Virus identification and the expression of Coxsackie-Adenovirus Receptor in heart-transplanted cardiomyopathic patients

Ph.D. Thesis

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INTRODUCTION I.

In our days, thanks to molecular biological methods, several viruses were detected in the myocardium, which might have role in the development of myocarditis (MC) and dilated cardiomyopathy (CM).

The important role of Coxsackie viruses in MC has been known for a long time. In the past years, there have been accumulating data on the detection of Adenovirus (AdV), Human Herpes Virus 6 (HHV6) and Parvovirus B19 (PVB19) sequences in the diseased myocardium.

The cardiotropic viruses cause mostly upper respiratory complaints, although in certain cases they can induce serious clinical symptoms. About 90 % of the population is affected by these virus infections without any complication in their hearts. MC in part stays unrecognized, whereas it partly causes flu symptoms forming upper respiratory or gastro-intestinal complaints. Theoretically, the inadequate immune response results in absence of virus elimination and the spread to other organs by circulation.

It is common in acute MC that the electrocardiogram (EKG) shows abnormal signs. The sensitivity of EKG is around 47 %, although its specificity is unknown.

Nowadays, virus serology is considered out of date owing to its low sensitivity and specificity. Moreover, this method did not confirm the actual virus infection in the myocardium.

Clinicians can make a decision of the adequate treatment method on the grounds of histological examination of the endomyocardial biopsy (EMB). The conservative approach where clinicians wait for a maximum of 2 days before performing EMBs is acceptable due to a high amount of MC patients who recover spontaneously, with this invasive procedure becoming unnecessary. Another point of view exists however, according to which all MC suspected patients should have an EMB because the sensitivity of the diagnostic examination is higher in the early phase of the illness. Treatment of the disease with known etiology is more efficient in the early phase than in case of inflammation resulting in irreparable damage.

The histological evaluation of EMB from patients with MC is based on the Dallas criteria, which has a number disadvantages:

- it might result in different pathological explanation;
- avoiding sampling error is difficult;
- it ignores the cause of pathological discrepancy.

For histological diagnosis, the presence of inflammatory infiltrate with or without necrosis is required. Sporadic inflammatory infiltrate without myocyte-necrosis suggests borderline MC. The sampling area is the right ventricular septum according to current practice.

Numerous viruses were identified in the myocardium by molecular biological methods (PCR, *in situ* hybridization*)*. According to the latest results, virus sequences were identified in a rather different proportion in the myocardium of MC and dilated CM patients. The cause of differences may be the clinical aspects of patient classification and the divergent sensitivity of the applied methods.

Most frequently, dilated CM affects middle-aged men. This is not in close relation with hypertension, cigarette smoking and alcohol consumption. Several patients are asymptomatic in spite of existing left ventricular dilatation for months or for years, which is only later recognised clinically, when symptoms get worse or the dilated heart appears on the X-ray. The most common symptoms are weakness, fatigue and reduced pump-function.

INTRODUCTION II.

The Coxsackie-Adenovirus Receptor (CAR), as a cell membrane protein, is a virus receptor for Coxsackie and Adenoviruses. CAR is a 46 kDa protein, belonging to CTX (*Cortical thymocyte marker in Xenopus*) proteins with two extracellular immunoglobulin domains. The CAR expresses in the tight junctions on the epithelial cells, although its physiological function is largely unknown.

First of all, CAR was found to be expressed at high levels during the embryonic development of the brain and the heart, suggesting its considerable role in the embryonic development of these organs through maintaining cell-to-cell communication. Contrarily, CAR was expressed at low level or was absent in the brain and the heart of adult mice.

CAR-deficient mouse embryos died around the 11^{th} day of embryonic development. In the embryonic heart, enlarged pericardium, smaller lumen of the ventricles, enlarged ventricular wall and only a single atrioventricular canal were observed whereas normal embryos of the same developmental stage already contained two atrioventricular canals. These observations represented a clear sign of delayed heart development. In wild-type cardiomyocytes, averages of

12-15 sarcomers are consecutively linked to each other while sections of CAR-deficient cells contained only 4-6 sarcomers. Deletion of CAR gene during embryonic development caused disorganisation of myofibrils, proliferation of myocytes and apoptosis.

OBJECTIVES

1. With what frequency could the examined virus sequences be identified in our heart-transplanted patients?

2. Could these virus sequences appear in ischemic CM as well?

3. Could the ischemic CM patient group be considered as a control group from the aspect of virus detection?

4. Does the virus detection show some kind of pattern in the sampling of different regions of the myocardium?

5. In the virus-positive regions, could the histological examination confirm the existence of inflammation?

6. Could we demonstrate high differences in mRNA expression of CAR in our patient population compared with healthy controls, and is the result verifiable by immunofluorescence?

MATERIAL AND METHODS

- Between November 2005 and March 2008, myocardial tissue and blood samples collections from 35 heart-transplanted CM patients (16 dilated, 17 ischemic and 2 inflammatory CMs).

- DNA and RNA isolation from blood and myocardial tissue.

- Amplification of EV, AdV2 and AdV3, HHV6 virus sequences by means of nested polymerase chain reaction (PCR). Analysis of PCR products by 1.75 % agarose gel electrophoresis.

- Direct sequencing of virus-positive samples by laser capillary electrophoresis.

- Histological evaluation of myocardial samples.

- Evaluation of mRNA expression of CAR with real-time PCR.

- Immunofluorescence examination of CAR protein.

- Sequencing of CAR exons.

RESULTS

1. Virus detection by PCR

We identified AdV3 in 20 % of CM patients. The AdV3-positive samples were localized to different areas of the heart, although positive right septal samples were double in amount compared with left septal samples. In 3 AdV3-positive patients more than one area presented AdV3 sequence.

Among the virus-positive patients 4 had dilated, 2 had ischemic and 1 had inflammatory CM. Besides the existence of ADV3 sequence in the right anterior- and left posterior walls, one young female's heart, with inflammatory CM, revealed HHV6 sequence in the left anterior wall as well. EV and AdV2 sequences could not be identified at all in any of the patients. None of the control and diseased myocardium and blood samples showed positive results for the examined viruses.

2. Sequencing of virus-positive samples

The obtained electropherograms by sequencing proved that the proper virus sequences were amplified.

3. Histological examination

No inflammatory infiltrates were detected in ischemic hearts.

In cases of dilated CM, the macroscopic characteristics were classical. 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).

4. CAR mRNA expression by real-time PCR

A total of 35 heart transplanted CM patient and 10 control myocardial samples were analysed. The mean C_T of β-actin, our endogenous control, showed almost the same expression in all sample types. In our results, significant differences were detected between control and diseased population. CAR in both dilated and ischemic CM was significantly up-regulated in comparison with the healthy control group, by 8.16-fold ($p<0.0002$) and 4.33-fold ($p<0.002$), respectively. Our two inflammatory CM cases showed higher CAR mRNA expression than healthy controls but lower than dilated and ischemic CM patients, although the

differences detected did not reach the p=0.05 significance level (p=0.12 and p=0.39, respectively). We could not demonstrate age-, or gender-related differences in the mRNA expression of CAR.

5. Immunofluorescence staining of CAR

To verify our CAR mRNA expression results, we applied immunofluorescence staining. In all myocardial samples from dilated, ischemic and inflammatory CM patients higher CAR expression was demonstrated compared with control samples. CAR was located in the intercalated disc (ICD) and sarcolemma in dilated CM, in contrast with the controls, where positive reaction could be detected only in the sarcolemma. In ischemic CM, the intercalated disc (ICD) and sarcolemma showed positive reaction similar to dilated CM. Furthermore, inflammatory CM showed similar result to dilated CM with perhaps stronger positivity in the sarcolemma, although their mRNA expression did not present significant differences compared with the controls.

6. Sequencing of CAR

During direct sequencing of the 7 exons of CAR, we did not identify any mutation or polymorphism compared with the reference sequences from the database, which may be a determinant factor in progression of dilated, ischemic and inflammatory CM.

CONCLUSIONS

1. Our examinations proved the demonstration of a new type of adenovirus, AdV3, in CM myocardium. According to our hypothesis, the lack of EV and AdV2 sequences in the examined myocardium may be the conclusion of sampling error or the genetic attribute of the population.

2. We identified AdV3 sequence in the myocardium of ischemic CM patients.

3. Based on the AdV3 sequence identified in the ischemic CM myocardium samples, the ischemic patient group could not be considered as control group in our examinations.

4. On the grounds of virus-positive results, the presence of virus can be supported by the biopsy of the septal region of the right ventricle.

5. The results of histological examination confirmed the previous observation, that the sensitivity of EMB is low.

6. High CAR mRNA expression was detected in dilated, ischemic and inflammatory CM patient groups, although

significance was reached only in dilated and ischemic CM compared with the control group. The immunofluorescence examination supported our mRNA results. Our results support the idea that CAR may have a role in cell-to-cell connection and tissue regeneration.

LIST OF PUBLICATIONS

1. **Tátrai E**, Bedi K, Kovalszky I, Ifj Hartyánszky I, Lászik A, Acsády Gy, Sótonyi P, Hubay M. (2011) No mutation but high mRNA expression of Coxsackie-Adenovirus Receptor was observed in both dilated and ischemic cardiomyopathy. Int Forensic Sci, doi: 10.1016/j.forsciint.2011.05.010.

IF: 2,104

2. **Tátrai E**, Ifj Hartyánszky I, Lászik A, Acsády Gy, Sótonyi P, Hubay M. (2011) The role of viral infections in the development of dilated cardiomyopathy. Pathol Oncol Res, 17 (2): 229-35.

IF: 1,152

3. **Tátrai E**, Ifj Hartyánszky I, Lászik A, Hubay M, Acsády Gy, Sótonyi P. (2007) Víruskimutatás molekuláris biológiai vizsgálattal cardiomyopathiás betegek szívizommintáiból. (Molecular biological virus identification in dilated cardiomyopathy). Orv Hetil, 148 (48): 2275-8.

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