Enterovirus-related activation of the cardiomyocyte mitochondrial apoptotic pathway in patients with acute myocarditis

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Aims
We examined the impact of enterovirus (EV) cardiac replication activity on the endomyocardial mitochondrial pathway in patients with acute myocarditis.

Methods and results
Levels of apoptotic cardiomyocytes were determined by TUNEL and ligation-mediated polymerase chain reaction (PCR) assays and EV replication activity was assessed by immunostaining of EV VP1 capsid protein in ventricular myocytes of patients with acute myocarditis (n = 25), and healthy heart controls (n = 15). Ratio of cytosolic/mitochondrial cytochrome c concentrations was determined by ELISA assay, levels of active caspase-9 were determined by western blot analysis and Bax/Bcl2 mRNA ratio was assessed by real-time reverse transcription–polymerase chain reaction (RT–PCR) in the same cardiac tissues. Patients with EV-associated acute myocarditis (n = 15) exhibited a significantly higher number of apoptotic cardiomyocytes than those with non-EV-associated acute myocarditis (n = 10) and controls (n = 15) (P < 0.001). Endomyocardial ratio of cytosolic/mitochondrial cytochrome c concentrations and levels of active caspase-9 protein were significantly increased in EV than in non-EV-related myocarditis patients (P < 0.001). Moreover, Bax/Bcl2 mRNA ratio was significantly increased in EV than in non-EV-related myocarditis patients (P < 0.001).

Conclusion
Our findings evidence an EV-related activation of the cardiomyocyte mitochondrial apoptotic pathway in patients with acute myocarditis. Moreover, our results indicate that this EV-induced pro-apoptotic mechanism could be partly related to an up-regulation of Bax expression, and suggest that inhibition of this cell death process may constitute the basis for novel therapies.

Keywords
Enterovirus • Cardiomyocytes • Apoptosis • Cytochrome c • Caspase-9 protein • Acute myocarditis • End-stage dilated cardiomyopathy (DCM)

Introduction
Enteroviruses (EVs), especially Coxsackieviruses B (CV-B), are considered a common etiological cause of myocarditis in children and young adults and have been implicated in the pathogenesis of dilated cardiomyopathy (DCM), which is the second most frequent clinical condition leading to heart transplantation.1–8 The concept that endomyocardial persistent EV infection is the etiological cause of a subset of idiopathic DCM cases is supported by the detection of EV genomic sequences and in situ synthesis activity of enteroviral capsid protein VP1 in about 35% of explanted heart tissues from end-stage DCM patients.56 However, the cellular and molecular mechanisms by which EVs cause human myocarditis and progression to DCM remains poorly understood, therefore limiting...
the development of specific therapeutic strategies against EV-induced acute and chronic heart diseases.\textsuperscript{7}

Several studies reported that CV-B3 and other picornaviral infections are capable of inducing apoptosis in cardiac cell cultures.\textsuperscript{8,9} The possible mechanisms responsible for these findings include the release of cytochrome c and other pro-apoptotic factors from the mitochondria.\textsuperscript{10,11} In experimental CV-B3-induced acute myocarditis, the over-expression of pro-apoptotic mitochondrial Bax protein family proteins in cardiomyocytes of transgenic mice was identified as a causal mechanism of heart failure.\textsuperscript{10,12–16} Similarly, several clinical studies indicated that the number of apoptotic myocytes was related to the stage and severity of EV-related myocarditis. Moreover, some of them reported that endomyocardial expression of Bcl-2 anti-apoptotic protein in DCM cases could be related with low disease progression.\textsuperscript{15,16} Altogether, these findings suggest that apoptotic cell death could be, at least in part, responsible for the disease progression from acute viral myocarditis to DCM and that acute EV infections might modulate the mitochondrial apoptotic pathway in human active myocarditis. However, the evidence of a direct link between cardiac EV replication activity and the modulation of mitochondrial apoptotic pathways remains to be established in clinically defined patient groups.

In the present study, we examined the impact of EV cardiac replication activity on the endomyocardial mitochondrial pathway in patients with acute myocarditis. Levels of apoptotic cardiomyocytes were determined by TUNEL and ligation-mediated PCR assays, and EV replication activity was assessed by immunostaining of EV VP1 capsid protein in ventricular myocytes of well clinically characterized patients. Moreover, in order to explore the mitochondrial apoptotic pathway, ratio of cytosolic/mitochondrial cytochrome c concentrations was determined by ELISA assay, levels of active caspase-9 were determined by western blot analysis, and Bax/Bcl2 mRNA ratio was assessed by real-time reverse transcription–polymerase chain reaction (RT–PCR) in the same cardiac tissues.

### Methods

#### Patients

Clinical details of the patients are given in Table 1. For each of the subjects recruited in this study, a part of the myocardial sample was quickly frozen in liquid nitrogen and stored at −80°C and the other part was fixed in 10% buffered formalin and paraffin-embedded for classical histopathological analyses, VP1 immunohistochemistry, and apoptotic marker detection assays. The Hospital Ethics Committee (CHU de Reims, Champagne-Ardonne, France) approved the study and informed consent was obtained from the patients at the time of heart transplantation or from the patients’ or subjects’ families at the time of patient or subject death.

#### Patients with acute myocarditis

Between March 1998 and October 2005, 25 adult patients living in the northeast of France were included in the present study because they were suffering from an acute myocarditis according to the Dallas histological criteria. A myocardial sample was obtained at the time of initial ventricular assistance setting up (n = 16) or at autopsy within 12 h after a sudden cardiac death (n = 9) related to a severe or fulminating myocarditis (Table 1).\textsuperscript{17} Of them, 15 patients were positive for the detection of EV RNA and VP1 and negative for the detection of other common DNA or RNA cardiotropic viruses in endomyocardial tissue (see Material and Methods) (Table 1). The remaining 10 patients were negative for EV markers, but positive for the genome detection of other DNA (adenovirus, n = 4; parvovirus B19, n = 4) or RNA (virus influenza A, n = 2) viruses in the cardiac samples (Table 1).

### Table 1  Characteristics of study patients

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Acute myocarditis</th>
<th>Healthy heart controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EV+ **</td>
<td>EV− **</td>
</tr>
<tr>
<td>Total number of patients</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Age in years, Mean (SD, range)</td>
<td>25 (15, 18–40)</td>
<td>34 (15, 19–55)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/5</td>
<td>4/6</td>
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<tr>
<td>Duration of heart failure weeks (range)</td>
<td>4 (2–12)</td>
<td>6 (2–16)</td>
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<tr>
<td>Cardiac index (l/min/m²) (SD)</td>
<td>1.1 (0.63)</td>
<td>1.73 (0.55)</td>
</tr>
<tr>
<td>Ejection fraction (I %) (SD)</td>
<td>23 (9.8)</td>
<td>28 (8.9)</td>
</tr>
<tr>
<td>LVEDD (mm)**</td>
<td>52 (5.6)</td>
<td>48 (2.5)</td>
</tr>
<tr>
<td>Histological score, Mean (SD)****</td>
<td>3.65 (0.75)</td>
<td>2.55 (0.55)</td>
</tr>
<tr>
<td>Ventricular assistance</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Sudden cardiac dead†</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Results of nested RT–PCR detection assay in four cardiac ventricular samples.\textsuperscript{5}

**Patients suffering from adenovirus (n = 4), parvovirus B19 (n = 4), influenza A (n = 2) related acute myocarditis.

***Left ventricular end-diastolic diameter (mm).

****Mean value of the severity of inflammatory lesions (cellular infiltrate), of necrosis and fibrosis; this value was separately graded by two pathologists using a scale of 0 to 4 per section.\textsuperscript{18}

†–, none.

For all the cases of sudden cardiac death, clinical parameters (duration of heart failure, cardiac index, ejection fraction, and LVEDD) were not available.
Healthy heart controls
For comparison, 15 adult organ donors living in the North of France and exhibiting neither known nor cardiac pathology or histological abnormalities were recruited between 1999 and 2003 (Table 1). For each of these controls, four ventricular biopsies had been sampled at the time of heart transplantation. None of these patients were positive for EV RNA and VP1 markers and for the genome detection of other common DNA or RNA cardiotropic viruses in endomyocardial tissues.

Methods

Histopathological analysis
For each heart sample, four transverse paraffin sections were performed at three levels and were examined for classical histopathology to confirm the clinical findings of either acute myocarditis (Dallas criteria) or end-stage DCM or to demonstrate the absence of cardiac lesions or abnormalities in control heart donors. Two pathologists (L.V. and G.L.G.M) separately graded the severity of inflammatory lesions (cellular infiltrate), necrosis and fibrosis using a scale of 0 to 4 per section; the mean value resulting from the two-blinded histopathology analyses was recorded as described previously.18

Extraction and amplification of viral RNA and DNA
DNA and RNA were simultaneously extracted using All Prep DNA/RNA-Protein Mini Kit (Qiagen, France) from all the cardiac tissue samples kept frozen at −80°C. For each specimen, the GAPDH mRNA and DNA were amplified in RT–PCR and PCR reactions, respectively, in order to verify the quality of nucleic acid extraction.5 The presence of specific EV sequences was tested by a semi-nested RT–PCR assay using three primers recognizing highly conserved motif of the N-terminal part of the capsid protein VP1, highly conserved sequences within the 5’ non-coding (5’NC) region. DNA sequences of cytopathic enterovirus, herpes simplex virus, varicella-zoster virus, and Epstein–Barr virus were simultaneously amplified and secondly identified by specific probe hybridization using Hybriwell16 Herpes Consensus Kit according to the manufacturer’s recommendations (Argene-Biosoft, Varailles, France).19 The detection of DNA sequences of parvovirus B19 and adenovirus were carried out using classical PCR amplification and southern-blotting procedures as described previously.20,21

Immunohistochemical detection of enteroviral VP1 protein
Sections of myocardial tissue fixed in PFA and, for EV-infected samples, adjacent in location to those found positive for EV by PCR test were tested by immunohistochemistry for the presence of the enteroviral VP1 protein using the mouse monoclonal antibody (mAb) 5D8/1 (IgG2a) (Dako SA, Trappes, France) directed towards a group-specific epitope of the VP1 protein using the mouse monoclonal antibody (mAb) 5D8/1 (IgG2a) (Dako SA, Trappes, France) directed towards a group-specific motif of the N-terminal part of the capsid protein VP1, highly conserved in the Enterovirus genus.6,21 Heat-mediated antigen retrieval procedures and immunohistochemistry detection with the polymer/peroxidase conjugate (EnVision®) were performed as previously described.6 The mAb was applied at a dilution of 1:500 (0.22 mg/mL). Two independent observers (L.V. and G.L.G.M) read the results blindly in two different laboratories.

Detection of apoptosis markers

DNA in situ end labelling
TUNEL assay was performed using the ApopTag kit/Oncor (Gaithersburg, Germany) on five consecutive tissue sections of myocardial tissue fixed in PFA and, for EV infected samples, adjacent in location to those found positive for EV by PCR test. Briefly, tissue sections were incubated in a humidified chamber with 20 μg/mL of proteinase K (Sigma chemicals) for 15 min at 23°C and then washed twice in distilled water for 5 min. Endogenous peroxidase activity was quenched by incubating sections in 3% H2O2 for 5 min followed by two washing steps in TBS for 4 min each and a 1 min incubation at room temperature in equilibration buffer. The medium was then replaced by terminal deoxynucleotidyl transferase (TdT) solution (33 μL of TdT + 77 μL of reaction buffer) and the section was incubated in a humidified chamber at 37°C for 1 h. All the other steps of the protocol were performed following the manufacturer’s instructions. Tissue sections were finally counterstained with haematoxylin, dehydrated, and mounted.

The number of cardiomyocytes was counted under a light microscope with an ocular grid (×250 magnification, area of the field 0.28 mm²). The cardiomyocyte origin of the cells was confirmed by the presence of myofibers that were stained by an antibody directed against cardiac myosin as described previously.16 For each section, two independent observers (L.V. and G.L.G.M) belonging to different laboratories read an average of 50 fields (6082 μm² in size). The results were expressed as a mean per 10³ myocytes.16

Ligation-mediated PCR assay
The Apopalert LM-PCR ladder assay kit (Clontech laboratories, Palo Alto, CA, USA) was used to confirm the detection of nucleosomal ladders in apoptotic cells according to the manufacturer’s recommendations.22 Briefly, 0.5 μg of total genomic DNA was extracted from frozen cardiac samples and, after a ligation step, submitted to 20 to 25 PCR cycles. The amplitcons were migrated on a 1.2% (W/V) agarose gel electrophoresis (Figure 1B). The results were interpreted independently of the TUNEL assay.

Immunohistochemical detection of cytochrome c protein
Serial sections of myocardial tissue fixed in PFA and, for EV-infected samples, adjacent in location to those found positive for EV by PCR test were used to perform the immunohistochemical detection of endogenous levels of cytochrome c protein in cardiac tissues. A polyclonal rabbit anti-cytochrome c antibody (Cell Signaling Technology, Beverly, MA, USA) was used following the manufacturer’s instructions. Heat-mediated antigen retrieval procedures and immunohistochemistry detection with the polymer/peroxidase conjugate (EnVision®) were performed according to the manufacturer’s recommendations.6 The antibody was applied at a dilution of 1:50. As a control, the primary antibody was replaced with diluent only or concentration-matched mouse IgG2a. Two independent observers (L.V. and G.L.G.M) read the results blindly in two different laboratories. Four sections per
cardiac sample were tested. Moreover, the data were interpreted independently of the VP1 immunohistochemistry detection and TUNEL assays.

### Elisa quantitative detection of cytochrome c protein

Briefly, 20 mg of frozen cardiac tissues were processed using the Qproteome cell compartment kit following the manufacturer’s recommendations (Qiagen, France). The cytosolic and mitochondrial fractions were then quantified by spectrophotometer, and tested for each sample in duplicate using the human cytochrome c ELISA Kit following the manufacturer’s instructions (Assay Designs, Ann Arbor, MI, USA). The measured optical density (405/570 nm) was directly proportional to the concentration of cytochrome c in either standards or samples. Fractions were run in the assay and the resulting picogram determinations were divided by the protein concentration. The resulting values were expressed as pg/mg of total protein from each cellular fraction.

### Western blot analysis

Western blot analysis was used to detect the presence of non-cleaved and cleaved active forms: (35 and 37 kDa) of caspase-9 protein in cardiac tissues. In brief, total proteins were extracted from 20 mg of frozen ventricular tissues of selected patients using All Prep DNA/RNA/Protein Mini Kit following the manufacturer’s instructions (Qiagen, France) and 30 μg of total extracted proteins were subjected to SDS/PAGE with appropriate concentrations of polyacrylamide and then transferred onto a Hybond-P membrane under semi-dry conditions by means of a Transfer-blot SD Semi-dry transfer cell (Bio-Rad, Marnes-la-coquette, France). The membranes were incubated with a rabbit anti-(human caspase-9) Ig (Cell Signaling Technology, Beverly, MA, USA), or rabbit anti-beta actin IgG (Santa Cruz Biotechnology, Heidelberg, Germany) for 16 h at 4°C. After washing, the membrane was incubated with 1:1000 diluted peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. After washing, the peroxidase activity on the membranes was detected by a chemiluminescence method using an ECL® (enhanced chemiluminescence) Plus kit (Amersham Biosciences) and exposed to X-ray films (Kodak, Rochester, NY, USA). The amounts of protein were quantified by densitometric scanning of X-ray films using a Gel doc analyser system (Bio-Rad, Marnes-la-Coquette, France). For each sample, the results were analysed using the quantity one 1-D analysis software (version 4.6.1, Bio-Rad, France, 2000) and expressed as the ratio of OD.

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**Figure 1** Levels of apoptosis in ventricular myocytes. (A) Mean number (± SD) of ventricular myocytes positive by TUNEL assay in the different subgroups of study subjects (see Introduction). *P < 0.001 vs. values from healthy heart controls. (B) TUNEL and PCR-mediated ligation assays in ventricular cardiac sections: (B1) positive TUNEL assay the ventricular heart tissue section of an acute myocarditis patient (magnification ×100). (B2) the arrows indicate the presence of positive stained apoptotic nucleus observed in several myocytes of the ventricular tissue section of a patient with an EV-related myocarditis (positive TUNEL assay; magnification ×400). (B3) Negative TUNEL assay in a healthy heart control patient (magnification ×100). (B4) Agarose gel electrophoresis (1.2% w/v) revelation of PCR-mediated ligation assay allowing to detect the presence of apoptotic DNA fragments (multiple of 200 bp) in endomyocardial tissues: T-, negative control; lane-1, non EV-related myocarditis case; lane-2, EV-related myocarditis case; lane-3, healthy heart control; lane-4, EV-related myocarditis case; lane-5, non-EV-related myocarditis case.
values of cleaved/uncleaved caspase-9 protein and were normalized comparatively to the OD values of β-actin.

**Detection of Bax and Bcl-2m RNA and protein expression levels in cardiac tissue sections**

Serial sections of frozen myocardial tissue and, for viral-infected samples, adjacent in location to those found positive for viruses by PCR test, were analysed. A quantitative detection of Bax/Bcl-2 mRNA ratio was assessed by real-time RT–PCR and the results were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as relative ratio. Onto the same cardiac tissues, the immunohistochemical detection of Bax and Bcl-2 proteins in cardiac tissues was performed as described previously. For each of the four tissue sections, the number of positive cells was counted and the results were expressed as a mean per 10^5 myocytes.

**Statistical analysis**

The Spearman’s rank correlation was used to evaluate linear associations between the number of VP1 positive cardiomyocytes and the number of TUNEL positive cardiomyocytes in the same tissue area. Linear regression analyses on log-transformed values were carried out using the Stat-View SE 1 software (Abacus Concepts, Inc., Berkeley, CA, USA). Student’s t-tests and Mann–Whitney tests were carried out when necessary with the SAS software, version 8.2 (SAS Institute, Cary, NC, USA). Results were considered as statistically significant for two-sided P-values < 0.05.

**Results**

**Clinical and histological features of patients**

In patients with acute myocarditis, we observed that the mean histological score of lesions was significantly higher in EV-positive than in EV-negative subgroup of patients (P = 0.03, Student’s t-test), whereas all of the clinical parameters were statistically equivalent between these two subgroups (Table 1).

**Levels of apoptosis in ventricular myocytes**

The number of apoptotic myocytes was determined by TUNEL assay and appeared to be significantly higher in EV-related than in non-EV-related myocarditis cases (205 ± 25 vs. 140 ± 15 per 10^5 myocytes; P < 0.001, Student’s t-test) (Figure 1A and B). Moreover, in EV-positive patients, the number of apoptotic myocytes was significantly higher in those with a sudden cardiac death related to a severe or fulminant myocarditis than in those with a ventricular assistance and who were alive at the time of cardiac tissue sampling (190 ± 20 vs. 95 ± 10 per 10^5 myocytes, P < 0.001, Student’s t-test) (data not shown). For each ventricular tissue sample tested by TUNEL assay, the presence or the absence of apoptotic ventricular myocytes was confirmed using the ligation-mediated PCR assay (Figure 1B).

**Enterovirus replication activity and apoptosis in ventricular myocytes**

This part of the study was focused on the subgroups of patients exhibiting an acute myocarditis (n = 15) associated with the presence of EV infection as demonstrated by the presence of EV genome by PCR assay. Figure 2B illustrates representative findings in heart sections from patients with EV-associated acute myocarditis by using markers of enteroviral replication (detection of VP1 protein) or apoptosis (TUNEL assay). In patients with EV-related acute myocarditis, the number of VP1 positive myocytes was correlated to the number of positive myocytes by TUNEL assay (r = 0.58, P = 0.003) (Figure 2B).

**Enterovirus replication activity and activation of cytochrome c/caspase-9 pathway in cardiac tissues**

To further assess the impact of EV cardiac replication activity on the endomyocardial mitochondrial pathway in patients with acute myocarditis, the efflux of cytochrome c from the mitochondria was assessed both by ELISA and immunohistochemistry assays. We observed that levels of cytochrome c release in cytosol were significantly higher in EV than in non-EV-related myocarditis cases as demonstrated by ELISA assay of the cardiomyocytes subcellular protein fractions (P < 0.001, Student’s t-test) (Figure 3A). Moreover, immunohistochemistry assay of cytochrome c in cardiac tissues sections evidenced a higher positive immunostaining in EV than in non-EV-related myocarditis patients (Figure 3B).

To explore the impact of cytochrome c cytosolic release from mitochondria and the consequence of the apoptosome cytosolic complex onto the activation of caspase-9 protein, levels of cleaved caspase-9 protein were determined by western blotting analysis of ventricular tissues of all study subjects (Figure 3C and D). Levels of cleaved caspase-9 protein were significantly increased in EV than in non-EV-related myocarditis patients (P < 0.001, Student’s t-test). These findings demonstrated an EV-related modulation of the caspase-9 protein activation levels in patients with active myocarditis.

**Detection of Bax and Bcl-2 mRNA and protein expression levels in cardiac tissue sections**

Finally, we assessed the impact of EV cardiac replication activity onto the Bax and Bcl-2 mRNA and protein expression levels in cardiac tissue sections of patients suffering from acute myocarditis (Figure 4). Interestingly, Bax/Bcl-2 mRNA ratio normalized with GAPDH was significantly increased in EV than in non-EV-related myocarditis patients (P < 0.001, Student’s t-test) (Figure 4A). Figure 4B shows an example of the distribution of Bax and Bcl-2 protein expression in the cardiac sections of EV-related myocarditis patients. The mean number of cardiomyocytes expressing the Bax pro-apoptotic protein appeared to be significantly higher in EV than in non-EV-related myocarditis cases (152 ± 25 vs. 90 ± 10 per 10^5 myocytes; P < 0.01, Student’s t-test) (data not shown).
Discussion

The pathogenesis of EV infection is based on the ability of these viruses to kill host cells directly or to establish a viral persistence in target cells. One of the potential mechanisms by which EV-infected cells die is apoptosis. Understanding how EVs are capable of modulating host apoptotic balance in human myocytes is of major interest for the development of new therapeutic strategies against acute EV-related cardiac diseases and therefore for the control of the viral spread in cardiac tissues. In the present study, we assessed for the first time the impact of cardiac EV replication activity on the mitochondrial (intrinsic) apoptotic pathway in cardiomyocytes of patients with an acute myocarditis related to an acute EV cardiac infection. For a specific evaluation of the impact of EV infection on the modulation of the mitochondrial apoptotic pathway of human myocytes, EV cardiac infected patients were compared with adult patients with non-EV-induced myocarditis and with healthy heart subjects who were young adult organ donors free of any known viral cardiac infection and heart disease (Table 1).

In the present study, 15 of 25 (60%) acute myocarditis patients were positive for EV genome detection by molecular assays, whereas the others were suffering from adenovirus (n = 4), parvovirus B19 (n = 4), and influenza A (n = 2) related acute myocarditis (Table 1); this high prevalence of EV cardiac detection could be explained because we used four cardiac tissue samples per patient for our viral PCR detection assays. In these patients with acute myocarditis, we observed that the number of apoptotic cardiomyocytes was significantly higher in EV than in non-EV-associated cases, and that this number was correlated with that of VP1 positive cardiomyocytes (r = 0.58, P = 0.003) (Figures 1 and 2). Moreover, endomyocardial ratio of cytosolic/mitochondrial cytochrome c concentrations and levels of active caspase-9 protein were significantly increased in EV than in non-EV-related myocarditis patients (P < 0.001) (Figure 3). Taken together, these findings evidenced the contribution of mitochondrial apoptotic pathway in the pathogenesis of EV-related myocarditis, and suggested that an EV cardiac active replication could directly enhance levels of cardiomyocytes apoptosis through an up-regulation of the cytochrome c/caspase-9 pathway in myocarditis patients.

In the subgroup of patients with EV-associated acute myocarditis, the mean histological scores and the total number of apoptotic myocytes were significantly higher in patients having experienced a sudden cardiac death related to a fulminating myocarditis, than in those who were alive at the time of tissue sampling (heart transplantation). This observation suggests a relationship between the severity of the histological lesions, the death of cardiomyocytes, and its impact on heart failure. In support of this finding, some clinical and experimental studies have demonstrated that the EV-induced death of myocytes has a deleterious effect on ventricular haemodynamic and could cause sudden cardiac death by heart failure. Moreover, murine models provided conclusive evidence that a diffuse cardiomyocyte death is capable of leading to DCM, suggesting that the number of apoptotic cardiomyocytes in the acute phase of myocarditis could be in part responsible for the disease progression from acute myocarditis to DCM. Taken together, these findings suggest that the apoptotic levels based on in situ DNA nick end-labelling assay on endomyocardial biopsies may be related with the severity of heart disease.

Figure 2. Viral and apoptosis markers in myocardial tissue of patients with EV-associated active myocarditis. (A) Viral and apoptosis markers in serial sections of myocardial tissue from a patient with acute myocarditis (panel 1, VP1 protein; panel 2, DNA fragmentation detected by TUNEL assay; magnification x400). (B) Correlation analysis between the number of VP1 positive cardiomyocytes and the number of TUNEL positive cardiomyocytes in the same ventricular tissue area of patients with EV-associated acute myocarditis (n = 15).
failure and predictive of the disease progression towards chronic myocarditis and DCM. This interpretation is supported by the statistical correlation observed in this study between the number of apoptotic cardiomyocytes and those found positive for VP1 capsid protein (Figure 2).

Interestingly, our results indicated that the degree of EV replication correlated very well to the degree of apoptosis (Figure 2), the ratio of cytosolic/mitochondrial cytochrome c concentrations (Figure 3A) and levels of active caspase-9 (Figure 3C). Enterovirus cardiac infection but also adenoviral and influenza viral myocardial infection can induce cardiac inflammation and related-cytopathic effects based on myocyte necrosis and apoptosis mechanisms. In addition, it has been shown that a lot of cardiotropic viruses (not only EV) activate pro-inflammatory processes within the myocardium for example via stimulation of toll-like receptors. This fits to the findings in this investigation, as in all subgroups of patients cardiac inflammation has been detected (Table 1). The cardiac inflammation is known to be an important trigger for apoptosis in patients with myocarditis. Because levels of apoptotic myocytes were significantly higher in EV than in non-EV-related myocarditis (Figure 1), we speculated that EV cardiac replication activities could directly and specifically impact on the apoptotic pathways in patients with acute myocarditis. Our findings evidence for the first time an EV-related modulation of the cardiomyocyte mitochondrial apoptotic pathway in patients with acute myocarditis (Figures 2–4). During EV-induced myocarditis, endomyocardial apoptosis can occur through the receptor- and mitochondrial-mediated pathways and it is likely that cardiomyocyte apoptosis can be triggered by immediate and direct receptor mediated pathway, by the expression of viral antigens or proteins or by the direct viral protease-induced cleavage of myocyte proteins. Therefore, direct or indirect EV-induced cellular or inflammatory mechanisms could explain the up-regulation of the mitochondrial apoptotic pathway in infected cardiomyocytes during the active myocarditis phase. In our study, we evidenced that the increases of the

**Figure 3** Detection of the efflux of cytochrome c from the mitochondria and of the activation of cytochrome c/caspase-9 pathway in cardiac tissues. (A) ELISA quantitative detection of cytochrome c protein in cardiac tissues of study subjects. The cytosolic and mitochondrial fractions were quantified by in duplicate using the human cytochrome c ELISA Kit following the manufacturer’s instructions (Assay Designs, Ann Arbor, MI, USA). Hela 229 cells were used as negative control; Hela 299 cells treated by actinomycin D were used as positive apoptotic cells control. The resulting values were expressed as pg/mg of total protein from each cellular fraction. *p < 0.001 vs. values from healthy heart controls. (B) Immunohistochemical detection of cytochrome c protein. Serial sections of myocardial tissue fixed in PFA and, for EV-infected samples, adjacent in location to those found positive for EV by PCR test were tested for the immunohistochemical detection of cytochrome c protein. (B1) EV-negative myocarditis, (B2) EV-positive myocarditis case, (magnification ×400). (C) After classical western blot analysis, the amounts of protein expression were quantified by densitometric scanning of X-ray films using a ‘Geldoc Analyser System’ (Bio-Rad, France). For each sample, the results were expressed as the ratio of OD values of cleaved/uncleaved caspase-9 protein and were normalized comparatively to the OD values of β-actin. *p < 0.001 vs. values from healthy heart controls. (D) Western blot analysis of cardiac tissues of study subjects: visualisation of β-actin protein and uncleaved and active forms (35 and 37 kDa) of caspase-9 protein in cardiac tissues of EV- and non-EV-infected myocarditis patients by SDS–PAGE analysis.
cytochrome c efflux in the cytosol of EV infected patients could be partly related to an up-expression of the Bax protein resulting finally into the cleavage of caspase 9 (Figures 3 and 4). These results are in agreement with previous experimental studies that identified a causal link between an endomyocardial over-expression of pro-apoptotic mitochondrial Bax family proteins and mechanism of heart failure in transgenic mice models.14,28 Altogether, these findings raise the possibility that inhibition of this cell death process may constitute the basis for novel therapies.

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Contributors

L.A. and T.B. designed the study: L.A. supervised the virological, histological, and immunohistochemical components, interpreted the data and their analysis; L.A. wrote the manuscript. L.V. supervised the histological components, performed the major part of the immunohistochemical analysis, and participated in the writing of the manuscript. F.R. performed the western blot analysis and the ELISA assays. F.D.-A. performed a part of the immunohistochemical and virological analysis. G.L.G.M. designed the study, selected, and classified the patients into the different pathological subgroups. J.-F.M. developed some of the immunohistochemical techniques used in this study to analyse the heart tissue samples. M.P. selected some of the patients’ cases and interpreted the immunohistochemical data. B.P. initiated the study, assisted in the study design, and contributed to the redaction of the manuscript.

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