No Evidence for Persistent Enterovirus Infection in Patients with End-Stage Idiopathic Dilated Cardiomyopathy

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Persistence of enteroviruses in heart tissue has been implicated in the etiology of idiopathic dilated cardiomyopathy (IDC). Therefore, we determined the prevalence of enterovirus RNA in heart tissue from patients with end-stage IDC. During heart transplantation, 287 transmural biopsy specimens were aseptically collected from the explanted hearts of 38 patients with IDC and of 39 patients with cardiac failure of known cause. A nested polymerase chain reaction with general specificity for enteroviruses was used to screen for the presence of enterovirus RNA in the heart tissue samples. No enterovirus RNA was detected in any of the 287 heart biopsy specimens. These findings lead to a conclusion that enteroviruses do not persist in heart tissue from patients with end-stage IDC, nor with other heart diseases of known cause.

Ischemic heart disease is the most common cause of cardiac failure, and the next most common is dilated cardiomyopathy, with an estimated annual incidence of 5/100,000. Dilated cardiomyopathy is characterized by dilation of one or both ventricles and impaired systolic function. Microbial infection, alcoholism, autoimmunity, genetic predisposition, toxins, drugs, and nutritional deficiencies have been implicated in the pathogenesis of dilated cardiomyopathy [1]. In the majority of patients, however, the cause of the disease remains unidentified, and the condition is referred to as idiopathic dilated cardiomyopathy (IDC). The disease affects relatively young people (average age, 45 years), and in the end stage of the disease, the only effective therapy is heart transplantation. An attempt to elucidate the etiology of IDC is therefore warranted.

It is believed that myocarditis precedes dilated cardiomyopathy. The most important cause of myocarditis is a viral infection, in the majority of cases caused by an enterovirus and in particular a Coxsackie B virus. The genus Enterovirus belongs to the family of picornaviruses: small, nonenveloped, cytoplasmic viruses that contain a 7.5-kb single-stranded RNA genome with positive polarity. Enteroviruses are common human pathogens that usually cause asymptomatic or mild infections. However, enterovirus infections may also cause severe disease, including myocarditis, meningoencephalitis, pancreatitis, hepatitis, and poliomyelitis [2].

Retrospective serologic data indeed support an involvement of enteroviruses in both myocarditis and in dilated cardiomyopathy [3]. However, isolation of (entero)viruses from patients with myocarditis is rare, particularly in a later stage of the disease. Numerous research groups have applied molecular techniques to detect enterovirus genomic RNA in heart tissue in order to provide more definitive proof for the role of (persistent) enterovirus infection in the pathogenesis of IDC. Unfortunately, the results from these studies were often variable and conflicting [4–12]. The discrepancies in the experimental data between research groups have been attributed either to a lack of specificity or sensitivity of the experimental designs or to actual differences in the various populations that were studied. The degree of specificity is largely determined by the choice of primer and probe sequences. Sequences selected from the highly conserved 5’-noncoding region of the enterovirus genome proved to have general specificity and are currently widely used for the detection of enteroviruses.

We have scrutinized hearts from IDC patients for the presence of enterovirus RNA. We used a sensitive nested polymerase chain reaction (PCR) assay with general specificity for enteroviruses [13] and tested multiple transmural heart biopsy samples obtained immediately after cardiac explantation. Viral cultures were performed on throat swabs, fecal swabs, and heart tissue samples from each patient to rule out the possibility that acute infections confounded the results.

Patients and Methods

Patients. Between February 1994 and July 1996, 77 patients with end-stage heart failure undergoing cardiac transplantation were included in this study. Thirty-eight patients had a diagnosis of IDC. The hearts of these patients had dilated, diffusely hypokinetic...
Table 1. Baseline characteristics of the heart transplantation patients.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient group (n = 38)</th>
<th>Comparison group (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnoses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic dilated cardiomyopathy</td>
<td></td>
<td>Ischemic heart disease (n = 32)</td>
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<tr>
<td>Hypertrophic cardiomyopathy (n = 3)</td>
<td></td>
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<tr>
<td>Congenital heart failure (n = 3)</td>
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<tr>
<td>Valvular heart disease (n = 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myxoma cordis (n = 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>22/16</td>
<td>32/7</td>
</tr>
<tr>
<td>Average age (range), years</td>
<td>47 (14–63)</td>
<td>52 (32–65)</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td>4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Median, years</td>
<td>1 month to &gt;10 years</td>
<td>3 months to &gt;10 years</td>
</tr>
<tr>
<td>Range</td>
<td></td>
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</table>

chambers, left ventricular ejection fraction <20%, and a normal or decreased wall thickness. These patients had no signs of obstructive coronary artery disease, alcohol abuse, congenital heart disease, primary valvular heart disease, systemic disease, or insulin-dependent diabetes mellitus. The comparison group consisted of 39 patients with cardiac failure of known cause. The baseline characteristics of all patients are summarized in table 1. None of the patients in either group showed clinical signs of an active microbial infection at the time of sampling.

Clinical specimens. Multiple transmural biopsy specimens were collected under aseptic conditions from explanted hearts during cardiac transplantation at the University Hospitals of Utrecht and Rotterdam. Two myocardial samples from the left ventricle and 2 samples from the right ventricle were cut out near the apex (average sample size, 0.3 cm³). Each specimen was split into 3 sections, representing the endocardial, intramural, and epicardial part of the ventricular tissue. These samples were directly snap-frozen in liquid nitrogen and subsequently stored at −80°C until further processing. Two additional heart biopsy samples from the left and right ventricles plus throat and fecal swabs were collected from each patient and placed in virus transport medium for routine virus isolation procedures.

RNA isolation, reverse transcription (RT), and PCR. To prevent cross-contamination, the set of heart biopsy specimens from each patient was processed individually, 1 set per day. Three rat heart specimens were processed in parallel with each patient set as a negative control for the enterovirus-specific PCR. From each heart tissue section, 20 mg was homogenized with a disposable tissue grinder. RNA isolation, RT, and subsequent nested PCR amplification (40 cycles in each assay) were performed as described [14].

To identify false-positive results, all enterovirus-specific amplification reactions were run in duplicate. Specimens exhibiting only a single positive reaction were suspected of being false-positive and were rerun. Two positive controls, coxsackievirus B3 (CVB3) RNA and a CVB3 cDNA clone, were included in each PCR assay. In addition, a β-actin PCR [15] was applied to each RNA sample to evaluate the quality and the amplifiability of the extracted RNA.

The amplification reactions were assessed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Southern blot analysis was subsequently performed with a nonradioactive probe that was 3'-end-labeled with digoxigenin-11-ddUTP (DIG-11-ddUTP; Boehringer Mannheim, Mannheim, Germany). The sequence of the oligonucleotide probe (5'-TGTGTCGTAACGGCAACTCTGCAGCGGA-3') was complementary to an internal portion of the amplified product. Hybridization and detection of the DIG-labeled probe was accomplished following the guidelines in the manufacturer’s manual.

Results

Throat swabs, fecal swabs, and left and right ventricle biopsy specimens from each patient were all negative by virus isolation for enteroviruses. Herpes simplex virus type 1 was isolated from throat swabs of 2 patients with ischemic heart disease. No other viruses were isolated.

RNA was extracted from a total of 287 myocardial tissue specimens collected from the explanted hearts of 77 patients with end-stage heart failure. β-actin mRNA was successfully amplified in all samples, indicating that the RNA isolates did not contain inhibitors (figure 1). In each PCR assay, amplification products were obtained from the positive control samples. All heart tissue samples from the 77 patients were negative for enterovirus RNA. Incidentally, an enterovirus-specific PCR product was obtained. However, such amplicons were never produced in both duplicate reactions of a sample or upon repeating the PCR with the same cDNA source. These inconsistent findings were attributed to contamination, which is stressed by the fact that sometimes a positive result was obtained from negative rat heart samples.

Discussion

The aim of this study was to investigate the possible association between enterovirus persistence and the development of IDC. We examined multiple transmural myocardial specimens from explanted hearts of 38 patients with IDC and 39 patients with cardiac failure of known etiology. No enterovirus RNA was detected in any of these samples.

Our findings are in contrast with experimental data reported by Kandolf [5] and Bowles et al. [6], who used in situ nucleic
acid hybridization and a slot blot hybridization assay, respectively, to detect enterovirus RNA in explanted heart tissue. They both found a positivity rate of 30% in patients with IDC and 15% in patients with other cardiac disorders. In numerous subsequent studies on explanted hearts, PCR was used. Weiss et al. [7] detected enterovirus RNA in cardiac tissue of 45% of patients with IDC, as well as in myocardium of 38% of patients with other cardiac conditions. Andreoletti et al. [8] obtained similar results; enterovirus-specific amplicons were produced in heart tissue samples from 11 of 19 patients with dilated cardiomyopathy and from 8 of 14 patients with chronic coronary cardiomyopathy. A low prevalence of enterovirus-specific sequences in myocardium from patients with end-stage dilated cardiomyopathy (4%) and other cardiac diseases (8%) was reported by Muir et al. [9]. The experimental data from these research groups suggest that, although enterovirus RNA sequences may be found in the myocardium of some patients with end-stage heart disease, its presence is not specifically associated with IDC.

Our experimental data, however, cast great doubt on the persistence of enterovirus RNA in heart tissue of patients with heart failure. Our results are in agreement with the findings of Grasso et al. [10], who did not detect enterovirus RNA in biopsy samples from explanted hearts of patients with IDC or patients with cardiac failure of other causes. Similarly, Griffin et al. [11] studied autopsy specimens from 28 patients with dilated cardiomyopathy and did not find enterovirus RNA sequences in any of these myocardial samples. Giacca et al. [12] patients with other cardiac disorders. In numerous subsequent studies on explanted hearts, PCR was used. Weiss et al. [7] detected enterovirus RNA in cardiac tissue of 45% of patients with IDC, as well as in myocardium of 38% of patients with other cardiac conditions. Andreoletti et al. [8] obtained similar results; enterovirus-specific amplicons were produced in heart tissue samples from 11 of 19 patients with dilated cardiomyopathy and from 8 of 14 patients with chronic coronary cardiomyopathy. A low prevalence of enterovirus-specific sequences in myocardium from patients with end-stage dilated cardiomyopathy (4%) and other cardiac diseases (8%) was reported by Muir et al. [9]. The experimental data from these research groups suggest that, although enterovirus RNA sequences may be found in the myocardium of some patients with end-stage heart disease, its presence is not specifically associated with IDC.

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Some of the positive findings reported in the literature could have resulted from acute infections that may have a confounding effect on the outcome of the PCR assay. In this study, virus isolation was performed on throat swabs, fecal swabs, and single left and right ventricle specimens from each patient. All 77 patients were negative for enterovirus, which makes it unlikely that acute enterovirus infections in patients with end-stage heart failure can explain positive PCR results as found in other studies.

The variability in detecting enterovirus RNA sequences in explanted heart tissue of patients with end-stage heart disease may be due to actual differences in the populations that have been studied. It is possible that the duration of illness from onset to heart transplantation affects the study. It might be that
enterovirus RNA can be found only in patients who rapidly progressed toward an end stage of disease. Unfortunately, the actual onset of the heart disease is often unknown, which makes it difficult to determine and compare the duration of illness between patients.

Our experimental approach was designed to achieve optimal conditions for the detection of minimum amounts of enterovirus RNA present in the explanted hearts. Upon estimating the sensitivity of our nested PCR assay, ~10 enterovirus genome equivalents were detectable. The general specificity of our PCR primers has previously been demonstrated [13]. Therefore, it is unlikely that the negative outcome of our study is due to a lack of sensitivity or specificity. This study is one of the few in which multiple transmural heart biopsy samples were tested from each patient. To further minimize the risk of sampling error, each biopsy specimen was subdivided into 3 sections for subsequent PCR analysis. Our PCR primers have been used by others who obtained positive results from a variety of clinical specimens, including single, small endomyocardial biopsy samples. Successful amplification of the internal control mRNA in all samples excluded the likelihood of negative PCR results caused by inhibition. We were able to recognize false-positive amplification reactions since we ran all PCRs in duplicate. Single positive reactions were not considered as true positives. This was confirmed by the fact that these results were not reproducible, even though the same cDNA source was used as in the first PCR assay.

In conclusion, we did not find any evidence for the persistence of enterovirus RNA in heart tissue from patients with end-stage IDC. This is in line with previous experimental data that demonstrated that enteroviruses do not persist in other chronic diseases, such as postviral chronic fatigue syndrome, polymyositis, and postpolio syndrome [4]. Although some research groups detected enterovirus RNA sequences in myocardium from patients with end-stage heart failure, these were not specifically associated with IDC. If it exists, persistent enterovirus infection cannot be held responsible for the maintenance of the disease process in IDC. It does not exclude, however, the possibility that an enterovirus infection may initiate a pathologic process that eventually will lead to IDC. An infection or another event that damages the heart may in some individuals lead to extensive, uncontrolled fibrotic changes that eventually cause dilatation of the heart. Pathohistologic characterization of the explanted heart tissue of our IDC patients is currently under investigation.

References