Complex Investigation of Heart Failure and its Non-pharmacological Therapy

PhD Thesis

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INTRODUCTION

Heart failure is an increasing medical and economic problem in the United States and all over the world. In the United States 5 million heart failure patients are registered, and 550,000 cases are diagnosed anually. Heart failure is responsible for 12-15 million doctoral inspections, and 6.5 million days in hospital a year. Despite the continous development of medical therapy, the number of deaths caused by heart failure shows a constant increase. The reasons are the increasing numbers of heart failure patients, and the increasing number of saved patients after myocardial infarction, due to better treatment. Heart failure occurs mainly in elderly patients, thus Heart failure patient population consists mainly of elderly patients: ageing of the population is also a factor in the increasing number of heart failures. Its incidence can exceed 1% above the age of 65 and more than 80% of people getting to hospital due to heart failure are above 65 years old.

AIMS OF OUR STUDY

Based on the suspicion of Pauschinger – namely that the virus genomes are located focally in the myocardium – our goal was to show that dilatative cardiomyopathy that was formerly thought to be idiopathic is actually caused by viral infection. This has never been confirmed before. It has already been verified that if a virus genome could be detected in the myocardium of an idiopathic cardiomyopathic patient, then the cause of the left side heart failure is a former viral infection. If that is true, and the viruses are located focally in the myocardium, then the diagnostic protocols are not correct at the moment: since endomyocardial biopsy is usually done from the same area, it is not appropriate for detecting viral origin in its current form. The conclusion might be that viral heart failures occur much more often, and most of those are not diagnosed. In these cases virus persistency is proven to worsen the prognosis of the left side heart failure. Treatment of recognised virus persistency can improve the life expectancy of patients, thus final state of heart failure, or even the transplantation could be avoided. An additional possibility during our research was that the progression of secunder (ischemic, valvular, etc.) cardiomyopathies could be aggravated by simultaneous persisting virus genome.

Our aims were:

1. **Molecular biological examination of dilitative cardiomyopathy caused by viral infections**
2. **Verification myocardial persistency of newer virus types**
3. **Verification of focal virus persistency in the myocardium**
4. **Examination of endomyocardial biopsy specificity used in diagnosis**
5. **Examination of the possible role of virus persistancy in the case of secunder cardiomyopathy**

METHODS

Patients and tissue samples

To verify our goals, we carried out two series of experiments in which we tried to isolate virus genomes from hearts explanted through a transplantation performed on patients who suffered from chronic heart failure: in their case the left side heart failure is not caused by possible acute infectious inflammation. We took samples from 5 different regions of the explanted heart, in which – after DNA and RNA isolation – we searched for typical virus sequences with nestedPCR technique. In our first experiment series we took samples from 28 patients searching for adenovirus 3, and in our second experiment series we examined 35 patients' samples as to local persistency of viruses. We searched for the genomes of the most common RNA viruses confirmed in the bibliography: the enterovirus and the two most common DNA viruses: adeno- and herpes viruses. The methods we used in the two series of experiments for taking samples and detecting viruses were similar, as described later. Meanwhile peripherial blood samples were also taken in order to rule out a systemic viral infection. Taking the myocardium sample took place right after the explantation of the heart, in sterile circumstances. At the same time, we also took blood samples from the patients. After taking the samples we stored the cardiac muscle pieces at -70 ^{o}C until usage. After the extraction of the DNA and RNA, we amplified the virus genome with

nested-PCR technique, then direct sequencing of the positive samples was done. We compared the resultant sequences with the virus sequences found in the database of the National Center for Biotechnology Information (NCBI) Genebank.

Patient population in the 1st series of experiments

Samples have been taken from 24 patients (14 dilatative cardiomyopathy, 2 inflammatic cardiomyopathy, 12 ischemic cardimyopathy). Distribution of the genders: 23 males and 5 females. The average age of patients was 45.71 ± 13.15 years.

Patient population in the 2nd series of experiments

We took samples from 35 patients altogether, from whom 17 had idiopathic dilatative cardiomyopathy (DCM-group), while 18 had ischemic/secunder origin of cardiomiopathy (Ischemic-group). Distribution of the genders: 13 males és 4 females were in the idiopathic group, while 14 men and 4 women were in the ischemic group. The average age of patients was 41,73 and 52,17 years in the idiopathic and the ischemic group, respectively. As control, we used the cardiac muscle samples of 20 healthy patients who died in accidents dissected at the Institution of Justice for both series of experiments. The plan for the research was authorized by the Semmelweis University's Committee of Regional, Institutional Scientific and Research-ethics under the number of 68/2005.

Techniques for defining virus sequences

After extracting the DNA and RNA from the frozen cardial muscle we multiplied the non-coding region of the Enterovirus 5, the Adenovirus 3 hexon and the Human Herpes Virus 6 alkalin-exonuclease using polimer chain reaction (PCR). For the sensitiveness and the specificity of the reaction we added a new polimer chain reactive step to the primer pair (Invitogen) that was planned inside the sequency which was multiplied in the first round. PCR products were checked by electroforesis with 1,75 % agarose gel and visualised with etidium-bromid. We compared the resultant fragmens sizes with molecule weight marker (100 bp ladder, BioRad)

Histological examination

During the processing of the explanted myocardial muscle samples for the histological examination, we fixed the samples in formaldehyde, then we cut 3um thick slices planted in paraffin. The samples were painted with haematoxylin-eosin, van Gieson, Azan and Phosphotungstic acid-haematoxylin (PTAH) methods and inspected by a Nicon Eclipse E400 light-microscope. The macroscopic and microscopic inspection of the explanted cardial muscle pieces have proved different levels of hipertrophy in the cardial muscle and the myocytes. Meanwhile thinning of the wall of the left ventricle was seen along with extensive intersticial fibriosis in connection with heart attacks and diffuse epicardial coronary disease. The results showed no inflammating infiltration in the myocardium at patiens belonging to the ischemic group.

Statistical analysis

We used a Fisher-exact test for determining the significant differences between virus persistency frequency found in the idiopatic DCM, the ischemic and the control samples. The significancy level was chosen to be 5 %.

RESULTS

We began sampling in November 2005. In the first series of the experiment we took and processed samples from 28 patients. The samples were investigated with PCR searching for adenovirus 3 sequences. Among the 28 patients there were 14 patients with dilated cardiomyopathy, 2 patients with with inflammatory etiology, and 12 patients with ischemic origin. We found positive samples at seven patients. From the seven patients one had inflammatory, two ischemic origin and 4 DCM etiology.

In the second series of experiment we took samples from 35 explanted hearts during the the cardiac transplantation from the recipients.The muscle samples were taken from five different regions: right ventricular antero-septal and postero-septal region, left ventricular anterior region, posterior region and left ventricular apex.The analyzation of the blood samples that we took from the patients, we could not find any sign of acute systemic inflammation, so we ruled

out the false positive results.The following table contains the patients' data. There was no significant difference between the groups in ejection fraction and in the average age.

Preop.EF(%): Preoperative ejection fraction

	DCM group	Ischemic group	Control Group
Number of pts	17	18	20
Gender (F/N)	13/4	14/4	13/7
Age (year±SD)	41,73±11,68	52,17±6,68	45,9±10,28
Preop. EF (%)	25,57	27,18	n.a.

SD: standard deviation

A total of 175 muscle samples and 35 blood samples were analyzed (from 18 ischemic and 17 DCM hearts). For control we analyzed100 samples from 20 patients.We found virus genomes in seven patients (5 with DCM and 2 with inflammatory origin). 11 samples showed positive result for viral infection: 10 samples for adenovirus and 1 for herpes virus. We did not manage to find any positive regions for enterovirus in any patient groups (including the control group). The blood samples were all negative for acute systemic viral infection.

Positive viral genom results

Patient identity number	**Right ventricle Antero-septal**	**Right ventricle Postero-septal**	**Left ventricle Anterior**	**Left ventricle Posterior**	**Left ventricle Apex**
DCM group (n=17)					
Adenoviral samples					
5.	positive				
13.	positive			positive	
16.	positive	positive	positive		
17.		positive			
18.	positive				
Herpes viral samples					
13.					positive
Enteroviral samples					
	No positive results				

Patient identity number	Right ventricle Antero-septal	Right ventricle Postero-septal	Left ventricle Anterior	Left ventricle Posterior	Left ventricle Apex
Ischemic group (n=18)					
Adenoviral samples					
11.			positive		
28.					positive
Herpes viral samples					
	No positive results				
Enteroviral samples					
	No positive results				
Kontroll csoport (n=20)					
Adenoviral samples					
	No positive results				
Herpes viral samples					
	No positive results				
Enteroviral samples					
	No positive results				

The table shows the numbers and regions of the positive samples by groups.The statistical analysis showed a significant difference between the DCM and the control group in the the case of adenovirus (p=0.014%). At the same time as we compared the ischemic and the control group, we found no significant difference in the incidence of the frequency of positive samples (p=0.21%).

DISCUSSION

1. Molecular biological examination of dilitative cardiomyopathy caused by viral infections

Our main goal in designing the experiment was –using molecular methods – to find viral origin in patients suffering from heart failure where previously viral genomes could not be found.The PCR and nested PCR techniques in detection of viral genomes are the most accurate methods. In the first part of the experiment the first and most obvious result was that the most common enterovirus genomes in the bibliography were could not be found. The explanation for that may be the different pathomechanism of the adeno- and enteroviruses. In the case of heart failure with enterovirus origin, for the development of the disease the eneterovirus genom must persist in the myocardium, but in the case of adenovirus, the virus genom just induces the process but is not requeired for the progression. Therefore, enterovirus can be detected with biopsy, so with appropriate therapy for these patients there is no need for transplantation.

2. Verification myocardial persistency of newer virus types

Previous studies have shown that adenovirus-2 and adenovirus type 5 have an etiologic role in myocarditis and in dilated cardiomyopathy. In the first part of our study we investigated the similar role of adenovirus-3. This virus subgroup often causes infection in the upper respiratory tract in children and in young adults. At the investigated patient group we found adenovirus type 3 in

seven cases: 4 patients with DCM, 1 patient with inflammatory and 2 patients with ischemic origin. We proved that this virus can persist in the myocardium and may have a etiologic role in heart failure.

3. Verification of focal virus persistency in the myocardium

In the second part of our study we analyzed a total of 175 samples and 100 control samples. 11 out of 175 (6.3%) were positive: 10 samples showed the presence of adenovirus and 1 sample showed presence of herpesvirus. In the control group we did not find any viral genome. Our goal in this part of the study was to prove that viral genomes can persist focally in the myocardium. In the case of adenovirus, at the seven patients who had positive samples, we could not find any patient who had all regions positive. In one patient there were 3 positive regions and in another one, there were two positive regions. The results back up the theory that adenovirus can persist focally in the myocardium. We found herpesvirus genome in only one patient (patient number 13 in the DCM group) with adenovirus positivity at the same time. With this one case we can only suspect that the virus may also persist focally. Since we have not been able to find find enterovirus genome, we cannot come to a conclusion about the virus distribution in the myocardium.

4. Examination of endomiocardial biopsy specificity used in diagnosing

A notable fact is that in our idiopathic group, at the 30% of the patients we could detect adenovirus genome, while previously performed endomyocardial biopsy could not give any evidence of viral etiology. It is known that EMB is routinely performed from the right atrial septum. We tried to determine the specifity of the EMB by deviding the right ventricle septum into anterior and posterior parts, and the samples were taken from both regions. The results show that in the idiopathic group only one patient had both right region positive. However, the fact is that in all positive cases one of the two right regions was always positive. Multiple samples should be taken from the right ventricle using biomolecular methods to clarify the viral etiology.

5. Examination of the possible role of virus persistancy in the case of secunder cardiomyopathy

The two positive cases in the ischemic group are to be given special attention. The adenovirus was located focally in this patient group as well, but in these cases the left ventricle regions were positive. Because the ischemic etiology was clear, no further investigations were done for viral genomes in these patients' heart muscle. However, these two cases show that the adenovirus infection can occur in this etiology as well. The question is whether viral infection can speed up the progression of heart failure. There is not enough data and further research is needed to prove this theory. If we are able to verify this theory, it may mean that in positive cases there is also need for screening and eradication of the virus in this group.

CONCLUSIONS

The basic novelty of our clinical survey was the method of sampling, with which we took heart muscle samples from five different topological zones of hearts explanted during transplantation from heart disease patients in the final stage. We wanted to detect cardiotypical viruses that can be the cause of developing dilatative cardiomyopathy. We have also succeeded in showing that the adenoviruses are able to persist in the myocardium of heart transplantation patients, furthermore we have reported myocardial persistence of a new subgroup of viruses, the adenovirus 3.

We have also proved that the adenovirus genome has a focal persistence pattern. There was not a single adenovirus positive patient whose heart muscle samples were all positive. The blood samples taken at the same time were never positive, which excludes the possibility of a fake positive result in the myocardium caused by simultaneous acute virus infection. In conclusion our results have proved that the adenovirus shows focal persistence in our transplant patient group, supporting Pauschinger's focal persistence theory. We have also found focal type persistence for the Herpes-virus, but we cannot draw conclusions based on only a single positive result.

Despite the fact that in existing literature enterovirus is listed as the most common etiological factor of virus induced cardiomyopathy, we have not found any positve enterovirus samples in the patient group. We think that the reason for this is that in the case of enteroviruses, for the impairment of the myocardium, continuous presence of the virus is required in all segments of the heart. In this case with the help of the earlier endomyocardial biopsies the viral origin was detectable, so these patients did not get into our group.

Kindermann et al. proved that in the case of acute myocarditis the presence and demonstrability of the adenovirus is not connected to the clinical outcome, so there is no need to demonstrate the virus genome from the heart muscle in case of acute myocarditis. These facts prove that it is not the presence of the adenovirus in the acute phase that matters in myocardium impairment, but long-term persistence of the virus is the key. Before the adjustment of any antiviral or immune suppressive treatment it is crucial to decide whether we are facing acute viral infection with cellular infiltration or harmless latent viral persistence without cellular infiltration. Based on this, repeated demonstration of the virus or immunohistology from a check-up endomyocardium biopsy might be needed even months after the passage of acute myocarditis.

The high (>30%) rate of adenovirus presence in our patient sample may draw clinians' attention to the low sensitivity of endomyocardial biopsy conducted only once. According to routine procedure the endomyocardial biopsies are taken from the septum of the right chamber, so during our survey we divided the septum of the right chamber into two – anterior, posterior – parts based on sample regions, so that we could demonstrate the contingent subregional virus genome distribution inside the septum, which was proved in our surveys. So we can conclude that one endomyocardial sample is not enough to exclude virus persistence. Yet based on the found adenovirus positive pattern – in each positive patient there was a positive sample from the right chamber as well – we can conclude that sampling from the right chamber is adequate, but the number of samples taken needs to be raised.

The two adenovirus positive patients from the ischemic patient group need to be highlighted. In regards to known ischemic etiology, in the case of these patients there has been no virus persistence survey before transplantion. These positive cases might suggest the theory that contingent virus infection might play a role in progrediation of ischemic heart failure and the decay of patients' status. Further experiments are needed to decide whether virus infections might play a role in secunder cardiomyopathies developing into final stage heart failures. It is also to be cleared whether in this group of patients the conducted virus eradication in case of contingent virus persistence can efficiently prevent the development of final stage heart failure.

Adenovirus positive patients may not only be of diagnostic but also of therapeutic importance. Kühl et al. proved that virus persistence can worsen the outcome of idiopathic left chamber failure. Recent surveys have shown that virus eradication conducted with interferone might weaken the clinical consequences of entero- and adenovirus cardiomyopathy. The effectiveness of interferone therapy in acute myocarditis is still doubtful.

All in all we have strong evidence for focal persistence in case of adenoviruses. Repeated multiple samplings from the septum of the right chamber are suggested after previous passage of viral myocarditis to demonstrate virus persistence and for the avoidance of fake negative results.

SUMMARY

According to the World Health Organization/ International Society and Federation of Cardiology 25 % of all heart failure patients suffered from DCM (dilated cardiomyopathy) in 1995, leading to 60,000 new patients every year in Europe. EMB (endomyocardial biopsies) were able to detect infectious aetiology in less than 30 % of DCM patients. Recent studies, however predict virus-mediated myocardial damage in 60 % of idiopathic DCM patients.

The pathway from viral myocarditis to end-stage heart failure is commonly accepted, but diagnosis of virus-mediated myocardial injury is still challenging. Virus persistency in the myocardium may accelerate ventricular failure, thus precise diagnosis of virus persistency may prevent the development of end-stage heart failure. The aetiology of viral infection is routinely diagnosed by endomyocardial biopsies taken from the right ventricle. There are hypotheses in the literature, that after certain infections viral particles could persist in the myocardium focally, thus endomyocardial biopsies taken only from one part of the heart could give false negative results.

We have performed a systematic investigation on the sampling error of viral diagnostics in heart transplant recipients: trans mural samples from five regions of explanted hearts from recipients during heart transplantation have been amplified using entero-, adeno-, and herpesvirus sequences and histological examination has also been performed.

In total 175 myocardium samples have been examined from dilated cardiomyopathy and 100 myocardium samples from 20 forensic medicine patients as a control group. Seven patients were positive for the examined viruses: 10 positive regions for adenovirus, and 1 positive region for herpes virus DNA. No positive region for enterovirus was found. No positive samples were found among the forensic medicine patients. For adenovirus a focal myocardial pattern was detected.

Our results with the patchy myocardial virus persistence may explain possible false negative results in virus-mediated aetiology in end-stage dilated cardiomyopathy patients. Therefore, repeated endomyocardial biopsies, and multiple cardiac samples are suggested to be taken to prove aetiology in heart failure patients, thus prevent end-stage heart failure and decrease the number of heart transplant recipients.

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