Low-Level Expression of a Mutant Coxsackieviral cDNA Induces a Myocytopathic Effect in Culture

An Approach to the Study of Enteroviral Persistence in Cardiac Myocytes

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Background—Enteroviral ribonucleic acids have been identified in heart muscle of a subset of patients with myocarditis and dilated cardiomyopathy as well as in a mouse model of persistent coxsackievirus B3 (CVB3) infection, suggesting that persistent viral infection along with activation of an immune response may contribute to the pathogenesis of ongoing cardiac disease and dilated cardiomyopathy in certain patients. It is still not known whether persistence of the viral genome contributes to the pathogenesis of dilated cardiomyopathy.

Methods and Results—To determine whether low-level enteroviral gene expression similar to that observed with viral persistence can induce myocytopathic effects without formation of infectious virus progeny, the full-length infectious cDNA copy of CVB3 was mutated at the VP0 maturation cleavage site. This prevented formation of infectious virus progeny. In myocytes transfected with this mutated cDNA copy of the viral genome, both positive- and negative-strand viral RNAs were detected, demonstrating that there was replication of the viral genome by the RNA-dependent RNA polymerase. The level of viral protein expression was found to be below limits of detection by conventional methods of protein detection, thus resembling restricted virus replication. Nonetheless, the CVB3 mutant was found to induce a cytopathic effect in transfected myocytes, which was demonstrated by inhibition of cotransfected MLC-2v luciferase reporter activity and an increase in release of lactate dehydrogenase from transfected cells.

Conclusions—This study demonstrates that restricted replication of enteroviral genomes in myocytes in a pattern similar to that observed in hearts with persistent viral infection can induce myocytopathic effects without generation of infectious virus progeny. (Circulation. 1998;98:450-457.)

Key Words: viruses ■ myocarditis ■ heart failure

Enteroviruses can induce viral heart disease in both adult and pediatric patients, leading to severe heart failure, arrhythmias, and sudden death. It has been shown in survivors of acute myocarditis that the histological features of acute myocarditis may progress to features consistent with idiopathic dilated cardiomyopathy. Recently, in situ hybridization and RT-PCR of myocardial tissue from patients with chronic dilated cardiomyopathy suggest that persistence of enteroviral ribonucleic acids may play a role in a subset of patients with dilated cardiomyopathy (for review, see References 2 through 4). Although it has been possible to detect low-level enteroviral RNA in a significant proportion of patients with dilated cardiomyopathy, it is not known whether and how the presence of enteroviral ribonucleic acids and/or proteins affects cardiac structure and function. It is possible that both a virus-mediated myocytopathic effect and activation of the immune response are important in certain cases of dilated cardiomyopathy.

Coxsackievirus is a member of the picornavirus family and the enterovirus genus that is commonly associated with myocarditis. Coxsackievirus is closely related to other enteroviruses, such as echovirus, poliovirus, and rhinovirus. The 7.4-kb positive-strand RNA genome is encapsidated by four structural proteins: VP1, VP2, VP3, and VP4. On entry into the host cell, the single positive-strand RNA is released from the capsid, and viral protein synthesis is initiated by host cell translational mechanisms. The viral genome is translated as a monocistronic polyprotein that is cleaved by the viral proteases 2A and 3C at specific proteolytic cleavage sites. One of the viral proteins is an RNA-dependent RNA polymerase that allows replication of the viral RNA through a negative-strand intermediate. Replicated positive-strand RNAs are encapsidated and usually released by cell lysis during acute infection. Formation of infectious virus progeny requires autocatalytic cleavage of the capsid precursor polypeptide VP0 to VP4 and VP2.
Selected Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
<th>Construct</th>
<th>Promoter</th>
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<tr>
<td>pcDNA3</td>
<td>Eukaryotic plasmid expression vector</td>
<td>CMV</td>
</tr>
<tr>
<td>pCB3-M1</td>
<td>Infectious coxsackieviral cDNA</td>
<td>None</td>
</tr>
<tr>
<td>pCMV CVB3ΔVP0</td>
<td>Mutated coxsackieviral cDNA cloned in plasmid vector pcDNA3</td>
<td>CMV</td>
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<tr>
<td>pCMV VP1</td>
<td>cDNA for capsid protein VP1 of CVB3 (pCB3-M1) in plasmid expression vector</td>
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<td>pMLC-2v L</td>
<td>Luciferase expression vector</td>
<td>MLC-2v</td>
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<td>pCMV β-gal</td>
<td><em>Escherichia coli</em> β-galactosidase expression vector</td>
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obtained by transferring supernatant from transfected cos-1 cells to HeLa cell monolayers.

Methods

Virus

All coxsackievirus used in this study were derived from the infectious cDNA copy of the cardiotropic Nancy strain of CVB3 referred to as pCB3 M1 in previous articles.20,21 Plasmid DNA was transfected into cos-1 cells by use of a modified calcium phosphate precipitation protocol.22 Large-scale preparations of virus were

Coxsackievirus B3 RNA can persist in the myocardium in a murine model of ongoing viral heart disease.19 These previous experiments demonstrated that persistent coxsackievirus infection in the murine heart is distinct from acute viral infection and is associated with the following characteristics. First, in acute viral infection, there is significantly more positive- than negative-strand RNA synthesis, whereas in persistently infected mice, the amount of negative-strand RNA is approximately equivalent to the amount of positive-strand RNA, indicating restricted virus replication. Second, there is evidence of ongoing myocardial damage in the myocardium where viral RNA is detected. Third, although infectious virus is easily isolated from acute infections, infectious virus is usually not recovered from in vivo tissue samples with persistent enteroviral infection. Fourth, in persistently infected mouse hearts, viral protein expression is difficult to detect by conventional immunohistochemistry or immunoblotting. Many of these characteristics are also typical of hearts from a subset of patients with dilated cardiomyopathy who have evidence of entero viral RNAs by in situ hybridization or RT-PCR.2

To understand the role of low-level coxsackievirus gene expression in the myocardium under conditions that mimic those that occur in persistent infection of cardiac myocytes, we generated a full-length CVB3 expression vector by mutating the autocatalytic cleavage site of VP0 in the 5′ flanking region of MLC-2v upstream from luciferase, RSV luciferase, or CMV β-galactosidase.22 The CMV CVB3ΔVP0 cDNA could be used to express viral proteins in a highly efficient expression system, recombinant VV containing the full-length CVB3ΔVP0 mutant cDNA was constructed.23,24 Expression of the appropriate CVB3-specific proteins was confirmed by immunoblot analysis with a rabbit anti-CVB3 antibody.

Generation of VV CVB3ΔVP0

To confirm that the CVB3ΔVP0 cDNA could be used to express viral proteins in a highly efficient expression system, recombinant VV containing the full-length CVB3ΔVP0 mutant cDNA was constructed.23,24 Expression of the appropriate CVB3-specific proteins was confirmed by immunoblot analysis with a rabbit anti-CVB3 antibody.

Myocyte Cell Culture Techniques and Immunocytofluorescent Staining

Neonatal rat ventricular myocytes were cultured from 1- to 2-day-old Sprague-Dawley rats by use of Percoll gradient as previously described.22,23 The myocytes were plated at a density of 3×10^5 to 4×10^5/cm². Forty-eight hours after plating, cells were either transfected or microinjected with the appropriate plasmid vector or infected with CVB3 or with recombinant VV by addition of virus directly into the DMEM with 5% heat-inactivated FCS and antibiotics.

Infected myocytes were identified by immunostaining with a rabbit polyclonal anti-CVB3 antibody.26 Myofilament architecture was assessed by staining of myocytes with a monoclonal mouse anti-myomesin antibody (generous gift from J.C. Perriard29). β-Galactosidase–expressing cells were identified by a monoclonal antibody (Promega).

Plasmid Expression Vectors

Cells were cotransfected or microinjected with the wild-type pCB3 M1,20 the CMV CVB3ΔVP0 or CMV VP1 expression vectors, or the empty backbone vector pcDNA3 (see Table for plasmid vector abbreviations). Reporter gene expression was determined by cotransfecting or microinjecting MLC-2v luciferase containing 250 bp of the 5′ flanking region of MLC-2v upstream from luciferase, RSV luciferase, or CMV β-galactosidase.22 The CMV CVB3ΔVP0 expression vector was generated by cutting the mutated full-length pCB3ΔVP0 cDNA plasmid with EcoRI, isolating the 7.5-kb fragment and inserting it into the CMV-driven eukaryotic expression vector pcDNA3.

Nested RT-PCR

RNA was isolated from myocytes 48 hours after transfection by use of RNeasy columns (Qiagen) and treated with DNase I (Life
Coxsackievirus-Mediated Myocytotoxic Effect

Technologies, Inc). Primers were designed corresponding to the 5′ untranslated region of the coxsackieviral RNA as previously described.30 The sequences of the primers were as follows: CP1, 5′-ACCTTTTGGCCGCTTGT-3′; CP2, 5′-CACGGACACCC AAAGTA-3′; CP3, 5′-AAGACTTCTGTTACC-3′; and CP4, 5′-ATTCAAGGGGCGGGAGGA-3′. Nested RT-PCR was performed as follows: 2 μg of RNA was used for reverse transcription. The appropriate primer (CP1 or CP2) was allowed to anneal 10 minutes at 70°C, and reverse transcription was then carried out at 42°C for 50 minutes with 1 μL of Superscript II reverse transcriptase (200 U/μL, Life Technologies, Inc). For positive-strand RNA, the CP2 primer was used; negative-strand RNA was initially primed with the CP1 primer. The samples were then amplified with CP1 and CP2 primers at 94°C (1 minute), 52°C (1 minute), and 72°C (2 hours) for a total of 36 cycles with VENT-DNA-Polymerase in a total volume of 100 μL. Five microliters of the first PCR was used in the nested PCR step with primers CP3 and CP4 at 94°C (1 minute), 50°C (1 minute), and 72°C (2 minutes) for a total of another 40 cycles, resulting in a final amplification of a 298-bp fragment. Appropriate controls were used for every step to control for sensitivity and specificity of the amplification. Aliquots (10 μL) of samples were then analyzed on a 1% agarose gel containing 10 μg/mL ethidium bromide.

LDH Assay
Cytotoxicity was measured with a colorimetric assay for the quantification of cell injury based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant with an LDH cytotoxicity detection assay (Boehringer Mannheim). Values were corrected against the background of the serum containing medium alone and plotted as cumulative absorbance at 490 versus 630 nm. Cells were transfected with either pCMV CVB3ΔVP0 and, as control, pcDNA3, or a mock transfection without DNA.

Microinjection
Myocytes were plated as described on 25-mm² etched square coverslips (BellaCo). Forty-eight hours after plating, the supernatant was changed to medium containing DMEM/medium 199 4:1, 10% horse serum, 5% FCS, antibiotics, 1% glutamine, and 20 mmol/L 2,3-butanedione monoxime. Microinjections were carried out with a semiautomated micromanipulator (Eppendorf) and a Zeiss Axiovert microscope as previously described.31,32

Statistical Analysis
Data are expressed as mean±SEM. To test for statistical difference in luciferase expression, a one-factor ANOVA was used. A Student’s t test with Bonferroni correction was used for two comparisons. To examine the effects on LDH release between control transfection and cells transfected with pCMV CVB3ΔVP0, a two-way repeated-measures ANOVA was used. Post hoc analysis for significant differences between the groups at specific time points was performed with a two-tailed t test with Bonferroni correction for five comparisons.

Results

Coxsackievirus-Mediated Myocytotoxic Effect
Figure 1. CVB3 infection induces a direct cytopathic effect. Cultured neonatal rat ventricular myocytes were infected with CVB3 derived from infectious cDNA pCB3 M1. Forty-eight hours after infection, cells were fixed and dual-immunostained for CVB3 (A) and myomesin (B). Arrows show two infected myocytes that stained positive for CVB3 (A). Same cells stained with an anti-myomesin antibody (B) have disrupted myofilaments in infected cells compared with typical well-organized striations of adjacent uninfected cell (arrowhead). Faint nonspecific CVB3 staining in uninfected cell is similar to that observed in myocytes to which no virus has been added (not shown).

Mutation of the VP0 Maturation Cleavage Site of the Wild-Type CVB3 cDNA Inhibits Generation of Infectious Virus Progeny
To simulate restricted CVB3 replication without generation of infectious virus progeny as observed in the murine model of persistent myocardial infection,19 we mutated VP0 at the VP4/VP2 autocatalytic cleavage site in the pCB3 M1 full-length infectious cDNA copy of the CVB3 genome. This mutated cDNA is referred to as CVB3ΔVP0. The Asn-Ser cleavage site was changed to Lys-Ala by site-directed mutagenesis of the AAC-TCC codons to AAG-GCC (Figure 2A). The full-length wild-type and mutant cDNA copies of the viral genome were inserted into pcDNA3. Wild-type
Plasmid-Based Expression of CVB3ΔVP0 in Cardiac Myocytes Is Associated With Low-Level Viral RNA and Protein Expression

One characteristic of persistent myocardial enteroviral infection in mice is the synthesis of low-level positive- and negative-strand viral RNA.\(^{19,33}\) This pattern is clearly distinct from acute myocardial infection characterized by predominantly positive-strand RNA synthesis and high-level expression of viral proteins. To determine whether expression of CVB3ΔVP0 in myocytes would have characteristics similar to those observed in persistent viral infection, myocytes were transfected with pCMV CVB3ΔVP0. Both positive- and negative-strand RNAs were consistently identified by nested RT-PCR of myocytes transfected with the CMV CVB3ΔVP0 plasmid expression vector (Figure 3A). There was at least as much negative-strand RNA amplified as positive-strand RNA, although this was not precisely quantified. This was in contrast to the large amount of positive-strand RNA amplified with identical primers when myocytes were infected with the wild-type infectious virus (Figure 3B). In contrast to myocytes infected with the VV vector expressing CVB3ΔVP0 (below), no viral protein was detectable by immunocytofluorescence or immunoblot after transfection with the CMV CVB3ΔVP0 plasmid expression vector (data not shown), reflecting the low sensitivity of conventional methods for protein detection. The presence of negative-strand RNA, however, provides evidence that the viral RNA-dependent RNA polymerase (CVB protein 3D) is expressed. These findings suggest that the level of viral protein expression is low after transfection with CVB3ΔVP0.

VV-Directed Expression of CVB3ΔVP0

To obtain definitive evidence that the CVB3ΔVP0 construct was able to express viral proteins, we used a VV expression system similar to that used with other picornaviruses.\(^7\) After purification of the recombinant VV containing the CVB3ΔVP0 cDNA (VV-CVB3ΔVP0), cultured neonatal rat ventricular myocytes were infected with either VV-CVB3ΔVP0 or a control VV that lacked the viral cDNA.

CVB3 protein expression was detected in infected myocytes by immunocytofluorescence with an anti-CVB3 antibody that recognizes primarily VP1 (Figure 4A and 4B) or by immunoblotting of infected myocyte protein extracts with the anti-CVB3 antibody (Figure 4C). These experiments clearly demonstrate that CVB3 capsid proteins can be expressed in myocytes by use of the CVB3ΔVP0 cDNA mutant.

Low-Level Expression of the Mutated cDNA Copy of CVB3 Is Sufficient to Inhibit MLC-2v-Directed Protein Expression

To determine whether CVB3ΔVP0 expression could affect expression of a reporter directed by a cardiac-specific promoter, the mutated viral cDNA, CVB3ΔVP0, ligated into the CMV expression vector (pcDNA3) was cotransfected into cultured neonatal rat ventricular myocytes with an MLC-2v luciferase reporter construct.\(^{22}\) As controls, the expression vector without insert and a CMV VP1 expression vector that expresses full-length VP1 from CVB3 were cotransfected with MLC-2v luciferase. Transfection of the CMV
CVB3ΔVP0 vector significantly decreased MLC-2v–directed luciferase expression compared with cotransfection with pcDNA3 or transfection with pCMV VP1 (Figure 5). Downregulation of reporter gene expression is not restricted to cardiac transcriptional promoters because pCMV CVB3ΔVP0 was able to significantly decrease luciferase expression when driven by the RSV promoter or human papilloma virus promoter (data not shown). These experiments provide evidence that low-level viral replication in cardiac myocytes can downregulate protein expression directed by cardiac promoters and other eukaryotic promoters that rely on typical TATA-dependent transcription and cap-dependent initiation of translation in cardiac myocytes.

To confirm that the CVB3ΔVP0 cDNA mutant can decrease eukaryotic protein expression at the single-cell level, experiments were carried out with microinjection of the CMV CVB3ΔVP0 plasmid expression vector into the nuclei of cardiac myocytes along with a CMV β-galactosidase plasmid expression vector. Control cells were injected with the CMV expression vector (pcDNA3) without insert and the CMV β-galactosidase expression vector.22 Although β-galactosidase expression was easily detected in cells microinjected with the empty pcDNA3 without insert, β-galactosidase was not detectable by immunostaining or histochemical stain in cells microinjected with CMV CVB3ΔVP0 (data not shown), indicating that expression of the mutated CVB3 cDNA interferes with cellular protein expression.

CVB3ΔVP0 Expression Is Sufficient to Induce a Myocytopathic Effect

To determine whether expression of CVB3ΔVP0 is associated with a significant cytopathic effect in cultured cardiac myocytes with release of endogenous myocyte proteins, we assayed the supernatant of cultured myocytes that were transfected with pCMV-CVB3ΔVP0 for LDH as a marker for cell death. Transfection of pCMV-CVB3ΔVP0 significantly increased the release of LDH into the myocyte culture supernatant from 72 hours after transfection compared with LDH levels after control transfection (Figure 6). Control LDH levels were similar when cells were transfected either with the empty vector pcDNA3 or with mock transfection. These data indicate that low-level viral replication and viral gene expression without formation of infectious progeny virus are sufficient to induce myocyte injury and that the effect of CVB3ΔVP0 expression is not restricted to reporter gene expression.

Conclusions

It is clear that enterovirus infections are associated with some acute forms of myocardial injury, such as myocarditis.34 In addition, enteroviral nucleic acids have been detected in a portion of patients with chronic dilated cardiomyopathy.3,34 However, the precise mechanism of tissue injury in virus-mediated cardiomyopathy is not clear. It is likely that both a direct viral injury and an associated immune response are important in the pathogenesis of viral heart disease.

Previous studies have shown that coxsackievirus can induce a direct cytopathic effect in fetal human14 and fetal35 and adult mouse ventricular myocytes in culture. In the present study, we show that coxsackievirus B3 derived from an infectious cDNA is able to induce a direct cytopathic effect in the well-characterized cultured neonatal rat ventricular myocytes.

To demonstrate that low-level expression of the viral genome, in the absence of maturation of infectious virus progeny, can induce myocardial injury, the full-length infec-
tious cDNA copy of the CVB3 genome was mutated at the maturation cleavage site of the VP0 capsid precursor protein. We have shown that mutation of this cleavage site prevents formation of infectious virus progeny when the mutated viral cDNA is transfected into cultured cells. However, when CVB3 VP0 was expressed by use of a highly efficient VV expression vector, coxsackieviral protein was expressed at levels that were easily detected by immunoblotting or immunocytofluorescence studies. The inability to form infectious virus progeny associated with improper cleavage of the capsid precursor polyprotein VP0 occurs with VP0 mutations in various picornaviruses and may result in accumulation of so-called provirions.6,7 To facilitate restricted viral gene expression in transfected myocytes, as observed in the murine model of myocardial CVB3 persistence, the CVB3 VP0 mutant was expressed in subsequent experiments under the control of human CMV-promoter sequences.

Transfection of the mutated full-length copy of the CVB3 genome, CVB3 VP0, in cultured neonatal rat ventricular myocytes is associated with many of the characteristics of persistent enteroviral infection in a mouse model of ongoing viral replication. These characteristics include (1) a lower ratio of positive- to negative-strand viral RNA than is present in acute infection, (2) evidence of a myocytopathic effect, (3) the inability to detect infectious virus progeny, and (4) low-level viral protein expression that cannot be detected by conventional immunohistochemistry or immunoblotting.19 Evidence of viral protein expression with transfection of the plasmid vector was obtained by detection of negative-strand...
end-stage cell death and that virus-induced alteration in host cell protein expression may lead to the direct cytopathic effect.

Data obtained in other cell types suggest that picornavirus proteins have a significant role in host cell cytopathic effects. The coxsackievirus proteinase 2A (2A Pro ) is not only important for cleavage of viral polyprotein but is also associated with cleavage of eukaryotic initiation factor 4G (eIF4G). The eukaryotic initiation factor complex has a key role in the initiation of cap-dependent translation of eukaryotic mRNA. Similarly, viral proteinase 3C (3C Pro ) has been shown to cleave the host cell transcription binding protein that is involved in initiation of TATA-dependent transcription. It is possible that expression of these viral proteins is sufficient to induce a potent direct cytopathic effect on cardiac myocytes by inhibiting both myocyte transcriptional and translational mechanisms. In addition, it is possible that double-stranded viral RNA may be able to activate intracellular signaling mechanisms that contribute to the effect of persistent viral infection on cardiac myocytes. Future experiments will determine whether proteinase 2A alone or proteinase 3C alone can induce an effect on cardiac myocytes similar to that which occurs with the full-length CVB3 ΔVP0 expression.

In summary, these results conclusively demonstrate that (1) cultured neonatal rat ventricular myocytes can be used to study the cytopathic effect of CVB3 on cardiac myocytes, (2) mutation of an infectious cDNA copy of CVB3 at the VP0 maturation cleavage site can be used to express viral proteins and viral cDNA in myocytes without formation of an infectious virus progeny, and (3) a mutant cDNA of the coxsackievirus genome expressed at low levels without formation of infectious virus progeny can induce cytopathic effects in cultured ventricular myocytes with a pattern of viral gene expression that has many of the characteristics that occur with CVB persistence in a murine model of ongoing enteroviral replication. This pattern of expression and induction of a cytopathic effect may be relevant to some forms of dilated cardiomyopathy in humans. Further use of this model system will allow dissection of the mechanisms by which CVB3 can induce myocardial injury without generating infectious virus progeny. In addition, cardiac myocyte targeted expression of the mutated CVB3 cDNA in a transgenic animal should provide the experimental paradigm to test whether chronic low-level viral protein expression can induce cardiomyopathy in the intact animal.

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References


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