Molecular Changes of the Angiotensin Converting Enzyme 2 Pathway in Myocardial Tissue from Heart Failure Patients Undergoing Heart Transplantation

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Abstract

Background: The angiotensin-converting enzyme homologue ACE2 and the peptide angiotensin 1-7 (Ang 1-7) have antifibrotic effects, but it is not understood whether they have a protective role in cardiac remodeling. We aimed to analyze the Ang 1-7 Mas receptor and ACE2 expression levels and their relationship with fibrotic and remodeling factors in human heart failure (HF) and compared them with non-pathological human hearts.

Methods: This was a human case-control study in which two groups of samples were analyzed. Controls were organ donors without cardiovascular pathology, but whose heart could not be used for transplantation. Cases were patients with end-stage HF and left ventricle samples were harvested right after the heart transplantation surgery. They were either kept with liquid nitrogen until their analysis or were processed for paraffin embedding. We quantified mRNA expression by real time PCR and ACE2 protein levels by western blot. The collagen deposition was stained by sirius red and quantified with morphometric analysis.

Results: Expression of the MMP3 stromelysin mRNA was only detectable in 10 out of 33 pathological samples and in 1 out of 13 control samples. Expression of components of the ACE2 pathway and fibrotic factors were higher in patients with detectable MMP3 expression than in patients with non-detectable MMP3. No differences were found between patients with MMP3 expression or no MMP3 expression regarding etiology, functional class, ventricle dilatation or medication.

Conclusions: Only a subset of myocardiums from HF patients is active in the remodeling process at the moment of heart transplantation, as indicated by the MMP3 expression and the higher levels of fibrotic factors. Angiotensin 1-7 Mas receptor expression and ACE2 protein levels are increased in this myocardium subset, suggesting a role for the ACE2 in this process. Overall, we encountered molecular differences in transplanted hearts from patients with similar clinical characteristics.

Keywords: Heart transplantation; Heart failure; Angiotensin converting enzyme 2; Metalloproteinase 3; Fibrosis; Remodeling

Abbreviations: HT: Heart Transplantation; HF: Heart Failure; ACE2: Angiotensin Converting Enzyme 2; Ang 1-7: Peptide angiotensin 1-7; MMP3: Metalloproteinase 3; N-MMP3: Patients with non-detectable MMP3; COL1 and COL3: Collagens I and III; NOS1 and NOS3: Nitric oxide synthases I and III; MAS17: Ang 1-7 Mas receptor; NYHA: New York Heart Association

Introduction

Heart failure (HF) constitutes the end-stage of many cardiopathies and is characterised by a ventricular dysfunction. The prevalence of HF in Europe is between 2-3% and rises sharply at 75 years of age [1]. Even though the prognosis of HF has improved substantially with the medical therapy recommended in the current guidelines, evolution towards worsening heart function frequently cannot be avoided and in some cases heart transplantation (HT) is the only therapeutic approach available. Left ventricular dysfunction is associated with ventricular dilatation and remodeling, which is a progressive and silent phenomenon [2].

The neurohumoral system is activated as a compensatory mechanism when there is a reduction in cardiac output. An increase in circulating levels of hormones such as norepinephrine, angiotensin II (Ang II) and aldosterone triggers a haemodynamic response to preserve cardiac output and blood pressure [3]. Although neurohumoral activation is very effective at short-term, over a long period of time, increases the workload of the heart and contributes to further deterioration of ventricular function. Besides the circulatory effects of these hormones, they are also very active at tissue level and they promote hypertrophy and fibrosis of the myocardium [4]. Concomitantly to the increase of circulating Ang II, Ang II cardiac production increases with the progression of HF [5]. ACE2 is a homologue of the angiotensin-converting enzyme (ACE) that cleaves Ang II to produce the aminoacid peptide Ang 1-7 [6,7]. Experimental results suggest that

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ACE2 has an important role in cardiac function [8,9]. Ang 1-7 seems to act as a cardiac protector and its synthesis might balance the deleterious effects of the renin-angiotensin system (RAS) activation [10,11]. Mas17 is a G-protein coupled receptor and its ligand is Ang 1-7 [12]. Lack of the receptor expression has been shown to lead to cardiac dysfunction in both in vivo and in vitro [13].

The cardiac extracellular matrix (ECM) is composed by a network of mainly fibrillar collagen of types I and III and provides the scaffolding for the cardiomyocytes. The balance between its synthesis and degradation has been considered to play a key role in maintaining left ventricular geometry and function. An alteration of both collagen synthesis and degradation in cardiac diseases has been described [14]. The stability of the collagen that constitutes the ECM depends on the equilibrium between matrix metalloproteinases (MMP) and their endogenous inhibitors (TIMP). A mechanistic role for MMPs activity and ventricular remodeling has been established with genetically modified animal models [15,16].

MMP3 is a stromelysin with a wide range of substrates: fibronectin, laminin, collagen types III, IV, IX and X and plays a critical role in the activation of other MMPs such as proMMP1 and proMMP9 [17]. In a transgenic model of HF with cardiac-specific over expression of TNF-α, MMP3 expression was higher than in WT mice and it increased with age and HF severity. MMP9, TIMP1 and TIMP2 levels were also examined in this transgenic animal but only MMP3 presented an expression pattern that paralleled the development of HF [18].

The explanted heart at the moment of cardiac transplantation offers a unique opportunity to study the pathways that take part in the process of ventricular remodeling and fibrosis. Altogether, we aimed to get a more detailed description of the expression of components of the ACE2 pathway and their relationship with the remodeling and fibrotic processes that take place in failing hearts.

Methods
Study group
Two groups of patients were required for the study. One group consisted of patients undergoing heart transplantation because of end-stage congestive heart failure, n=33, secondary to either idiopathic dilated cardiomyopathy or ischemic heart disease. A sample of the explanted heart was harvested during the surgical intervention on the day of transplant.

Two groups of control hearts were studied, in one group mRNA and protein levels were quantified (n=13, control group 1) and in the other group histology assays were performed (n=15, control group 2). Control groups consisted of multi-organ donors without cardiovascular pathology, whose heart could not be used for transplantation because they did not match the requirements of the potential heart receptors. Both control groups did not differ in their characteristics and were similar in age and gender percentage to the patient’s group (Table 1A).

The total number of groups finally analyzed was three because the patient’s group was later divided in two groups: patients with detectable myocardial expression of MMP-3 and patients with non-detectable MMP-3 expression.

The protocol was approved by the Ethics Committee of our institution and an informed consent was obtained from all patients.

Sample collection and RNA isolation
At the time of heart transplantation, 1 cm³ myocardial biopsies were obtained from the left ventricles of the explanted hearts and immediately immersed in liquid nitrogen or in formol. Samples used for mRNA and protein levels quantification, were kept in liquid nitrogen until their analysis. Samples used for histology were kept in formalin for at least 24h and they were later embedded in paraffin blocs. A similar protocol was used for the control hearts.

Total RNA was isolated from left ventricular myocardium as previously described [19]. The integrity of the resulting RNA was assessed by electropherograms using the microfluidic platform Agilent 2100 bioanalyzer. The RNA concentration and purity were determined by absorbance at 260 and 280 nm using Ultraspec 3300 pro (Amersham Biosciences). A reverse transcription protocol with random primers was applied to 1μg of total RNA for cDNA synthesis with the addition of RNAse inhibitors (High capacity cDNA RT kit, AB, Applied Biosystems) with the MJ Research PTC 200 thermal cycler (MJ Research).

**Real time polymerase chain reaction (Real Time PCR)**

Messenger RNA (mRNA) expression was assayed with the real-time PCR 7900 thermal cycler (AB, Applied Biosystems), using TaqMan Universal PCR Master Mix with AmpErase UNG and the following TaqMan one-gene expression assays: Matrix Metalloproteinase-3 (Hs00968305_m1, MMP3), G-protein coupled receptor Mas (Hs00267157_s1, MAS1), Collagen 1 (Hs00164004_m1, COL1A1), Collagen 3 (Hs00164103_m1, COL3A1), Nitric Oxide Synthase III (Hs00167166_m1, NOS3) and Nitric Oxide Synthase I (Hs00167223_m1, NOS1). All mRNA quantification results are relative to a cDNA pool with sequential dilutions, units are given as ng-equivalents of cDNA (ng-equ). Variation in the number of control and pathological samples analyzed is due to shortage of samples in some cases and we were not able to perform all the assays with the total n.

MMP-3 expression was very low with the minimum treshold cycle (Ct) of 30. Two separate assays of MMP-3 expression by real time were performed. If a sample had fluorescence detectable in both assays was classified as having MMP-3 expression detectable (MMP3). If a sample had no detectable fluorescence in any assay or fluorescence in only one assay with a Ct>37 was classified as MMP-3 expression non-detectable (N-MMP3).

**Western Blot analysis of ACE2 protein**

Whenever enough tissue was available, myocardial proteins were also extracted from the left ventricle biopsies. Sliced frozen tissue was submerged in 1ml of ice-cold protein lysis buffer containing: 50 mM Tris HCl pH 7.5 (T5941, Sigma), 150 mM NaCl (1.06404.5000, Merck), 1% Nonidet p-40 (Tergitol solution, NP40S, Sigma), 0.5% Sodium deoxycholate (D6750, Sigma), 0.1% SDS (Sodium Dodecyl Sulfate, L5750, Sigma), 1 mM PMSF (phenylmethanesulfonyl fluoride, P7626, Sigma), 1mM sodium orthovanadate (S6508, Sigma), 1 mM Pefabloc (11249868001, Roche) and complete mini inhibitors (11836153001, Roche). Samples were homogenized with the Omni TH homogenizer (Omni International Inc.). After 1h of rotation at 4°C samples were centrifuged at 10.000 g at 4°C for 30 min. The upper phase was collected and the total protein concentration was quantified.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age(years)</th>
<th>Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group 1</td>
<td>13</td>
<td>52 ± 5</td>
<td>66%/33%</td>
</tr>
<tr>
<td>Control group 2</td>
<td>15</td>
<td>55 ± 3</td>
<td>66%/33%</td>
</tr>
<tr>
<td>HF patients</td>
<td>33</td>
<td>54 ± 2</td>
<td>82.4%/17.6%</td>
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Table 1A: Age and gender of controls and patients.
with the Pierce BCA protein Assay method (23227, Thermo Scientific, Pierce) relative to a BSA standard curve. 30 μg of total protein extract were loaded to Novex®4-12% Tris-Glycine Mini Gel (EC60352) and a western blot was performed with the Novex® gels methodology (Invitrogen). Proteins were transferred from the gel to a nitrocellulose membrane using a blot gel transfer (IB3010-01) and the iBlot® Dry Blotting System. After 1h blockade of the membrane, it was incubated overnight at 4°C with the ACE2 primary antibody ab15347 diluted 1/1000 (Abcam). Afterwards, the membrane was incubated during one hour with an HRP-goat anti-rabbit secondary antibody diluted 1/1000 (Abcam). Afterwards, the membrane was incubated during one hour with an HRP-goat anti-rabbit secondary antibody diluted 1/1000 (111-035-1440, Jackson ImmunoResearch Europe). Final detection of the ACE2 protein band was accomplished with the ECL kit Supersignal West Pico Chemiluminescent Substrate (34080, Thermo Scientific). Quantification of the integrated density of the band around 120 kDa was done with the Image J program (NIH, Maryland, USA). Each blot was loaded with 30 μg of a standard pool of human myocardial protein for calibration between blots. Results are given in relative units (RU) as the ratio between the integrated density of each sample divided by the integrated density of the standard.

**Collagen fraction quantification**

Four-micron sections of paraffin-embedded left ventricular tissue were stained with picrosirius red. Four sequential pictures at 40X fold were taken with an OLYMPUS BX51 microscope camera, and a composite image was created from aligning the four pictures. Six composite images were taken per sample and the morphometric program Analysis was used for quantification of the interstitial collagen fraction.

**Statistical analysis**

All variables are expressed as mean ± standard error of the mean (SEM). Statistical calculations were performed with the SPSS software, version 16.0 (SPSS Institute Inc, Cary, NC, USA). A Chi-Square test was used to analyze for differences between categorical variables. The non-parametric Kruskall-Wallis test was applied to compare between the 3 groups of individuals. When only two groups had to be compared the non-parametric U Mann-Whitney test was used. A p value <0.05 was considered to be statistically significant.

**Results**

**Clinical characteristics of the patients**

Two groups of controls were used for the assays and were similar in age and gender characteristics as the group of patients (Table 1A). HF etiology was ischemic in 17 patients and non-ischemic in the remaining 16. The clinical characteristics of patients and their medical therapy before heart transplantation are summarized in Table 1B.

**Ventricular expression of MMP3 mRNA**

MMP-3 mRNA expression was very low (Ct>30) or non-detectable in left ventricle samples. Samples were classified as either having MMP-3 expression detectable (MMP3) in 10 out of 33 patients and in 1 out of 13 controls or as having MMP-3 non-detectable (N-MMP3) in the remaining samples. Despite the higher percentage of samples with MMP-3 detectable in patients (30%) compared to the percentage of samples with MMP-3 detectable in controls (7%), the difference was not statistically different.

No significant differences were found between patients with detectable MMP3 expression or N-MMP3 regarding etiology, NYHA class, sex or medication. To analyze whether there were differences upon heart remodeling we also compared the frequency of MMP3 expressed in patients with left ventricular end-diastolic diameter larger or smaller than 70 mm or in patients with left atrial diameter larger or smaller than 45 mm, or whether the ejection fraction was higher or lower than 20%. No differences were found in any of these subgroups of patients.

**Differences in MAS17 mRNA expression and in ACE2 protein levels in samples with or without detectable MMP-3 expression**

MAS17 mRNA expression was higher in patients with detectable MMP3 expression when compared to patients with N-MMP3 (74.7 ± 12.2 ng-equ, n=10 vs 40.0 ± 3.6 ng-equ, n=22 *p<0.01). MAS17 expression was also higher in patients with detectable MMP3 mRNA expression (74.7 ± 12.2 ng-equ) than in controls (60.0 ± 8.0 ng-equ, n=12) but the difference was not statistically different. MAS17 mRNA levels were lower in patients with non-detectable MMP3 than in controls (40.0 ± 3.6 ng-equ vs 60.0 ± 8.0 ng-equ, *p<0.05, Figure 1A).

A tendency to higher ACE2 protein amounts was found in patients with detectable MMP3 (0.93 ± 0.26 RU, n=7) compared to patients with non-detectable MMP3 (0.64 ± 0.12 RU, n=14, Figure 1B), and to controls (0.58 ± 0.11 RU, n=10) but the differences between groups were not statistically different. Representative ACE2 protein bands from the western blot are shown in Figure 1C.

**Differences in COL1 and COL3 mRNA expression in samples with or without detectable MMP-3 expression**

COL1 expression was higher in patients with detectable MMP3 expression vs patients N-MMP3 (41.9 ± 5.9 ng-equ, n=10 vs 27.2 ± 2.7 ng-equ n=20, *p<0.05 and vs controls (25.2 ± 2.4 ng-equ, n=11, *p<0.05). The COL1 expression in patients with N-MMP3 was similar to controls (27.2 ± 2.7 ng-equ vs 25.2 ± 2.4, Figure 2A).

COLIII expression presented a tendency to increase from controls (33.3 ± 3.4 ng-equ, n=13) to patients with N-MMP3 (41.9 ± 4.0 ng-equ, n=21) and to patients with detectable MMP3 (55.8 ± 6.3 ng-equ, n=10), but the differences were only statistically significant between controls and patients with detectable MMP3 (**p<0.01, Figure 2B).
Differences in fibrosis in samples with or without detectable MMP-3 expression

Percentage of collagen fibers measured with Sirius red staining also presented a tendency to increase from controls (4.0 ± 0.6%, n=15), to patients with no MMP-3 detectable (N-MMP3) (8.7 ± 1.1%, n=21) and to patients with detectable MMP-3 (12.1 ± 2.6%, n=9). Both subgroups of pathological samples had higher collagen deposition than control samples (*p < 0.05 versus N-MMP3 and **p < 0.01 controls versus MMP3, Figure 3).

NOS3 and NOS1 mRNA expression in samples with or without detectable MMP-3 expression

NOS3 expression was similar in both groups of patients (16.6 ± 1.4 ng-equ, n=23 in N-MMP3 patients vs 16.8 ± 2.1 ng-equ, n=10 in MMP3 patients). In both cases, expression was significantly lower than in controls (24.2 ± 1.9 ng-equ, n=13, **p < 0.01 controls versus N-MMP3 and *p < 0.05 controls versus MMP3, Figure 4A). NOS1 expression was lower in pathological samples with detectable MMP-3 (39.6 ± 10.6 ng-equ, n=10) than in pathological samples with N-MMP-3 (52.7 ± 14.7 ng-equ, n=23) or than in controls (53.2 ± 22.0 ng-equ, n=13), but the differences were not statistically significant (Figure 4B).

Discussion

Evidence of active remodeling stage in some heart transplantation samples

In this study we have found that only a subset of hearts is in an active remodeling state at the moment of heart transplantation. In these hearts, the stromelysin MMP-3 mRNA levels are high enough as to be detected with a Real Time assay and their collagen I and collagen III mRNA levels and deposition of collagen fibers are higher than in the remaining hearts. Furthermore, two key proteins of the ACE2 pathway, ACE2 protein itself and the Ang 1-7 receptor MAS17 have increased levels in these active remodeling hearts, suggesting a role for this pathway in the molecular changes involved in this process. Most notably, failing hearts that had non-detectable MMP-3 expression had mRNA levels of collagen I, collagen III, MAS17 and ACE2 protein levels closer to controls than to patients with detectable MMP-3. These results seem to indicate that, in this group of hearts, remodeling is not currently active.

Role of MMP-3 stromelysin in heart remodeling

Alterations in various MMPs and TIMPs expression in HF have been reported in the literature, but the results are controversial [14]. MMP-3 circulating levels are increased after acute myocardial infarction (AMI) and are inversely correlated with left ventricular ejection fraction. MMP-3 levels are also higher in patients that develop HF left ventricular remodeling [20]. In other reports, they found higher MMP-3 myocardial levels in patients with idiopathic HF but not in ischemic HF [21]. In the setting of a left ventricular assist device, where heart remodeling also takes place, analysis of MMPs/TIMPs myocardial...
changes after implantation found that while MMP1 and MMP9 levels decreased, no changes were observed in MMP2 and MMP3 levels [22].

Most probably, some of the contradictory results found in the literature arise from the analysis of samples taken from patients that are in a different remodeling stage. In our case, in a previous report with fewer patients analyzed with idiopathic HF, we found no differences in MMP-3 expression between HF patients and controls [23]. In most of these cases, MMP-3 expression was very low and undetectable.

Our attempts to find a clinical feature that distinguished both groups of patients have been unsuccessful. We found no differences between MMP3 and N-MMP3 patients according to etiology, NYHA class, sex and medication. We also hypothesized that patients with different left ventricular or atrial dimensions or left ventricular ejection fraction would have differences in MMP-3 expression, but no differences were found.

Role of the ACE2 pathway in heart remodeling

The increased MAS17 mRNA levels and ACE2 protein that we encounter in the MMP3 samples is in agreement with the reported increased Angiotensin-(1-7)-forming activity in myocardium from patients with end-stage idiopathic dilated cardiomyopathy and patients with primary pulmonary hypertension [24]. Treatment with Ang 1-7 intravenous infusion with osmotic minipumps prevented deterioration of cardiac function in an animal model of HF, after left coronary artery ligations in rats. It produced a 40% reduction in the left ventricular end-diastolic pressure, almost full preservation of coronary flow and preserved aortic endothelial function [25].

The beneficial and anti-fibrotic effects of both ACE2 and Ang 1-7 and the higher levels of ACE2 protein and Ang 1-7 receptor that we document in failing hearts with active remodeling process, suggest that the ACE2 branch of the RAS pathway has a role in the remodeling.

Role of fibrosis in heart remodeling

Collagen I and collagen III are the main structural collagens in the heart and their expression increases with fibrosis [14]. In the Syrian Hamster, an animal model of cardiomyopathy, an increase in collagens I and III expression has been quantified in different stages of the cardiomyopathy [26].

In our analysis we found that only samples with detectable MMP-3 had higher levels of collagen I and collagen III mRNA than controls. Also, collagen I levels in MMP3 patients were higher than in N-MMP3 patients, which surprisingly had collagen I mRNA levels similar to controls. Collagen III mRNA levels in N-MMP3 patients were in between controls and MMP3 patients values. Analysis of the collagen fraction in histological samples of these hearts stained with picrosirius red showed that both groups of failing hearts had higher values of collagen fraction than controls, being the highest in MMP3 patients. Altogether these results indicate that the fibrotic process is less aggressive and not currently active in the group of samples with non-detectable MMP3 as shown by the lack of differences in collagen I and collagen III mRNA expression compared to controls.

Role of nitric oxide synthases in heart remodeling

In the heart, three nitric oxide synthase (NOS) isoforms can be present: neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2). Either NOS1[27,28] and NOS3[29] deletion leads to higher mortality and remodeling in experimental models of MI suggesting a protective role of both enzymes in heart remodeling. Chronic inhibition of NOS3 has been shown to increase ACE activity and angiotensin-induced fibrotic effects [30]. Because of the relationship between NOS3 expression and the ACE pathway and the putative role of NOS3 and NOS1 in cardiac remodeling, we decided to analyze the mRNA levels of these two constitutive nitric oxide synthases. In our samples, all pathological samples had lower levels of NOS3 mRNA levels than in controls, as we have previously reported [19] but no differences were observed within both groups of patients. And no differences were found in NOS1 mRNA in any case. These results suggest that NO does not account for the differences in remodeling stages that we find in these two subgroups of patients.

Conclusions

In this study we have analyzed the left ventricle remodeling stage from a group of end-stage HF patients and found that at a molecular level hearts could be distinguished in two groups; hearts that were currently active in remodeling and hearts that were not. We have analyzed proteins whose role in either fibrosis or cardiac dilatation has been well established in animal models. Published results of expression of these proteins in human samples are sometimes contradictory. We feel that the contradictory results may arise from the complexity of end-stage HF patients and also for the presence of samples that are at different remodeling stage. The division of pathological cardiac samples according to MMP3 expression has allowed us to find clear differences between those two groups and compare them to controls. We have not found any clinical differences between these two groups of patients but future research will be needed to further elucidate their characteristics and whether a different therapy regime should be given to each group of patients.

Limitations of the study

Analysis of the explanted heart at the moment of cardiac transplantation describes the remodeling process at the terminal stage of HF and is not representative of the process in earlier phases. Since endomyocardial biopsy is the only means of obtaining samples of myocardial tissue in the earliest phases of HF, and because this is an invasive process with an inherent risk, ethical considerations prevents us from collecting endomyocardial biopsies at different time points to study the sequential changes. We could not perform all the analysis in one control group because we lacked frozen samples in one group and lacked paraffin embedded samples in the other group. Nevertheless, in both groups, controls had a similar age and gender percentage and they were all subjects with no cardiovascular diseases.

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