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Active Coxsackieviral B Infection Is Associated With Disruption of Dystrophin in Endomyocardial Tissue of Patients Who Died Suddenly of Acute Myocardial Infarction

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Objectives In this study, we evaluated the potential direct role of enterovirus (EV) cardiac infections in the pathogenesis of myocardial infarction (MI).

Background Enteroviruses (*Picornaviridae*) have been suspected to play a role in the development of acute MI.

Methods The presence of EV ribonucleic acid (RNA) sequences and capsid viral protein 1 (VP1) and the virus-mediated focal disruption of dystrophin were retrospectively investigated by reverse transcriptase-polymerase chain reaction and immunohistochemistry assays in endomyocardial tissues of patients who died suddenly of acute MI by comparison with similar samples of control patients matched for gender, residence area, and year of death.

Results Enterovirus infection markers were detected in 20 (40%) of 50 patients who died suddenly of MI, 2 (4%) of 50 matched subjects without cardiac disease ($p < 0.001$), and 4 (8%) of 50 matched patients exhibiting a noncoronary chronic cardiopathy ($p < 0.001$). All of the EV RNA-positive patients exhibited VP1, which provided evidence of viral protein synthesis activity. The VP1 gene sequences amplified after cloning from myocardial or coronary samples of 8 of the MI patients and showed a strong homology with sequences of coxsackievirus B2 and B3 serotypes. Moreover, in the endomyocardial tissue of these 8 patients, immunohistochemical analyses demonstrated that there was disruption of the sarcolemmal localization of dystrophin in the same tissue areas that were infected by coxsackieviruses.

Conclusions Our findings demonstrate a significantly higher proportion of active coxsackievirus B cardiovascular infections in patients who suddenly died of MI compared with matched control subjects, suggesting that these EVs may significantly contribute to the pathogenesis of acute MI by a focal disruption of the dystrophin-glycoprotein complex. (J Am Coll Cardiol 2007;50:2207–14) © 2007 by the American College of Cardiology Foundation

Myocardial infarction (MI) results from an acute coronary syndrome that can occur during the natural course of atherosclerosis (1). The atherosclerotic process is related to endovascular chronic inflammatory mechanisms that could be potentially initiated or sustained by infectious agents (2).

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Several reports have suggested the potential role of human enteroviruses (EVs) (*Picornaviridae*), particularly coxsackievirus B (CV-B) serotypes (belonging to the species human *Enterovirus B*), in the pathogenesis of MI (2,3). The CV-B serotypes are known to possess a strong cardiac tropism and to persistently infect human myocytes and vascular endothelial cells (4,5). Moreover, the CV-B protease 2A is capable

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Abbreviations and Acronyms

CV-B = coxsackievirus B
EV = enterovirus
MI = myocardial infarction
VP1 = capsid viral protein 1

of cleaving dystrophin in the hinge 3 region, leading to disruption of the dystrophin–glycoprotein complex in EV-infected human myocytes, therefore contributing directly to the pathogenesis of EV-induced human cardiac pathologies (6–8). At the present time, knowledge of the general involvement of EV in human coronary heart disease is largely based on serologic studies demonstrating a concomitant increase in antibodies to CV-B at the time of diagnosis of MI (9–12). These studies have limitations to their interpretation, and the results remain controversial (13). Several published reports have demonstrated the molecular detection of EV ribonucleic acid (RNA) genomes in myocardial tissues of patients with end-stage cardiac chronic coronary diseases requiring heart transplantation (14,15). Moreover, the EV genome was detected in sections of thrombotic coronary segment of a young patient who died suddenly of acute MI (16), whereas another study identified the presence of EV RNA in 17% of 128 atherosclerotic plaque samples, but not in any of the 20 normal arterial wall samples (17). Taken together, these findings suggest that EV infection of cardiovascular tissues may play a role in the pathogenesis of coronary disease leading to MI.

In the present study, the presence of EV 5′-noncoding sequences and capsid viral protein 1 (VP1) was investigated in cardiovascular tissues of patients who died suddenly of acute MI compared with similar samples from control patients. To assess the direct role of EV endomyocardial infections in the pathogenesis of MI, we investigated the presence of virus-mediated focal disruption of the sarcolemmal localization of dystrophin in the endomyocardial tissue of study patients.

Patients and Methods

Patients. Fifty subjects who died suddenly of acute MI (sudden cardiac deaths further referred to as MI patients according to pathologic findings) and whose autopsies were performed within 24 h of death in 3 French university hospitals (Paris, Reims, and Saint-Etienne) between September 1998 and December 2003 were selected among 85 initial sudden death cases originating from a series of 1,834 French autopsied patients. According to consensual definitions of the Joint European Society of Cardiology/American College of Cardiology Committee (18), 28 and 22 of these patients demonstrated pathologic findings of inaugural and massive acute MI and of acute MI consecutive to a previous history of chronic coronary heart disease, respectively. For comparison, 50 adult subjects without any known cardiac pathology or histologic abnormalities and who died suddenly from accidents or suicide (control subjects without cardiac disease), and 50 patients suffering from noncoronary-linked heart diseases (congenital cardiac disease [n =15], obstructive dilated cardiomyopathy [n = 15], or chronic valvulopathy [n = 20]—control subjects with other chronic cardiac disease) were selected among 1,895 autopsied patients (Table 1). Each of the 50 subjects of the 2 control groups was selected because he or she was matched for gender, residence area, and year of death with an MI patient. For each of the subjects entering the study, 5 large ventricular tissue fragments (4 from the left ventricle and 1 from the right ventricle) had been sampled within 24 h of death, fixed in 10% neutral buffered formalin, and paraffin embedded. For each of the 50 patients who died suddenly of acute MI, sections of thrombotic coronary segment had been concomitantly sampled and fixed in the same conditions. Demographic features of the 50 coronary heart disease patients and 100 control patients are presented in

Table 1 Demographic Features and Markers of Enterovirus Infection in Patients Who Died of Acute MI and in 2 Control Groups of Subjects Matched for Gender and Year of Death With MI Patients

Subject Group	Total, n	Age, mean (SD, Range), yrs	Gender, M/F	Patients Positive for, n (%)	
				VP1	5′-NCR RNA
Patients with acute myocardial infarction					
Inaugural acute myocardial infarction	28	53 (14, 21–81)	20/8	12 (43)*	10 (36)
History of chronic coronary heart disease	22	60 (15, 29–92)	20/2	8 (36)	8 (36)
Total	50	56 (14, 21–92)	40/10	20 (40)	18 (30)
Control subjects without cardiac disease	50	50 (14, 15–73)	40/10	2 (4)†	2 (4)
Control subjects with other chronic cardiac disease					
Chronic valvulopathy	20	43 (8, 18–65)	16/4	0	0
Congenital cardiac disease	15	25 (9, 15–45)	11/4	2 (13)	0
Obstructive cardiomyopathy	15	44 (10, 35–63)	13/2	2 (13)	2 (13)
Total	50	44 (9, 15–65)	40/10	4 (8)‡	2 (4)

*$p = 0.64$ by Pearson chi-square test comparing the 2 groups with acute myocardial infarction (MI). †$p < 0.0001$ by McNemar chi-square test compared with the 50 patients with acute MI, on paired data. ‡$p = 0.0002$ by McNemar chi-square test compared with the 50 patients with acute MI, on paired data.

5′-NCR = 5′-noncoding region; RNA = ribonucleic acid; VP1 = capsid viral protein 1.

Table 1. For each study patient, additional data concerning concomitant acute or chronic pathologies were obtained by classic macroscopic and microscopic analyses of the organs by the pathologists. However, because all of the study patients were not included in this study during a hospitalization stage, some information, such as left ventricular function, the time delay between MI and death, and comorbidities, was not available. The hospital ethics committee approved the study, and informed consent was obtained from the patients' families.

Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of EV genomes. Embedded myocardial samples were dewaxed, and total RNA was extracted from cardiac tissue using Tri-Reagent (Sigma-Aldrich Chimie, L'Isle d'Abeau Chesnes, France) (19). For all of the myocardial specimens, glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid (mRNA) was amplified by RT-PCR and used to check the quality of the RNA extraction (19). The presence of specific EV 5′-noncoding sequences was assessed by a seminested RT-PCR assay. To obtain a broad specificity for the genus *Enterovirus*, 3 primers (P2: nucleotide position of CVB3-Nancy genome 164 to 184; 5′ CAAGCACTTCTGTTTCCCCGG 3′; P3: nucleotide position of CVB3-Nancy genome 599 to 580: 5′ ATTGTCACCATAAGCAGCCA 3′; and EV1: nucleotide position of CVB3-Nancy genome 526 to 512: 5′ CTTGCGCGTTACGAC 3′) described to anneal to the highly conserved sequences within the 5′-noncoding region (5′NC) were used (20). Complementary deoxyribonucleic acid (cDNA) for plus-strand RNA was synthesized by using the antisense (P3) primer (annealing temperature 45°C). Classic PCR amplification of cDNA was carried out using primers P2 and P3 as described elsewhere (20). A second PCR run was carried out using primers P2 and EV1 (18). Positive-control viral genomic RNA was transcribed from a complete CVB3-cDNA sequence (CVB3-M1) that was inserted in a pBluescript (SK+) vector (Stratagene, Palo Alto, California) (20). Each heart sample was PCR tested in duplicate. An aliquot of each amplified RT-PCR product was subjected to agarose gel electrophoresis with ethidium bromure staining (20). Extensive precautions were taken to prevent molecular contaminations, and 2 different virology laboratories independently confirmed positive results using the same technique. Moreover, positive and negative results had been run together in serial assays, and they were always interpreted independently of the immunohistochemistry results.

Immunohistochemical detection of EV VP1. Sections of myocardial tissue fixed in paraformaldehyde (PFA) and adjacent in location from where the EV-positive section samples were obtained, were used to detect the enteroviral VP1 by immunohistochemistry (19). The mouse monoclonal antibody 5D8/1 (IgG2a, Dako, Trappes, France) was directed toward a group-specific motif of the N-terminal part of the VP1, which is highly conserved in the *Enterovirus* genus (21). The normal mouse IgG2a, blocking reagent, antibody diluent, and detection system EnVision/horseradish peroxidase were also purchased from Dako. Heat-mediated antigen retrieval procedures and immunohistochemistry detection with the polymer/peroxidase conjugate (EnVision, Dako) were performed according to published protocols (22). The monoclonal antibody was applied at a dilution of 1:500 (0.22 mg/ml). As a control, the primary antibody was replaced with diluent only or concentration-matched mouse IgG2a (19). Two nonbiased observers read the results blindly in 2 different laboratories. Moreover, the immunohistochemistry results were always interpreted independently of the RNA viral detection by RT-PCR assay.

Immunohistochemical staining of human cardiac dystrophin. Serial sections of myocardial tissue fixed in PFA and adjacent in location from where the EV-positive section samples were obtained, were used to perform the sarcolemmal staining pattern for dystrophin by immunohistochemistry (7). The mouse monoclonal antibody NCL-DYSA (IgG1, Novo Castra, New Castle, United Kingdom) was used following the manufacturer's instructions (7). Heat-mediated antigen retrieval procedures and immunohistochemistry detection with 3 amino-9 ethyl carbazol (ACC kit, Vector Laboratories, Peterborough, United Kingdom) were performed according to the manufacturer's recommendations (22). The monoclonal antibody was applied at a dilution of 1:10. As a control, the primary antibody was replaced with diluent only or concentration-matched mouse IgG2a. Two nonbiased observers read the results blindly in 2 different laboratories. Moreover, these immunohistochemistry results were interpreted independently of the VP1 immunohistochemistry detection assay.

Molecular identification of EVs by RT-PCR and amplicon sequencing of the VP1 gene. For each sample that tested positive for EV RNA or antigen, a typing of the strain was attempted by partial PCR amplification, TA cloning, and sequencing of a part of the VP1 gene (23). The nucleotide sequence of the EV RT-PCR products was determined by double-strand DNA cycle sequencing using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Roissy, France) following the manufacturer's instructions. Electrophoresis and analysis of DNA sequence reactions were performed with an automated DNA sequencer (Model 377-A, Applied Biosystems). The sequencing primers were those used for PCR amplification. Before sequencing, the PCR products were purified using a Qiaquick gel extraction kit (Qiagen, Courtaboeuf, France) and were cloned using the Topo-TA cloning kit (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instructions. Both strands of the DNA fragments were sequenced. Usedit and Sequence Navigator programs (Applied Biosystems) were used to analyze the sequence data (24). Serotype was determined from the sequence by comparison with a database of all EV (or picornavirus) VP1 sequences using "Gap" (Wisconsin Sequence Analysis Package, Genetics Computer Group, San Diego, California) as described elsewhere (23). The highest identity score, if

>75%, indicated the serotype. The VP1 sequences obtained from the myocardial tissues of 8 patients are available in the European Molecular Biology Laboratory database under the accession numbers AM159194 to AM159201.

Statistical analysis. Quantitative data are presented as mean, SD, and range. Qualitative data are presented as number of observations and percentages. Comparisons between groups were performed with the Pearson chi-square test, Fisher exact test, or McNemar chi-square test (using the Yates correction when necessary). A conditional logistic regression was used for odds ratio (OR) and 95% confidence interval (CI) estimations. Results were considered to be statistically significant for 2-sided p values of <0.05. The statistical analysis was performed with the SAS software version 8.2 (SAS Institute, Cary, North Carolina).

Results

Immunohistochemical detection of EV VP1 in endomyocardial tissue of patients who died suddenly of acute MI. To determine whether the EV cardiac infection was associated with viral protein synthesis activity, the 5 heart tissue samples taken from each MI patient or control subject at the time of autopsy were examined by an immunostaining assay specific for VP1. This marker was found to be positive for at least 1 out of the 5 tested samples in 20 of the 50 patients who died of MI, and in 2 and 4 patients of the 2 control groups without cardiac disease and with other chronic cardiac diseases, respectively. The difference was highly significant by using the McNemar test when MI patients were compared with noncardiac control subjects and control subjects with other cardiac diseases, with ORs of 15.6 (95% CI 3.4 to 71.2; $p < 0.001$) and 7.5 (95% CI 2.35 to 24; $p < 0.001$), respectively. As shown in Figure 1, illustrating typical results obtained from 3 patients, viral capsid antigens appeared to be primarily localized in the cytoplasm of clustered myocytes adjacent to necrosis in cases of inaugural acute MI (Fig. 1A) or to necrosis and healing lesions in cases of patients with a previous history of chronic coronary heart disease (Fig. 1B).

Enterovirus genomic RNA detection in cardiovascular tissues of patients who died suddenly of acute MI. In parallel, the 5 heart tissue samples taken from each patient or control subject were tested for the presence of RNA by RT-PCR using primers located in the 5′-NC of the EV genome. This marker was found to be positive for at least 1 of the 5 tested samples in 18 of the 50 patients who died of acute MI and in 2 and 2 subjects of the 2 control groups, respectively (Table 1). Figure 2 illustrates typical results obtained for 8 MI patients; as shown in Figure 2B, the quality of the extraction step was systematically checked by the concomitant detection of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene.

As reported in Table 1, summarizing the results of the detection of EV RNA and VP1 in the 3 groups of subjects, all of the patients positive for viral genomic RNA detection were also positive for VP1 detection in cardiac tissues, whereas 4 patients appeared to be positive only for VP1 antigen detection in cardiac samples (2 in the MI group and 2 in the control group of patients with other cardiac diseases). However, the difference was not statistically significant ($p = 0.48$ for patients with acute MI, no discordant pairs for control subjects without chronic cardiac disease; and $p = 0.48$ for control subjects with other chronic cardiac diseases) (Table 1). The EV markers were detected in 12 (43%) of the 28 patients with inaugural acute MI and 8 (36%) of the 22 patients with history of chronic coronary

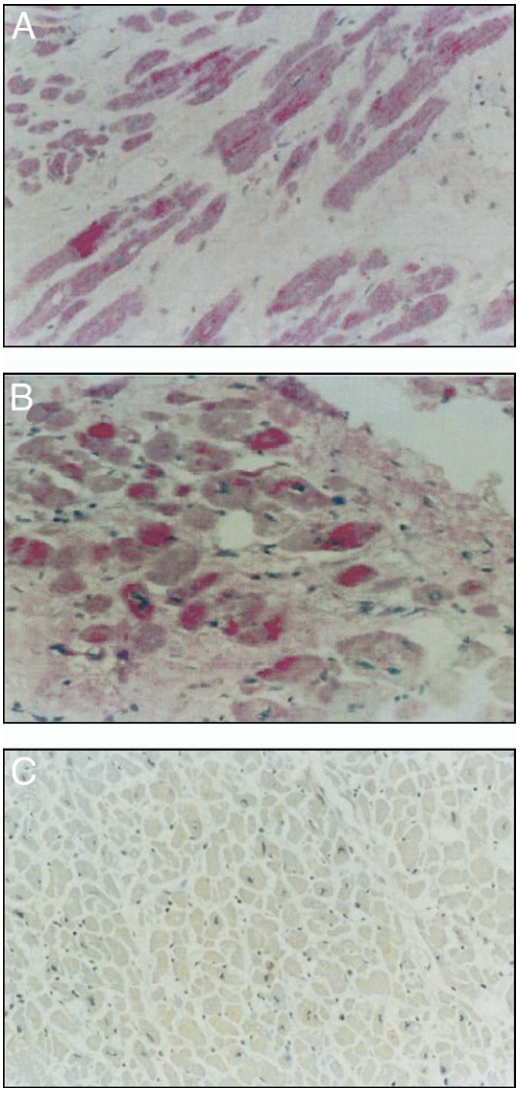

Figure 1 **Immunostaining of VP1 in Heart Tissue of Patients With MI and Control Patients**

Immunostaining **(red)** of capsid viral protein 1 (VP1) in heart tissue of a patient with an inaugural acute myocardial infarction (MI) **(A)**, a patient with acute MI in the context of chronic coronary heart disease **(B)**, and a negative healthy heart patient **(C)**. The sections were counterstained with hematoxylin. Magnification ×400.

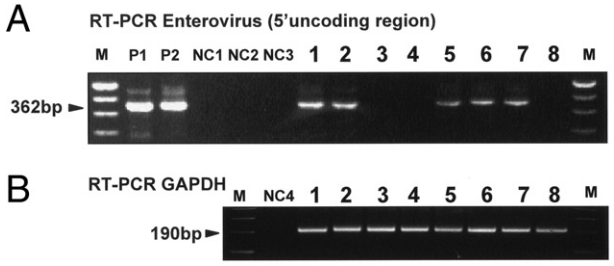

Figure 2 **Gel Electrophoresis of PCR Products**

(A) Polymerase chain reaction (PCR) products generated by nested reverse transcriptase (RT)-PCR using primers located in the 5′-noncoding region of the enterovirus genome (360 bp). **Lanes 1 to 4** correspond to patients who died of an inaugural acute myocardial infarction (MI); **lanes 5 to 8** correspond to patients with a previous history of chronic coronary disease and who died of an acute MI; P1 = positive control obtained by RT-PCR amplification of positive ribonucleic acid (RNA) strands (2×10^{10} copies), which were transcribed from a coxsackievirus B3 (CV-B3) complementary (c) deoxyribonucleic acid (DNA) sequence; P2 = positive control obtained by direct PCR amplification of single-strand copies of CV-B3 cDNA; NC1 = negative control of RT assay and nested PCR amplification; NC2 = negative control (sterile water) of the first PCR amplification assay; NC3 = negative control of the second PCR amplification assay; M = molecular size marker (100-bp DNA ladder). **(B)** PCR products generated by RT-PCR using primers located in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene (190 bp). **Lanes 1 to 8** correspond to a positive GAPDH messenger RNA detection in cardiac tissues from patients 1 to 8. NC4 = RT-PCR negative control (sterile water); M = molecular size marker (100-bp DNA ladder).

heart disease (p = 0.64) (Table 1). Among the 50 study patients who died suddenly of MI, the EV markers were detected in 11 (61%) of the 18 transmural MI cases and 9 (29%) of the 32 subendocardial infarction cases (p = 0.02; data not shown).

Epidemiologic, biologic, and clinical features of EV-positive study patients. Of the 20 EV-positive patients with acute MI, 7 died between May and October, whereas 2 of the 6 EV-positive control patients died during the same period, which corresponds to the seasonal epidemic period of EV infection in Europe (35% vs. 33%; p = 0.70 by Fisher exact test). No statistically significant difference was observed in the gender distribution of patients with acute MI exhibiting a positive detection of EV markers in the cardiac tissues (data not shown). All of the EV-positive cardiac patients who died of acute MI demonstrated at least 1 of the following cardiovascular risk factors for the development of coronary disease: cigarette smoking, diabetes, hypertension, hyperlipidemia, and obesity. The 2 subjects from the healthy heart control group with stigmata of EV cardiac infection demonstrated the presence of atherosclerotic plaques in aorta and other large arteries. The cardiac pathologic results indicated that the subendocardial infarction cases died likely from ventricular arrhythmia (1). No distinct or specific pattern of inflammatory cell infiltrate was observed in EV-infected patients compared with the EV cardiac-negative subjects (data not shown).

Detection of common cardiotropic viruses other than EVs in endomyocardial tissue of study patients. None of the 20 EV-positive patients with acute MI and none of the 2 EV-positive subjects from the healthy heart control group were positive for the detection of other cardiotropic viruses, including Epstein-Barr virus, herpes simplex viruses, cytomegalovirus, varicella-zoster virus, adenoviruses, and parvovirus B19, in their myocardial tissues using previously published PCR techniques (data not shown) (25). These results demonstrated that the EVs were the only cardiotropic viral agents detected by molecular approach in the endomyocardial samples of these patients. Interestingly, in 15 (75%) of the 20 EV-positive patients who died of acute MI, the presence of EV RNA was also detected by specific RT-PCR in sections of thrombotic coronary segment (data not shown). Moreover, all of the coronary death patients who were negative for the detection of EV markers (n = 30) were also negative for the detection of EV RNA in their coronary samples.

Molecular identification of enteroviruses by RT-PCR and amplicon sequencing of the VP1 gene. Using RT-PCR targeting the VP1 region and thymidine adenine cloning, a partial sequencing of cloned amplicons was possible for 8 of the MI patients found to be positive for the presence of markers of EV infection in endomyocardial tissues and for 4 of these patients in sections of thrombotic coronary segment. It showed an amino acid homology of more than 85% with CV-B2 for 4 strains or with CV-B3 for 4 other strains, therefore allowing a reliable viral serotyping identification by this genetic approach (Fig. 3) (23).

Disruption of the sarcolemmal localization of dystrophin in EV-infected patients. To assess whether there was a potential EV-mediated cleavage of dystrophin, the presence of a morphologic disruption of the organized dystrophin staining of the heart was investigated in heart tissues of 8 CV-positive patients who died of acute MI, 8 EV-negative MI patients, and their 16 matched healthy heart control subjects. Serial heart sections of each patient and control subject were immunostained with antibody against EV VP1 and with antibody against human dystrophin (Fig. 4). The typical dystrophin staining was normal in uninfected controls (n = 16) and in EV-negative MI patients (n = 8) (Figs. 4A and 4D), whereas it was disrupted in several areas of heart tissue sections of each of the MI patients infected by CV-B serotypes (n = 8) (Figs. 4B and 4E). Immunohistochemical analyses onto serial cardiac sections demonstrated that there was focal disruption of the sarcolemmal organization of dystrophin (Fig. 4F) in the same areas of cardiac tissue that were positive for EV VP1 (Fig. 4C).

Discussion

This study shows for the first time the presence of an active EV cardiovascular infection in a high proportion of patients who suddenly died of MI, and suggests that this viral

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Strain	Amino acid number 32–90
CV-B2	TAVETGHTSQVTPSDTMQTRHVHNYHSRSESSVENFLARSACVFYTTYTNSKNAAKEKK
CV-B1	--A--------V----------K---------I----C-----Y-A--N...-NS.--G
CV-B4	-----------D--------------------I----C-----I-IK-SSAESNN.L-R
CV-B3	--A--------V-G--------K--------TI----C-----YF-E-E...-SG.A-R
Case 1	*---------------------R------------------------------------
Case 2	*-A-------------------R-------P----------------------------
Case 3	*-A-------------------R------------------------------------
Case 4	***-------------------R------------------------------------
Case 5	**A--------V-G--------R--------TI----C-----YF-E-E...-SG.A-R
Case 6	**A--------V-G--------K-------PTI----C-----YF-E-E...-SG.A-R
Case 7	**A--------V-G--------R--------TI----C-----YF-E-E...-SG.A-R
Case 8	**A--------V-G--------K--------TI----C-----YF-E-E...-SG.A-R

Strain	Amino acid number 91–146
CV-B2	FATWKVSVRQAAQLRRKLELFTYLRCDIELTFVITSAQDPSTATNLDVPVLTHQIM
CV-B1	Y-E-VINT--V---L-RKLE-----F-I----------E-----SV-A--Q-Q---
CV-B4	Y-E-VINT--V------M-M---I---M--------H-EM-----S----Q-----
CV-B3	Y-E-VITP-----------F---V-F-I--------T-Q---TQ-Q-AQI------
Case 1	-------------------------------*************************
Case 2	--
Case 3	--
Case 4	--
Case 5	Y-E-VITP-----------F---V-F-I--------T-Q---TQ-Q-AQI------
Case 6	Y-E-VITP-----------F---V-F-I--------T-Q---TQ-Q-AQI------
Case 7	Y-E-VITP-----------F---V-F-I--------T-Q---TQ-S-AQI------
Case 8	Y-E-VITP--V--------F---V-F-I--------T-Q---TQ-Q-AQI------

Figure 3 **Alignment of Amino Acid Sequences of Partial Capsid Viral Protein of Cardiac EV Isolates From 8 Patients Who Died of Acute MI**

Amino acid sequences of partial capsid viral protein region of cardiac enterovirus (EV) isolates from 8 patients who died of acute myocardial infarction (MI), and of reference coxsackievirus B (CV-B) sequences retrieved from GenBank (M16560, AF085363, M16572, and X05690 for CV-B1, -B2, -B3, and -B4, respectively). The alignments were performed with reference to the CV-B2 sequence. The homology was >85% between sequences of strains from patients 1 to 4 and CV-B2 and from patients 5 to 8 and CV-B3. Differences are printed in full. **Dash** = identical amino acid; **dot** = amino acid deletion; **star** = undetermined amino acid.

infection may directly contribute to the pathogenesis of coronary diseases leading to MI. The EV markers of infection (genomic RNA or VP1 antigen) were detected at a very low rate in cardiac tissues of matched control subjects, which suggests that the heart tropism of CV-B is not responsible for an opportunistic infection in immunocompetent subjects, even in those with a chronic cardiac dysfunction (Table 1). An alternative explanation would be that patients with coronary heart disease are more prone to EV cardiovascular infection. However, this last hypothesis is unlikely, because equivalent infection rates were observed between patients with an inaugural cardiac ischemic event and those with a chronic coronary heart disease (Table 1). The possibility of cardiac superinfection by opportunistic viruses in patients critically ill with MI is also invalidated by the absence of molecular detection of DNA cardiotropic viruses in myocardial tissues of the 20 EV-positive patients with acute MI (data not shown). Taken together, the

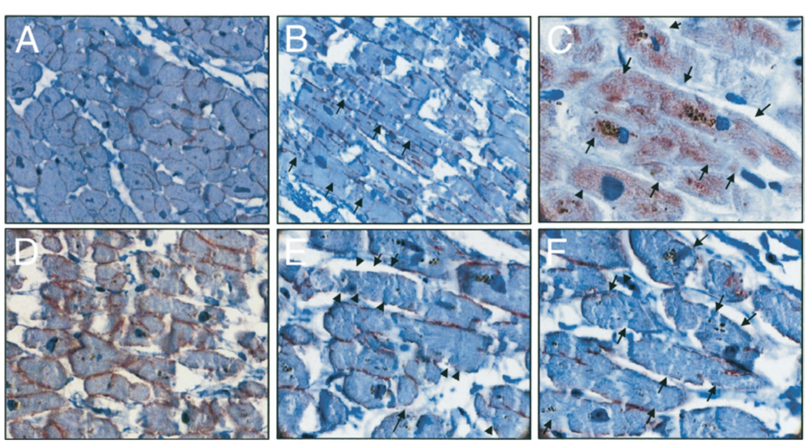

Figure 4 **Immunohistochemical Analyses Showing the Sarcolemmal Staining Pattern for Dystrophin Using Antidystrophin Antibody**

Dystrophin pattern was conserved in cardiac tissue sections of **(A)** healthy heart controls (immunostaining in **red**) and of **(B)** enterovirus (EV)-negative patients who died of acute myocardial infarction (immunostaining in **red**). Example of focal areas of myocardium displaying a loss of the sarcolemmal staining pattern in cardiac tissue sections of EV-positive patients **(B and E, arrows)**. In several study cases, immunohistochemical analyses of serial cardiac sections demonstrated that there was focal disruption of the sarcolemmal organization of dystrophin **(F, arrows)** in the same areas of cardiac tissue that were positive for the EV capsid viral protein 1 **(C, arrows)** (immunostaining in brown). The sections were counterstained with hematoxylin. Magnification: ×400 **(A and B)**; ×1,000 **(C to F)**.

present findings provide support for the controversial concept that a viral cardiac infection may contribute to the pathogenesis of coronary disease leading to MI in a high proportion (approximately 40%) of patients (26). Previously, there was not a strong direct linkage of EV cardiac infections with human MI except for anecdotal reports indicating that viral myocarditis may masquerade as clinical MI (27,28). In the present study, our severe cardiac histopathologic criteria allowed us to exclude the inclusion of viral myocarditis cases potentially mimicking acute MI.

In the present study, the rate of VP1 antigen detection appeared to be slightly higher than that obtained by the RT-PCR detection of EV genomes in the cardiac tissue samples of patients with acute MI as well of control subjects with other chronic cardiac diseases (Table 1). This could be explained by the fragility of single strands of viral genomic RNA present in PFA-fixed myocardial samples, which could be increased by the delay between the clinical death and the time of autopsy. In this study, the endomyocardial detection of VP1 by immunohistochemical technique was found to be the best tool for the diagnosis of EV-associated heart diseases, particularly in cases of sudden cardiac death (29). As previously shown for other cardiac diseases linked to EVs (3,5), all of the MI patients with stigmata of EV infection demonstrated the presence of viral capsid protein in their heart tissue samples. Those findings provided direct evidence of an ongoing viral replication activity compatible with an acute or a persistent infection and excluded the presence of a latent viral persistence in cardiac tissues at the time of sampling (14). The endomyocardial detection of VP1 antigens in 2 subjects without any cardiac disease suggests that this infection might have contributed to the development of coronary disease if they had survived. Taken together, these immunohistologic findings provide evidence of EV endomyocardial infection with a viral protein synthesis activity which could be involved in the pathogenesis of coronary heart disease leading to MI (14,19).

The use, after cloning, of nucleic acid sequencing of the VP1 region allowed us to perform, in some of these patients, a reliable identification of the serotype involved in the chronic infection of cardiac tissues. In addition, the small differences observed within sequences related to the same serotype are indicative that no cross-contamination occurred during PCR experiments. Not surprisingly, sequences close to CV-B2 and -B3 were identified in cardiac tissue samples of 8 patients (Fig. 3). Indeed, CV-B can persistently infect myocytes or vascular endothelial cells, inducing endothelial dysfunction, microvascular injury, or coronary spasms (4,5,30). Thus, by producing a subclinical acute or a persistent infection of cardiovascular tissues, these viruses could exacerbate not only the inflammatory process of chronic atherosclerosis but also acute ischemia and myocardial damage at the time of MI.

To assess the direct role of EV endomyocardial infections in the pathogenesis of MI, we investigated the presence of virus-mediated focal disruption of the sarcolemmal localization of dystrophin in endomyocardial tissue of some of the study patients. We demonstrated that in 8 CV-B cardiac-infected patients who died of acute MI, a focal disruption of the dystrophin-glycoprotein complex could occur in the same tissue areas that were infected by the virus (Fig. 4). The high VP1 immunostaining levels observed in the myocardial tissues of these EV-positive patients (Fig. 4C) suggested a viral protein synthesis activity that may result in high intracellular expression of EV protease 2A. Viral protease 2A is able to cleave human dystrophin, therefore decreasing the transmission of mechanical force from the sarcomere to the extracellular matrix and increasing sarcolemmal permeability of the human myocytes (6–8,31). This virus-mediated molecular mechanism may significantly enhance the cardiac failure resulting from ischemia, thereby contributing to the extension of cardiac necrosis and to the occurrence of cardiac fibrillation and ventricular arrhythmias at the time of MI (1). Moreover, CV-B may facilitate its propagation into the endomyocardial tissue through proteolysis of dystrophin, thereby contributing to the pathogenesis of virus-mediated heart disease in EV-infected patients. The present findings suggest that one of the direct roles of EVs in the development of severe ischemic lesions during MI may concern the disruption of dystrophin by protease 2A, as previously demonstrated in acute fulminant myocarditis (31). However, our results do not exclude the possibility that other virus- or immune-mediated mechanisms may contribute significantly to the pathogenesis of chronic coronary diseases leading to MI. Further retrospective case-control studies testing heart, artery, and atherosclerotic plaque samples in different populations and additional investigations using animal models are needed to investigate the other pathogenic mechanisms underlying the association between EV cardiovascular tissue infection and the development of coronary disease leading to MI.

Conclusions

Our findings demonstrate a significantly higher proportion of active CV-B cardiovascular infections in patients who suddenly died of MI compared with matched control subjects and suggest that these EVs may significantly contribute to the pathogenesis of acute MI by a focal disruption of the dystrophin–glycoprotein complex. Our study strongly suggests that EV infections may be one of the environmental and nontraditional risk factors for the development of coronary heart disease. These findings may be of major interest for the development of further therapies or preventive strategies to fight against the development of EV-mediated MI cases.

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