Inducible Cardiac-Restricted Expression of Enteroviral Protease 2A Is Sufficient to Induce Dilated Cardiomyopathy

Dingding Xiong, MD, PhD; Toshitaka Yajima, MD, PhD; Byung-Kwan Lim, PhD; Antine Stenbit, MD, PhD; Andrew Dublin, MD; Nancy D. Dalton, RDMS; Daphne Summers-Torres, BS; Jeffery D. Molkentin, MD; Herve Duplain, MD; Rainer Wessely, MD; Ju Chen, PhD; Kirk U. Knowlton, MD

Background
Enterovirus infection is a cause of cardiomyopathy. We previously demonstrated that enteroviral protease 2A directly cleaves the cytoskeletal protein dystrophin. However, the direct effect of protease 2A in enteroviral cardiomyopathy is less clear because other viral proteins are also expressed with viral infection.

Methods and Results
A transgenic mouse with inducible cardiac-restricted expression of enteroviral protease 2A was generated. In the transgenic mouse, a tamoxifen-regulated Cre-loxP system, MerCreMer (MCM), was used to induce genetic recombination in cardiac myocytes, which led to protease 2A expression. Protease 2A and MCM double transgenic (2AxMCM) mice were treated with tamoxifen; the controls included 2AxMCM mice treated with diluents for tamoxifen and tamoxifen-treated MCM littermates. Protease 2A activity was significantly induced after tamoxifen in the 2AxMCM mice compared with controls. Echocardiographic analysis demonstrated an increase in left ventricular end-diastolic and end-systolic chamber size, with decreased fractional shortening in tamoxifen-treated 2AxMCM mice. There was an increase in heart weight-to-body weight ratio in 2AxMCM mice treated with tamoxifen. Only a small increase in interstitial fibrosis and inflammation was found in tamoxifen-treated 2AxMCM mice; however, ultrastructural analysis demonstrated myofibrillar collapse with abnormalities of intercalated discs and sarcolemmal membranes. Evans blue dye–positive myocytes with disruption of dystrophin were present in 2AxMCM mice treated with tamoxifen. Disruption of dystrophin was also found in cultured myocytes isolated from 2AxMCM mice with Cre in the nucleus.

Conclusions
Protease 2A has a significant role in enteroviral cardiomyopathy and alone is sufficient to induce dilated cardiomyopathy, which is associated with disruption of the sarcolemmal membrane and cleavage of dystrophin with protease 2A expression. (Circulation. 2007;115:94-102.)

Key Words: cardiomyopathy ■ enterovirus ■ heart failure ■ myocarditis ■ protease

Dilated cardiomyopathy is a multifactorial disease that results in heart failure and premature death. Enterovirus infections, including coxsackie B viruses (CVB) are associated with a subset of acute and chronic forms of cardiomyopathy. Infection of the heart by coxsackievirus induces a direct myocytotoxic effect and can cause cardiomyopathy.1-4 In addition, low-level expression of full-length, replication-defective coxsackieviral genomes in the mouse heart is sufficient to induce dilated cardiomyopathy with characteristics similar to those observed in human cardiomyopathy.5,6 In both of these models, all viral proteins are expressed, which makes it difficult to determine the role of a single viral protein in viral mediated cardiomyopathy.

CVB are members of the picornaviridae family, enterovirus genus. They have a 7.4-kb positive-stranded RNA genome that is translated as a monocistronic polyprotein. CVB infection results in expression of capsid proteins (VP1 to VP4) and 7 nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) that are involved in viral replication.7 Two viral proteases, 2A and 3C, cotranslationally cleave the viral polyprotein into mature peptides.8 In addition to the cleavage activity of protease 2A (2APro) on the viral polyprotein, it also directly cleaves dystrophin, a cytoskeletal protein associated with Duchenne muscular dystrophy and X-linked dilated cardiomyopathy. CVB3 infection results in loss of the sarcolemmal localization of dystrophin, which suggests a role for 2APro–mediated cleavage of dystrophin.9 Recently, we also demonstrated a link between dystrophin and susceptibility to viral-
mediated cardiomyopathy by infection of dystrophin-deficient mice and showed that dystrophin deficiency increases enterovirus-induced cardiomyopathy with an increase in enteroviral replication and propagation in the heart. In sum, these findings suggest that 2APro-mediated cleavage of dystrophin during CVB3 infection contributes to a cascade of viral-mediated events that leads to dilated cardiomyopathy. However, it is not known whether 2APro alone in the absence of other viral proteins and activation of the viral-mediated immune response is sufficient to induce cardiomyopathy in the adult heart.

Therefore, to address this hypothesis, we generated a transgenic mouse with inducible cardiac-restricted expression of 2APro. Our results indicate that inducible expression of entero viral 2APro in the adult cardiac myocyte at levels comparable to those observed with enteroviral infection is sufficient to cause dilated cardiomyopathy. In addition, the present study also demonstrated that 2APro expression increased disruption of the sarcolemmal membrane with disruption of sarcolemmal localization of dystrophin in a subset of cells. These results add significantly to the evidence that an interaction between enteroviral 2APro and dystrophin contributes to the development of cardiomyopathy after entero viral infection and suggests that strategies directed at inhibition of 2APro are likely to have a significant, beneficial effect on enteroviral-mediated cardiomyopathy.

Methods

Construction of Transgene

The transgenic construct (α-MHC-IREs-FloxedLacZ-2APro) was created as follows: (1) an encephalomyocarditis virus internal ribosome entry site (IRES)11 from pCITE4b (Novagen) was inserted downstream from a cardiac-specific murine α-myosin heavy chain (α-MHC) promoter12,13 to allow expression of the transgene in the presence of 2APro; (2) consensus translation initiation sequences were positioned upstream from a loxP site followed by a β-galactosidase (LacZ) cDNA with a stop codon; and (3) another loxP site followed by a 2APro cleavage site and the 2APro cDNA were inserted downstream from the β-galactosidase gene (Figure 1A).

Generation of Transgenic Mice

The 10-kb expression cassette of α-MHC-IREs-FloxedLacZ-2APro construct was isolated after cleavage with BamH1 and microinjected into the male pronuclei of fertilized eggs from superovulated C57BL/6 × Balb/c mice. Injected eggs were transferred into the oviduct of pseudopregnant CD1 mice. Founder mice were bred with Balb/c mice and maintained in a pathogen-free environment. The α-MHC-IREs-FloxedLacZ-2APro mice were bred with α-MHC- MerCreMer (MCM)13 to create double transgenic mice (2AxMCM).

4-Hydroxytamoxifen Treatment and Recombination Analysis

We dissolved 4-hydroxytamoxifen (4OHTAM, Sigma, St Louis, Mo) in peanut oil (Sigma) at 5 mg/mL. Adult transgenic mice (6 to 8 weeks of age) were treated with 4OHTAM by intraperitoneal injection once daily for 5 days at a dose of 20 mg/kg per day. In separate experiments, adult cardiac myocytes were isolated from 2AxMCM mice with the protocol described in the Appendix. The cells were then treated with 10 μmol/L 4OHTAM for 36 hours.

Polymerase chain reaction (PCR) primers were designed to assess transgene recombination at the loxP sites in the DNA extracted from the hearts of transgenic mice. In the first PCR, the sense primer used was located within the IRES sequence (P1: 5′-CAATTGGCTACCTCAAGCG-3′). The antisense primer was in the 2APro cDNA (P2: 5′-CGTGGTACACCCGACATCGC-3′). After excision of the LacZ sequence, PCR with these primers yielded a 561-bp band. The theoretical 4-kb product was not detected. The second set of primers annealed to the IRES and the floxed LacZ sequence: sense primer is P1 and reverse primer is within LacZ (P3: 5′-AGGGAGATCGCACTCCAGCC-3′). The intact nonrecombined transgene yields a PCR fragment of 496 bp with the second set of primers, and no amplification occurs after Cre-mediated excision.

Assay for 2APro Activity

Heart protein extracts from either transgenic mice or CVB3-infected C3H/HeJ mice were analyzed for 2APro activity. The C3H/HeJ mice were inoculated by intraperitoneal injection with 5 × 10⁶ plaque-forming units of CVB3 at 4 weeks of age. The infected mice were euthanized 6 days after infection; the tamoxifen-stimulated mice were euthanized 12 days after initiation of tamoxifen and their hearts were harvested. Total proteins were extracted from the hearts of tamoxifen-treated transgenic mice (n = 3), control mice (n = 3), and CVB3-infected C3H/HeJ mice (n = 3). Five μg total protein from each heart were incubated with 50 μmol/L Ac-LSTT-AFC (LSTT-
7-amino-4-trifluoromethylcoumarin, custom-made by Enzyme Systems Products, now MP Biomedical) in modified 2APro cleavage buffer as described. The activity of 2APro was measured by a fluorometric assay based on the specific hydrolysis of synthetic peptide substrate Ac-LSTT-AFC.

Histochemistry
Heart tissue was embedded in OCT Tissue Tek (Sakura Finetechnical, Torrance, Calif) and snap-frozen in isopentane chilled in dry ice, and 6-μm sections were cut by cryosection. LacZ was detected with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) staining. In addition, the number of cells that expressed X-Gal was quantified with adult myocytes isolated from tamoxifen- or peanut oil–treated 2AxMCM and expressed as the percentage of the total number of cells.

Fluorescent staining with Evans blue dye (EBD) was used to assess the impairment of sarcolemmal membrane integrity as previously described. Dystrophin in the hearts of transgenic mice was detected by immunofluorescence with polyclonal antibodies that recognize epitopes that map toward the N-terminus of dystrophin (sc-15376) and at the C-terminus (sc-7461) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Dystrophin levels of cultured cardiac myocytes were detected by immunofluorescence with a monoclonal antibody that recognizes the mid-domain of dystrophin (Dy4/6D3; NovoCasra, Newcastle, UK). Cre protein in the hearts of transgenic mice and cultured cardiac myocytes was detected by immunofluorescence with a rabbit polyclonal antibody against Cre (BABCO). Cell membrane glycoproteins were visualized with FITC-labeled wheat germ agglutinin. The specimens used for conventional electronmicroscope were fixed and embedded as described previously.

Echocardiography
M-mode echocardiograms were performed as described previously.

Statistical Analysis
Data are expressed as mean±SE unless otherwise noted. Statistical significance was evaluated with the unpaired Student t test for comparisons between 2 means. For multiple comparisons, a 1-way ANOVA with Tukey-Kramer post hoc test was used. To test mean changes in left ventricular end-diastolic and end-systolic dimension as well as percent fractional shortening among 3 groups from baseline to 22 days after 4OHTAM administration, repeated-measure ANOVA was used. Probability values <0.05 were considered significantly different. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Generation of Transgenic Mice With Cardiac Restricted Expression of 2APro Regulated by a Cre-LoxP-Inducible System
To assess the effect of enteroviral 2APro in the adult heart, a tamoxifen-inducible Cre-LoxP system was used to regulate 2APro expression. To accomplish this, we generated transgenic mice that harbored a floxed LacZ-2APro cDNA expression vector. This construct contained a cardiac-specific murine α-MHC promoter followed by a picornaviral IRES. The viral IRES was inserted to facilitate translation in the presence of 2APro expression. A translation initiation start site and a loxP-flanked LacZ gene with a stop codon were inserted downstream of the IRES. Downstream of the LacZ expression cassette was placed a cDNA that contained loxP DNA sequence, a 2APro cleavage site, and the 2APro coding sequence followed by a stop codon. The construct terminates with an SV40 polyA signal sequence (Figure 1A, a). Of a total of 6 genotype-positive founder transgenics, 2 were identified that expressed LacZ in the hearts at baseline. Both were healthy, bred normally, and had phenotypically normal hearts (data not shown). The 2APro transgenic mice were bred with α-MHC MCM transgenic mice to establish double-transgenic mice with 2APro and MCM transgenes (referred to as 2AxMCM mice). MCM transgene expresses a Cre-recombinase fusion protein with mutant estrogen-receptor domains (Mer) under the control of the α-MHC promoter (Figure 1A, b).

In the absence of tamoxifen, the MCM fusion protein is sequestered in the cytoplasm of cardiomyocytes, and in the unstimulated 2AxMCM mice, LacZ is expressed without out expression of 2APro (Figure 1B). Conversely, stimulation of the mice with tamoxifen causes translocation of MCM fusion protein to the nucleus, where the LacZ cDNA is excised by Cre-mediated recombination (Figure 1C, a) and 2APro is expressed (Figure 1C, b). The 2APro then cleaves the amino terminus of the protein at the cleavage site to generate an exact copy of the enteroviral 2APro (Figure 1C, c).

To determine the extent of translocation of MCM after tamoxifen administration, immunofluorescence analysis was performed on ventricular sections from 2AxMCM mice 12 days after initiation of tamoxifen administration. Antibody to Cre protein and DAPI were used to visualize the MCM fusion protein and cell nuclei, respectively. In the absence of tamoxifen, the MCM fusion protein was seen diffusely throughout the cytoplasm of ventricular myocytes. In contrast, tamoxifen induced detectable nuclear localization of MCM in 38±4% of total nuclei (Figure 2A). Cre-mediated recombination was analyzed by PCR at the same time point. Recombination of the transgene was confirmed in 2AxMCM mice treated with tamoxifen (Figure 2B). The efficiency of tamoxifen-inducible recombination was also analyzed by quantitative assessment of LacZ-positive cells with X-gal staining at 12 days after initiation of tamoxifen administration. There was very little or no LacZ expressed after stimulation with tamoxifen when compared with LacZ expression in the absence of tamoxifen in either tissue section (Figure 2C) or in isolated adult myocytes (Figure 2D). These data clearly demonstrate that tamoxifen induced Cre-recombinase-mediated DNA recombination in this transgenic mouse model.

Expression of 2APro was determined 12 days after initiation of tamoxifen administration with a substrate-based fluorescent cleavage assay as described in Methods. No 2APro activity was detected in the hearts of the MCM mice treated with tamoxifen. Minimal 2APro activity was detected in the hearts of the 2AxMCM mice in the absence of tamoxifen, whereas administration of tamoxifen increased 2APro activity. Activity of 2APro in the tamoxifen-treated 2AxMCM mice was compared with 2APro activity of CVB3-infected heart extracts 6 days after infection (Figure 2E). These results demonstrated that the presence of tamoxifen can significantly induce 2APro expression in...
the hearts of 2AxMCM mice, with minimal expression of 2APro in the hearts of 2AxMCM mice in the absence of tamoxifen. The level of 2APro expression in the whole heart was comparable to that observed during the acute phase of CVB3 infection.

**Induction of 2APro Expression in Adult Heart Leads to Dilated Cardiomyopathy**

To determine whether 2APro expression is sufficient to induce dilated cardiomyopathy, male double-transgenic 2AxMCM mice 6 to 8 weeks of age were treated with tamoxifen. Two types of control mice were evaluated; ie, 2AxMCM mice treated with the diluent peanut oil, and MCM mice that lacked the 2APro transgene were treated with tamoxifen. All the mice, 5 in each group, underwent echocardiography at days 0, 9, and 22 after initiation of tamoxifen administration. Their hearts were harvested for analysis as described below at 22 days after initiation of treatment.

The 2AxMCM mice treated with tamoxifen had a time-dependent increase in left ventricular end-diastolic dimension and end-systolic dimension (Figure 3A and 3B). Abnormal systolic performance was observed because of a corresponding decrease in the fractional shortening of the left ventricle (Figure 3B); however, there was no significant left ventricular dilation or decrease in the fractional shortening of the left ventricle in control mice. There were no significant differences in average body weight and heart rate between 2AxMCM mice treated with tamoxifen and the controls (data not shown). These results indicate that cardiac-restricted enteroviral 2APro expression is sufficient to induce dilated cardiomyopathy.

Dilated cardiomyopathy was also demonstrated in the 2AxMCM mice treated with tamoxifen in terms of heart weight-to-body weight ratio and heart morphology. The heart weight-to-body weight ratio significantly increased in the 2AxMCM mice 22 days after administration of tamoxifen as compared with controls (Figure 4A). There were no significant differences in the body weight of the 2AxMCM mice treated with peanut oil when compared with the tamoxifen-treated MCM mice. In addition to severe ventricular enlargement, there were biaxial enlargement, pleural effusions, and ascites in the 2AxMCM mice treated with tamoxifen (data not shown). Transverse sections of hearts were stained with hematoxylin and eosin and Masson trichrome (Figure 4B). Minimal cellular infiltrate and a small amount of interstitial fibrosis were noted in the tamoxifen-treated 2AxMCM mice. The controls were normal. Ultrastructural analysis with electron microscopy revealed striking structural alterations. Compared with controls, the 2AxMCM mice treated with tamoxifen displayed “fuzzy” Z-lines and shortened sarcomeres (Figure 4C). In addition, cardiac cells also demonstrated severe cytoskeletal changes, such as myofibrillar collapse with a haphazard arrangement, concomitant with a decrease in myofibril number; the increased presence of swollen mitochondria were often observed. Although it is difficult to assess sarcolemmal membrane integrity with electron microscopy, the 2AxMCM mice treated with tamoxifen demonstrated consider-
able irregularity in the spacing between cells in the intercalated discs and irregularity of the sarcolemmal membrane when compared with control mice. (Figure 4C)

Cardiac 2APro Expression Induces Sarcolemmal Disruption and Loss of Sarcolemmal Localization of Dystrophin

Because we have previously shown that coxsackieviral 2APro cleaves dystrophin in vitro and that there is cleavage of dystrophin and disruption of the sarcolemma in CVB3-infected cultured myocytes and infected mouse hearts, we hypothesized that the dilated cardiomyopathy induced by 2APro expression would be associated with sarcolemmal disruption and loss of dystrophin localization to the sarcolemma.

To determine whether disruption of the sarcolemmal membrane occurred in the intact heart with enteroviral 2APro expression, we evaluated sarcolemmal membrane integrity in vivo by injecting EBD, a large tracer molecule that can only enter into the myocytes with a disrupted sarcolemma. There was uptake of EBD in scattered myocytes of the 2AxMCM mice treated with tamoxifen. Disruption of the sarcolemmal membrane, however, was completely absent in the MCM mice treated with tamoxifen and 2AxMCM mice treated with peanut oil (Figure 5A). To compare this with a mouse model of cardiomyopathy from a different cause, muscle lim protein knockout mice with cardiomyopathy and heart failure were also injected with EBD. In contrast to tamoxifen-treated 2AxMCM mice, there was only very weak, diffuse EBD staining that was clearly distinct from the bright staining that was detected in the 2AxMCM mice (Figure 5A). It is also notable that the pattern of EBD staining in the 2AxMCM mice was distinct from that seen in CVB3-infected mice in that the staining occurred in scattered individual myocytes as opposed to the positive staining of foci of adjacent myocytes observed after CVB3 infection. This finding indicates that sarcolemmal disruption in the 2AxMCM mice treated with tamoxifen was associated with protease 2A expression and not an indirect effect of cardiomyopathy alone.

To determine whether the disruption of the sarcolemma in mice with 2APro expression was associated with 2APro-mediated disruption of dystrophin at the sarcolemma as seen in virally infected hearts, mice were injected with EBD and sections were immunostained for the presence of dystrophin using antibodies directed against the C-terminus and toward the N-terminus of dystrophin. This demonstrated that EBD uptake occurred in the myocytes with a disrupted C-terminal dystrophin-staining pattern (Figure 5B). The disruption of dystrophin was similar, with the antibody directed nearer the N-terminus (data not shown). Double staining with wheat germ agglutinin, which stains membrane glycoproteins in general, and EBD demonstrated that loss of the dystrophin in the sarcolemma of the myocytes with EBD-positive staining was not the result of general disintegration of plasma membrane, as evidenced by preserved wheat germ agglutinin stain in the cells with sarcolemmal disruption (Figure 5C). However, there was partial disruption of caveolin-3 staining at the sarcolemma in EBD-positive cells (data not shown). These data indicate that impairment of sarcolemmal membrane in the mice with 2APro expression is associated with disruption of dystrophin.

In addition, to further demonstrate that 2APro expression in cardiomyocytes is sufficient to disrupt dystrophin, adult myocytes from 2AxMCM mice were isolated and treated with tamoxifen for 36 hours. Co-immunostaining of the adult myocytes for MCM and dystrophin showed that the loss of the sarcolemmal dystrophin-staining pattern oc-
curred in myocytes in which MCM was localized to the nucleus (Figure 5D). The result indicated that expression of enteroviral 2APro in isolated cardiomyocytes is sufficient to induce disruption of the sarcolemmal localization of dystrophin.

Discussion

It has been clearly demonstrated that coxsackieviral 2APro can directly cleave dystrophin, that CVB3 infection of the heart is associated with disruption of the dystrophin glycoprotein complex, and that dystrophin deficiency can have a role in determination of susceptibility to enteroviral-mediated cardiomyopathy.9,10 Because enteroviral infection is accompanied by expression of all viral proteins and activation of a potent immune response,3 it is not known whether expression of 2APro alone is sufficient to induce dilated cardiomyopathy or whether it would be associated with disruption of sarcolemmal membrane integrity. Therefore, the present study demonstrates for the first time that the presence of coxsackieviral 2APro in the cardiac myocyte is sufficient to induce cardiomyopathy with disruption of the sarcolemmal membrane and loss of localization of dystrophin in the intact heart. In addition, the present study also demonstrates that expression of 2APro in isolated adult myocytes is able to disrupt dystrophin localization to the cell membrane, which suggests that 2APro-mediated cleavage of dystrophin plays an important role in 2APro-induced dilated cardiomyopathy. These findings contribute to our understanding of the pathogenesis of viral-mediated dilated cardiomyopathy.

Hallmarks of dilated cardiomyopathy in humans include a decrease in systolic ventricular function and an increase in ventricular chamber size. Microscopic features include variable degrees of interstitial fibrosis, degeneration of myocytes, and occasional clusters of lymphocytes.24 Because the major objective of this study was to determine whether cardiac restricted expression of enteroviral 2APro could induce cardiomyopathy, we sought to determine whether some or all the characteristics of dilated cardiomyopathy were present in the transgenic mice with 2APro.
The manifestations of dilated cardiomyopathy and heart failure found in the 2AxMCM mice treated with tamoxifen included an increase in left ventricular end-diastolic and end-systolic chamber size in tamoxifen-treated transgenic mice, which was associated with decreased fractional shortening, a marker of systolic dysfunction. Other findings consistent with cardiomyopathy included atrial enlargement and an increased ratio of heart weight to body weight, as well as pleural effusions and ascites. Histopathological analysis of transgenic mice demonstrated mild fibrosis and degeneration of myocytes in the absence of a significant cellular immune response. Ultrastructural analysis of transgenic mice demonstrated atrophy of myofibrils and irregularities in the sarcoplasmic membrane and intercalated discs. These results clearly indicate that cardiac-restricted expression of enteroviral 2A Pro is sufficient to induce dilated cardiomyopathy in the absence of other viral proteins required for viral replication.

In the present study, we used a tamoxifen-regulated Cre-loxP system to induce enteroviral 2A Pro expression in adult mouse hearts. In the absence of tamoxifen in 2AxMCM mice or in the presence of tamoxifen in MCM mice without the 2A Pro transgene, there was no evidence of echocardiographic or histologic abnormality with exception that <10% of 2AxMCM mice had evidence of cardiomyopathy, presumably secondary to occasional “leaky” expression of 2A Pro in the absence of tamoxifen. Stimulation of the mice with tamoxifen resulted in a significant induction of 2A Pro activity and development of cardiomyopathy in all treated mice. These results demonstrate that the dilated cardiomyopathy observed in tamoxifen-treated 2AxMCM mice results from expression of 2A Pro induced by a tamoxifen-regulated Cre-LoxP system rather than tamoxifen or MCM fusion protein.

To establish a reference for the level of 2A Pro activity in the transgenic hearts, we compared them to the activity of 2A Pro in CVB3-infected hearts. The level of 2A Pro activity in the whole heart was similar to that observed in coxsackieviral-infected hearts 6 days after infection. Despite the similarity in 2A Pro expression, it should be noted that are significant differences in the development of the disease process between the transgenic mice and mice infected with CVB3. These differences include the absence of expression of all viral proteins and lack of a potent cellular immune response in the transgenic mice. In addition, there is a difference in the time course and localization of 2A Pro expression between the transgenic mice and mice infected with CVB3. Activity of 2A Pro is transient in acute viral infection secondary to host immune responses against viral infection. After the virus is completely cleared by host immune responses, 2A Pro expression will be decreased. However, the transgenic mice represent
a more chronic pattern of 2A<sup>pro</sup> expression because it is driven by the α-MHC promoter. This is more reminiscent of persistent viral infection of the heart. In addition, although we were not able to stain directly for 2A<sup>pro</sup> in the tissue, the EBD staining was dispersed throughout the myocardium rather than in focal areas of adjacent myocytes as occurs with CVB infection. This finding, in combination with the known pattern of expression of genes driven by the α-MHC promoter,12,13 indicates that transgenic expression of 2A<sup>pro</sup> is more diffuse than that observed after CVB infection. Because the total 2A<sup>pro</sup> activity is similar in the transgenic and CVB-infected hearts, it is likely that the level of 2A<sup>pro</sup> activity in each myocyte in the transgenic mice is lower than that of CVB3-infected myocytes.

We have previously shown that expression of a replication-defective enteroviral genome in the heart can also lead to cardiomyopathy. Although there are similarities between the previously reported model and that reported in the present article, there are also significant differences. Similarities include deterioration in ventricular function and the development of fibrosis without a significant proportion of patients with dilated cardiomyopathy.25–27 In addition, enteroviral RNAs may play a role in a subset of patients with dilated cardiomyopathy.28 However, although low-level enteroviral RNA has been detected in a significant proportion of patients with dilated cardiomyopathy, little is known with regard to whether or how the presence of enteroviral RNAs and/or proteins affects cardiac structure and function.

In summary, we have shown that cardiac-restricted expression of enteroviral 2A<sup>pro</sup> is sufficient to induce dilated cardiomyopathy associated with disruption of sarcolemmal membrane and 2A<sup>pro</sup>-mediated loss of dystrophin at the sarcolemma. This model will facilitate further understanding of the mechanisms by which enteroviruses can cause cardiomyopathy and the role for acquired disruption of the cytoskeleton.

**Acknowledgments**

We thank Sharon Reed, MD, for providing expertise related to measurement of protease 2A activity.

**Sources of Funding**

Dr Xiong was supported by an American Heart Association postdoctoral fellowship grant (AHA-0225166Y) and Dr Knowlton was supported by a National Heart, Lung, and Blood Institute grant (5R01HL066424–04).

**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Coxsackievirus infection is one of the common causes of viral myocarditis. Activation of the cellular immune system has a role in the pathogenesis of viral heart disease. However, it has recently been shown that viral proteases may be important in the pathogenesis of viral-mediated heart disease by cleaving proteins that stabilize the cell membrane. This article demonstrates that when the coxsackieviral protease 2A alone is expressed in the adult cardiac myocyte, it is sufficient to induce severe cardiomyopathy. This indicates that viral proteases are potentially important therapeutic targets for the treatment of viral-mediated heart disease and that protease inhibitors would likely limit the viral-mediated damage caused by proteases in the host cell.
Inducible Cardiac-Restricted Expression of Enteroviral Protease 2A Is Sufficient to Induce Dilated Cardiomyopathy
Dingding Xiong, Toshitaka Yajima, Byung-Kwan Lim, Antine Stenbit, Andrew Dublin, Nancy D. Dalton, Daphne Summers-Torres, Jeffery D. Molkentin, Herve Duplain, Rainer Wessely, Ju Chen and Kirk U. Knowlton

_Circulation_. 2007;115:94-102; originally published online December 26, 2006; doi: 10.1161/CIRCULATIONAHA.106.631093

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/115/1/94

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/