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RESEARCH

The Role of Viral Infections in the Development of Dilated Cardiomyopathy

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Abstract Enteroviruses (EVs) are the most frequent pathogens in myocarditis and in the subsequently developing dilated cardiomyopathy as well. Furthermore, persistence of other viruses might play a pathogenic role in the evolution from myocarditis to dilated cardiomyopathy. Explanted heart of 28 patients, who underwent heart transplantation were screened for EV, AdV3 and HHV6 sequences in order to assess the incidence of cardiac viral infection that may be implicated in the pathogenesis of cardiomyopathy, and estimate viral distribution in the myocardium. Viral sequences were extracted from five different regions of the hearts. Nested PCR was used to amplify conservative regions of AdV3, HHV6 and EVs. Histological examination was performed on routinely processed myocardial samples. AdV3 was verified in one fourth of the patients. ADV3 and HHV6 sequences coexisted in one case with inflammatory cardiomyopathy. Some patients had more than one positive area of their heart. AdV3 positive right ventricular samples were double in amount compared to the left ones. None of the patients had positive result for EV. This is the first occasion to identify AdV3 (a mainly respiratory infective virus) sequence in explanted hearts of cardiomyopathy patients. Though the clinical importance of our results is still unclear, AdV3 could be a new member of the viral group with possible pathogenic effect on the myocardium. Regional distribution of viral sequence location confirmed that the right ventricular wall as a biopsy sampling site might be adequate for endomyocardial biopsy pro diagnostic purposes.

Keywords Cardiomyopathy · Endomyocardial biopsy · Myocardium · Polymerase chain reaction · Viruses

Abbreviations

AdV2	Adenovirus type 2
AdV3	Adenovirus type 3
AdV5	Adenovirus type 5
CM	Cardiomyopathy
DCM	Dilated cardiomyopathy
DNA	Deoxyribonucleic acid
EMB	Endomyocardial biopsy
EV	Enterovirus
HHV6	Human Herpes Virus type 6
MC	Myocarditis
NCBI	National Centre for Biotechnology Information
neg.	negative result
PCR	Polymerase chain reaction
PTAH	Phosphotungstic acid-haematoxylin
PVB19	Parvovirus B19
RNA	Ribonucleic acid
WHO	World Health Organization

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Introduction

Cardiomyopathies (CMs) were first classified by Goodwin in 1972, based on a functional view, resulting in three main categories: hypertrophic, congestive/dilated and restrictive/obliterative CMs [1]. The World Health Organization

(WHO) defined CMs in 1980 as "heart muscle diseases of unknown cause," to distinguish cardiomyopathy from cardiac dysfunction due to known entities [2]. In 1995, WHO redefined CMs as diseases of the myocardium associated with cardiac dysfunction and classified them by the dominant pathophysiology or aetiological/pathogenic factors, separating dilated, hypertrophic, arrhythmogenic right ventricular, restrictive, unclassified and inflammatory (including myocarditis) CMs. The term specific CM was introduced, including ischaemic, valvular, hypertensive, metabolic etc. CM [3].

Accumulating data has revealed an important inflammatory component in the pathogenesis of dilated CM, and there is increasing evidence, that myocarditis (MC) and dilated CM is related [4]. Predominantly, viral infections may be responsible for acute MC in children and young adults [5]. EVs and in the past 10 years AdVs belonging to C serotype have been considered the most common pathogens of inflammatory CM (MC) [5–8]. Later on, other viruses, as HHV6 and Parvovirus B19 (PVB19), have been implicated in dilated CM as potential infective agents [9]. Both viral infections could lead to fatal MC [10–12]. Species C of AdVs are commonly involved in respiratory diseases in the paediatric population [13], as well as the B serotype AdV3, which may lead to clinical symptoms as conjunctivitis, pharyngitis, gastrointestinal signs [14], and may increase the risk for chronic lung disease [15].

In the majority of cases MC remains hidden without showing any specific symptoms [6] and still its pathophysiology remains incompletely understood [16]. Virus infection may induce ventricular dilatation and insufficient pump function as a late sequel of dilated CM by viral persistence [8, 17], or by chronic immune process [8, 17].

The diagnosis of MC has been based on serology and viral culture, that methods have low specificity or sensitivity in general [6]. Additionally, endomyocardial biopsy (EMB) was introduced as a diagnostic tool. Currently, due to deoxyribonucleic acid (DNA) diagnostics, the threshold of virus detection has considerably increased using in situ hybridization techniques and polymerase chain reaction (PCR) [6, 18].

Most of the earlier studies used EMB samples to demonstrate virus sequences in the myocardium [5, 19–21], although only a small percentage of multiple samplings were performed on explanted hearts [22, 23].

Recent studies, using PCR and more specific probes, reported around 35% of viral presence in the myocardium, although these data might underestimate the viral incidence as a result of possible sampling error [4]. Consequently, the question arouse, whether there is any approach to specify precisely the best sampling area.

The present retrospective study aims to screen for the conservative regions of AdV3, HHV6, and EV, and examine the viral distribution in various regions of the CM hearts.

Materials and Methods

Permission to conduct this study was granted by the Regional Scientific and Research Ethical Committee of the Semmelweis University, # 68/2005.

Patient Population

Myocardial samples from explanted hearts of 28 patients (14 dilated, 2 inflammatory and 12 ischaemic CM), who underwent heart transplantation at the Vascular- and Cardiac Surgery Clinic of the Semmelweis University, were obtained between November 2005 and July 2007. Among the patients 23 were male and 5 were female, their mean age was 45.71±13.15 years (Table 1). Myocardial samples from five regions of the hearts (right and left anterior and posterior wall, and left apical region) were stored at −80°C until further processing.

Control Population

The control group consisted of 20 individuals who died suddenly as a result of accident or suicide.

DNA Extraction

DNA extraction from the myocardial samples was performed using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Approximately 20 mg of the myocardial tissue were placed into 1.5 ml sterile tube with 180 µl ATL buffer and 20 µl Proteinase K. Overnight incubation at 55°C was performed until complete digestion occurred, and further steppes followed the manufacturer's instruction.

RNA Extraction

Frozen myocardial samples were homogenized in liquid nitrogen then total ribonucleic acid (RNA) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described in the user manual. Reverse transcription of RNA samples were performed with Superscript kit (Invitrogen), according to the provided description.

Viral Sequence Detection by Nested PCR

The AdV3 hexon, the HHV6 alkaline exonuclease and the EV 5′ non-coding regions were amplified by nested PCR (Maxim Biotech. Inc., Rockville, MD, USA). The amplification was performed in a volume of 25 µl, using Applied Biosystems GeneAmp PCR System 9700. Electrophoresis was used to detect PCR products on 1.75 % agarose gels stained with ethidium bromide. Kodak Image Station 4000 MM apparatus took the gel picture.

Table 1 Clinical data and AdV3 results of CM patients

	Sex	Age (Years)	Origin of CM	RAW	RPW	LAW	LPW	LVA
1	Male	57	dilated	neg.	neg.	neg.	neg.	neg.
2	Male	51	dilated	neg.	neg.	neg.	neg.	neg.
3	Female	33	ischaemic	neg.	neg.	neg.	neg.	neg.
4	Male	37	inflammatory	neg.	neg.	neg.	neg.	neg.
5	Male	51	dilated	neg.	neg.	positive	neg.	neg.
6	Female	53	dilated	neg.	neg.	neg.	neg.	neg.
7	Male	35	dilated	neg.	neg.	neg.	neg.	neg.
8	Male	17	dilated	neg.	neg.	neg.	neg.	neg.
9	Female	53	ischaemic	neg.	neg.	neg.	neg.	neg.
10	Male	40	ischaemic	neg.	neg.	neg.	neg.	neg.
11	Male	49	ischaemic	positive	neg.	neg.	neg.	neg.
12	Male	41	ischaemic	neg.	neg.	neg.	neg.	neg.
13	Male	59	dilated	neg.	neg.	neg.	neg.	neg.
14	Female	25	inflammatory	positive	neg.	neg.	positive	neg.
15	Male	54	ischaemic	neg.	neg.	neg.	neg.	neg.
16	Male	28	dilated	positive	positive	positive	neg.	neg.
17	Male	56	dilated	neg.	positive	neg.	neg.	neg.
18	Male	56	dilated	positive	neg.	neg.	neg.	positive
19	Male	49	dilated	neg.	neg.	neg.	neg.	neg.
20	Male	51	ischaemic	neg.	neg.	neg.	neg.	neg.
21	Male	47	dilated	neg.	neg.	neg.	neg.	neg.
22	Male	62	ischaemic	neg.	neg.	neg.	neg.	neg.
23	Male	62	ischaemic	neg.	neg.	neg.	neg.	neg.
24	Male	38	dilated	neg.	neg.	neg.	neg.	neg.
25	Female	19	dilated	neg.	neg.	neg.	neg.	neg.
26	Male	53	ischaemic	neg.	neg.	neg.	neg.	neg.
27	Male	65	ischaemic	neg.	neg.	neg.	neg.	neg.
28	Male	54	ischaemic	neg.	neg.	neg.	neg.	positive

LAW left anterior wall, *LPW* left posterior wall, *LVA* left ventricular apex, *RAW* right anterior wall, *RPW* right posterior wall

Direct Sequencing

To verify the positive results, PCR products were purified using High Pure PCR purification kit (Roche Co., Basel, Switzerland) according to instructions. Afterwards, 3 μl of amplification product was added to 20 μl of final volume sequencing PCR (5 μl of forward primer, 4 μl of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 2 μl of 5X sequencing buffer and 6 μl of ddH_2O). Following the sequencing reaction, DyeEx 2.0 Spin Kit (Qiagen) repeated purification. ABI PRISM 310 Genetic Analyser was used for running and Sequencing Analysis 3.7 software for assessing the results. The virus-sequence database of the National Centre for Biotechnology Information (NCBI) GeneBank was used for comparison.

Histological Evaluation

Myocardial samples of the explanted hearts after formaldehyde fixation were routinely processed, and embedded in paraffin. Afterwards 3 μm thick sections stained by Haematoxylin-eosin, van Gieson, Azan and Phosphotungstic acid-haematoxylin (PTAH) slides were examined by Nikon Eclipse E400 light microscope. Microphotographs taken with Nikon Coolpix E4500 digital camera were adjusted with ACDSee and Photoshop software to publish.

Results

PCR Analysis

AdV3 summarized results are demonstrated in Table 1. AdV3 sequence was identified in one fourth of the patients. Samples with present AdV3 sequence was scattered in different regions of the heart, and positive right ventricular wall samples were double in amount compared to the left ones. Three patients had more than one positive area of their heart. Most of the patients with positive viral results had dilated CM, two had ischaemic and one had inflammatory CM.

One young female's heart, with inflammatory CM, beside the existence of AdV3 sequence in the right

anterior- and left posterior walls, revealed HHV6 sequence in the left anterior wall as well. In any of the patients, EV sequence had not been identified at all. None of the control myocardium confirmed any positive results for the probed viral sequences. Some details for AdV3 study are demonstrated on Fig. 1.

Histological Examination

Macroscopic and microscopic features of explanted ischaemic hearts were various degree of hypertrophy of the myocardium and of the myocardial cells, focal thinning of the left ventricular wall with extensive interstitial fibrosis replacing infarcted areas of the myocardium, and signs of diffuse epicardial coronary artery disease. No inflammatory infiltrates were detected in ischaemic hearts.

In cases of dilated CM, the macroscopic characteristics were classical. Microscopic examination of explanted CM hearts revealed distinctive appearance such as various degrees of pericellular fibrosis, focal interstitial fibrosis, endocardial fibrosis and myocytolysis. Chronic, mainly lymphocytic inflammatory infiltration was seen in only one case with a rather unique manifestation of endocardial thickening (loose connective tissue, capillary rich granulation tissue, focal mild chronic inflammatory infiltrate). Other findings were myocardial bridging; intramyocardial small vessel disease and hypercontraction necrosis in some cases (Fig. 2).

Discussion

Our aim in the present retrospective study was to screen for the conservative regions of AdV3, HHV6, and EV, and examine the viral distribution in various regions of the CM hearts.

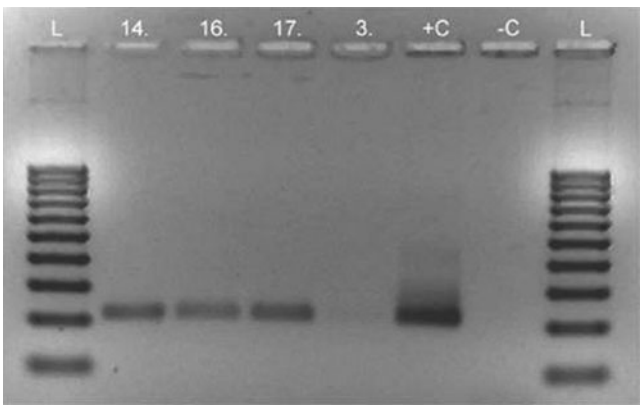

Fig. 1 Detection of AdV3 sequence (hexon region) in myocardial samples by nested PCR. PCR products were detected on 1,75 % agarose gel stained by ethidium bromide. A 100 base pair ladder (L) is in the first and last the lanes. AdV3 positive control (+C) is showed as 220 base pair amplimer, and lanes signed as 14, 16, 17 illustrate the AdV3 positive patients. Lane numbered 3 showed negative results from a patient, similar to the negative control lane (-C)

Based on studies to date, AdV2 and AdV5, HHV6 and EVs might have a role in the possible viral aetiology of MC and dilated CM [5, 20]. AdV3 that frequently occurs primarily in the respiratory tract of infected children and young adults [14], was identified for the first time in one fourth of explanted hearts of CM patients in our study. After a thorough research of the literature, we did not find any previous data described by others indicating the presence of AdV3 in heart diseases although early viral results of our first 14 cases had been published in a local paper to inform cardiologist about the newly available diagnostic method [24].

Case history of patient # 14 started with acute viral enteritis that followed by severe cardiac insufficiency in short period of time leading to urgent heart transplantation in 3 month after the onset of intestinal symptoms. She was the only one patient with two viral sequence (AdV3 and HHV6) identified. This result suggests a possible relationship between preceding viral infection (enteritis) and subsequent dilated CM. It had been already described that HHV6 is able to cause fatal MC by itself [10, 12]. In addition, when HHV6 coexist with other virus, such as PVB19 in acute MC, the clinical course could be much worse than compared with single HHV6 or PVB19 infections [25]. Others, frequently detected HHV6 B genome in explanted hearts from children with dilated CM in constantly low viral loads that suggest latent infection [9]. We suppose that in case # 14, the coexistence of the two virus-sequences in the myocardium may be responsible for the rapid development of heart failure indicating transplantation.

Among the other six AdV3 positive patients, 4 had dilated and two ischaemic CM. In patients with dilated CM, there was no histological sign of myocardial inflammation. According to Kühl and co-workers, patients with heart failure may show viral sequences in the myocardium in high prevalence without evidence of any inflammation. Although they emphasise, that routine histological analysis of EMB is too insensitive to detect myocardial inflammation accurately in the chronic phase of the dilated CM, or if only a few samples are used for routine analysis [26, 27]. Possible sampling error may explain our negative histological result for myocardial inflammatory infiltrates, although five different areas were selected. The detected uneven distribution of viral sequences at various sites of the hearts may be explained with the similar theory as well, although the question arises whether previous interferon-β treatment and consequent partial virus elimination might play an additional role [28].

Pathologically three phases separate in MC [4]. Through the first phase the myocytes are being destructed [4] by

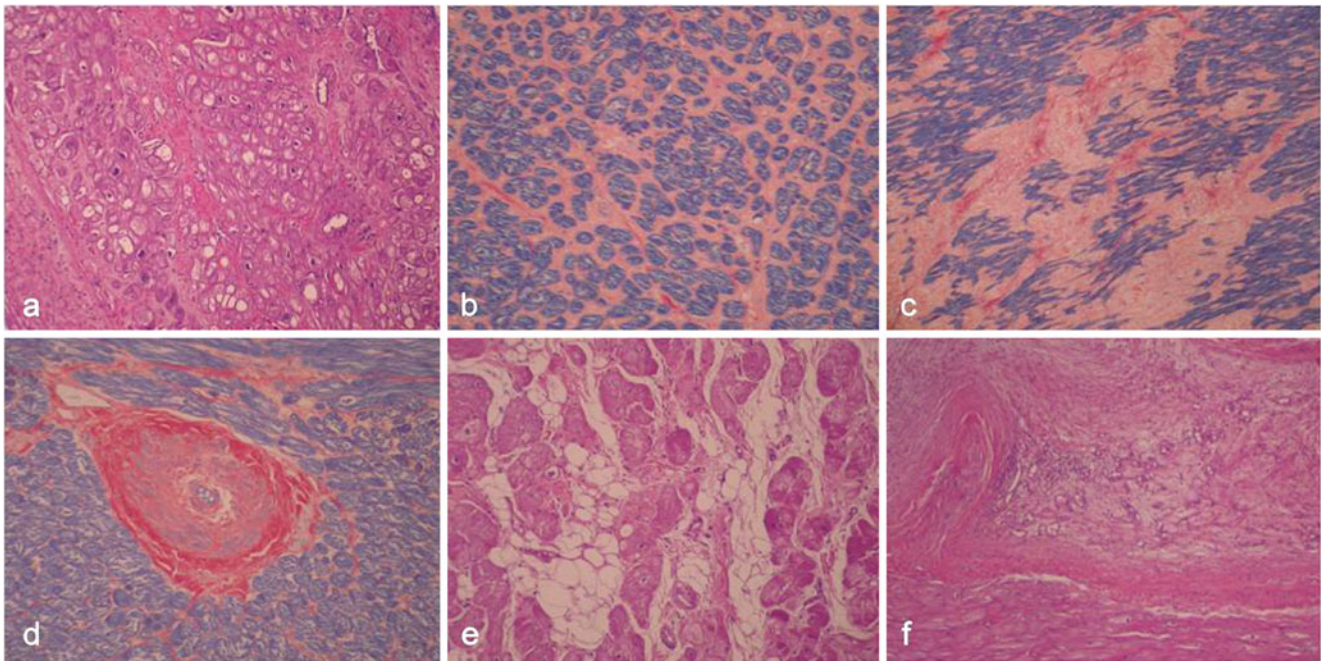

Fig. 2 Histological features of dilated CM. **a** Subendocardial myocytolysis, Haematoxylin-Eosin stain, original magnification 10×. **b** Mild interstitial/pericellular fibrosis, PTAH stain, original magnification 10×. **c** Moderate interstitial fibrosis, PTAH stain, original magnification 4×. **d** Intramyocardial small vessel disease with fibromuscular hypertrophy of the media, PTAH stain, original magnification 10×. **e** Fatty infiltrates in the left ventricular myocardium, Haematoxylin-Eosin stain, original magnification 10×. **f** Endocardial chronic inflammation, Haematoxylin-Eosin stain, original magnification 4×

virus mediated lysis and cardiac dilatation develops [29]. The second phase evolves from the inadequate cellular and humoral immune responses induced by molecular mimicry [30], or virus-induced cardio-myocyte injury generates intracellular cardiac protein release leading to myocardial damage, reparative fibrosis and final cardiac dysfunction by inflammatory cells activation [31]. Autoantibodies against cardio-myocytes were demonstrated in DCM serums [17]; most of them were directed against cardiac α- and β-myosin heavy chains [32]. Finally, in the third phase typical features of dilated CM develop from the extensive myocardial injury [4]. The described pathomechanisms suggest the importance of inflammatory and autoimmune responses in dilated CM.

On the grounds of viral sequence locations (positive right ventricular anterior and posterior wall samples were double in amount compared to the left), our results showed that around the right ventricular septum as a biopsy sampling site might be adequate for diagnostic purposes since EMB is routinely obtained from the septal region of the right ventricle as a consequence of practical considerations.

Two ischaemic CM patients showed positive result for AdV3 sequence. There are accumulating evidences that existing viral infections might have potential role in the development of atherosclerosis, for the reason that possible latent persistency in host cells and presence of viral DNA in atherosclerotic lesions [33–35]. Furthermore, EV sequence was detected in patients undergoing heart transplantation as a result of end-stage chronic coronary disease [36, 37], as well as in patients with ischaemic cardiac disease [37] that might suggest a positive relationship between ischaemic heart disease and viral infection.

In our study there was no EV sequence detected in any of the myocardial samples. This is in accordance with the results of those, who were not able to detect EV sequence in dilated CM and in patients with cardiac failure of known cause (ischaemic, hypertrophic, congenital, valvular, myxoma cordis) [38, 39]. Nonetheless, our finding for EV is contradictory to some results from the past [36, 40].

Dilated CM could have other than viral origin as well, like genetic, autoimmune or toxic . Familiar dilated CM is present approximately in 20–35 % of the dilated CM cases and genetically it is a heterogeneous group [41]. High numbers of genes are involved, mainly sarcomeric proteins as α-cardiac actin, α-tropomyosin, cardiac troponin T, I, C, β and α myosin heavy chain, myosin binding protein C. Most of the familial dilated CM cases demonstrate dominant inheritance, but X-linked and mitochondrial gene mutations exist as well [42]. Furthermore coexistence of more than one etiologic factor might lead to dilated CM, such as viral infection and genetic mutations. It is among our future plans to study the possible genetic background of these CM patients nevertheless we still have to collect more samples for statistical significance.

Moreover, whether if the identified AdV3 and HHV6 viruses have importance in the development of dilated and/or ischaemic CM, that should be carefully assessed in a prospective further study.

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