collagen, which was distributed around inflammatory loci in the acute phase and extensively proliferated across the ventricular interstitium in the chronic phase. Col I and Col III are the main components of the myocardial ECM. During collagen proliferation, Col I and Col III mRNA (especially Col III) was significantly increased. The differential increase of Col I and Col III mRNA led to a decreased Col I-to-Col III mRNA ratio in the myocardium. The increased ICTP and decreased relative proportion of Col I and Col III mRNA indicated that in ECM remodeling, properly cross-linked Col I was increasingly degraded and replaced with poorly cross-linked Col III. Col I provides substantial tensile and stiffness to prevent slippage and overstretching of myocytes, whereas Col III has greater elasticity (54). These changes might promote ventricular myocyte slippage and chamber dilation that contribute to wall thinning and systolic dysfunction in the development of DCM.

The reasons for the differential increase in Col I and Col III expression after IAV infection might be different molecular mechanisms. IL-6, IL-1β, and TNF-α were persistently upregulated during acute and chronic phases after IAV infection. Previous studies (10, 32) have indicated that IL-1β and IL-6

Fig. 4. Cytokine induction in the myocardium and suppression by trypsin inhibitor. A: time courses of induction of IL-6, IL-1β, and TNF-α from days 0 to 60 postinfection by Western blot analysis. β-Actin was the internal control. B–D: densitometry showed that Apr significantly inhibited the induction of IL-6 (B), IL-1β (C), and TNF-α (D) during acute and chronic phases of myocarditis. Data are means ± SD of 3 independent experiments from 7–10 mice/group. *P < 0.05 and **P < 0.01 vs. day 0; △P < 0.05 and △△P < 0.01 vs. no Apr at the same time after IAV infection.
Fig. 5. Increased collagen type I (Col I) degradation, upregulation of collagen synthesis, and effect of trypsin inhibitor. A: Col I degradation as measured by plasma Col I cross-linked carboxy-terminal telopeptide (ICTP) during acute and chronic phases (n = 7–10 mice/group). Apr effectively prevented degradation. B: proliferation of collagen in the myocardium as identified by van Gieson staining from days 0 to 60 without (1) and with (2) Apr treatment. Magnification: ×100. C: quantification of collagen as percent area (n = 5 mice/group). D: synthesis of Col I and collagen type III (Col III) as assessed by Col I and Col III mRNA using RT-PCR. β-Actin was the internal control. E–G: densitometry showed significantly upregulated Col I (E) and Col III (F) mRNA and a decreased Col I-to-Col III mRNA ratio (G) in IAV-induced acute and chronic myocarditis. Data are means ± SD of 3 independent experiments from 7–10 mice/group. *P < 0.05 and **P < 0.01 vs. day 0; △P < 0.05 and △△P < 0.01 vs. no Apr treatment at the same time point.
induce collagen synthesis through upregulation of Col I and Col III genes. In contrast, TNF-α reduces Col I mRNA levels and the steady state of Col I mRNA but has no effect on Col III mRNA (30, 43). In our study, differential regulatory effects of cytokines on Col I and Col III mRNA might have resulted in the shift of the Col I-to-Col III mRNA ratio in the development of DCM after IAV infection.

Trypsin is an efficient activator of a prourokinase cascade (33). Plasminogen is the primary physiological substrate of urokinase. Plasminogen is activated in patients with acute viral myocarditis at all stages of the clinical course (7). Plasmin can directly degrade basement collagen type IV (27) and activate MMP-9 (12). The combined upregulation of trypsin and plasmin activity causes synergistic proteolytic degradation of the vascular basement and the myocardial ECM. In addition to trypsin, aprotinin also inhibits plasmin activity, thus effectively protecting the heart from inflammatory injury and myocardial remodeling.

No specific treatment is currently available for the prevention of IAV-induced myocarditis and subsequent DCM. Heymans et al. (12) reported that suppression of MMP-9 activity reduces the cardiac inflammatory response, protecting mice against cardiac injury, dilatation, and failure during viral myocarditis. However, Cheng et al. (3) indicated that MMP-9 is

Table 1. Effects of the trypsin inhibitor Apr on kinetics of cardiac function and LV posterior wall thickness after influenza A virus infection

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Day 0</th>
<th>Day 9</th>
<th>Day 20</th>
<th>Day 40</th>
<th>Day 60</th>
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<tbody>
<tr>
<td>Ejection fraction, %</td>
<td>70.51 ± 9.06</td>
<td>49.94 ± 8.26</td>
<td>60.36 ± 6.46</td>
<td>52.35 ± 11.01</td>
<td>61.89 ± 8.87</td>
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<tr>
<td>Stroke volume, µl</td>
<td>44.56 ± 3.74</td>
<td>31.42 ± 3.43</td>
<td>37.42 ± 4.34</td>
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<td>38.25 ± 3.31</td>
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<tr>
<td>Cardiac output, µl/min</td>
<td>24.06 ± 3.63</td>
<td>18.22 ± 2.45</td>
<td>21.55 ± 3.08</td>
<td>18.27 ± 2.60</td>
<td>21.89 ± 2.01</td>
</tr>
<tr>
<td>LV posterior wall thickness at end diastole, mm</td>
<td>0.74 ± 0.07</td>
<td>0.76 ± 0.08</td>
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<tr>
<td>LV posterior wall thickness at end systole, mm</td>
<td>1.15 ± 0.07</td>
<td>1.17 ± 0.07</td>
<td>1.16 ± 0.06</td>
<td>1.13 ± 0.06</td>
<td>1.15 ± 0.07</td>
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Values are means ± SD; n, number of surviving mice. *P < 0.05 and †P < 0.01 compared with day 0; ‡P < 0.05 and §P < 0.01 compared with no aprotinin (Apr) treatment at the same time of observation.
essential for viral clearance by promoting immune cell recruitment in the early phase of infection. Therefore, MMP-9 inhibition might impair host defense and enhance infection dissemination. In our study, administration of the trypsin inhibitor aprotinin effectively suppressed IAV replication and the inflammatory response throughout acute and chronic phases by inhibiting trypsin-mediated activation of IAV hemagglutinin and interrupting the trypsin-MMP-9-cytokine cycle. Thus, inhibition of MMP-9 activity by aprotinin will not impair the host defense against viral infection. In addition, recent studies (2, 34, 52) have reported that PAR-2 is involved in innate immune responses during RNA virus infection and enhanced cardiac remodeling in the injured heart. Activation of PAR-2 negatively regulates the Toll-like receptor-3-dependent antiviral pathway with reduced expression of interferon-β (34). PAR-2 knockout mice are protected from H1N1/PR8 IAV virus-induced lethality and coxsackievirus B3-induced myocarditis (34, 52). Overexpression of PAR2 in mice induced cardiac fibrosis, inflammation, and heart failure (2). As trypsin is one of the most potent activators of PAR-2, aprotinin can partially abrogate the pathological roles of PAR-2 induced by trypsin.

Study limitations. Our findings suggested that trypsin is a key factor in acute and chronic stages of myocarditis after IAV infection. An experiment in which aprotinin treatment started from day 9 postinfection could further confirm the protective effects of trypsin inhibitor during chronic stage but was not performed. Thus, we cannot exclude the contribution of reduced acute myocarditis by aprotinin treatment for the prevention of DCM.

Conclusions. The results of the present study suggested that ectopic trypsin in the myocardium was involved in acute and chronic myocardial inflammation by promoting IAV infection and initiating a trypsin-MMP-9-cytokine cycle and promotes progressive cardiac dilation through mediation of collagen remodeling. Thus, trypsin might play important roles in the development of DCM after IAV infection. Aprotinin prevented the progression of myocarditis to DCM by suppressing IAV infection, interrupting the trypsin-MMP-9-cytokine cycle and restoring collagen metabolism. Our findings suggest that inhibition of trypsin activity might be a promising therapeutic approach for the prevention of DCM after IAV infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

TRYPsin IN THE MYOCARDIUM PROMOTES DILATED CARDIOMYOPATHY


