**BASIC RESEARCH**

Cardiac remodelling in end stage heart failure: upregulation of matrix metalloproteinase (MMP) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ACE inhibitors on MMP

D Reinhardt, H H Sigusch, J Henße, S C Tyagi, R Körfer, H R Figulla

Objective: To investigate matrix metalloproteinases (MMP-2 and MMP-9) in heart failure caused by ischaemic and idiopathic dilated cardiomyopathy, and the impact of angiotensin converting enzyme (ACE) inhibition on MMP.

Design and main outcome measures: MMP were extracted from myocardium of patients with heart failure (coronary artery disease, n = 13; idiopathic dilated cardiomyopathy (IDCM), n = 16) and from controls (n = 6). The active form of MMP-2 and MMP-9 was measured by enzyme linked immunosorbent assay; activity of MMPs by zymography; mRNA expression of MMPs by reverse transcriptase polymerase chain reaction.

Results: Active MMP-9 was significantly increased in coronary artery disease (mean (SD) 1.6 (0.35) ng/ml) and IDCM (2.11 (0.54) ng/ml) in comparison with controls (0.53 (0.15) ng/ml). Increased MMP-2 was only found in IDCM (3.68 (0.41) ng/ml). There were corresponding increases in MMP activity but no upregulation of mRNA expression was found. The ACE inhibitors captopril and ramiprilate inhibited MMP-2 and MMP-9 activity in vitro (inhibitory capacity (IC50), in mmol/l: MMP-2: captopril 2.0 (0.16), ramiprilate 2.1 (0.3); MMP-9: captopril 1.65 (0.18), ramiprilate 2.0 (0.3)). Lisinopril inhibited MMP-9 significantly but did not inhibit MMP-2 in vitro (IC50 MMP-2: 7.4 (0.88); MMP-9: 7.86 (2.23)). Inhibition of MMP activity by ACE inhibitors was blunted by zinc excess.

Conclusions: Upregulation of MMP-9 activity is common in the failing myocardium, independent of the underlying disease. Missing upregulation of transcription suggests that activation of latent forms of MMP is the source of increased MMP activity, rather than increased de novo synthesis. Some ACE inhibitors may influence MMP activity by a direct effect.

Recent results from the ELITE-2 (evaluation of losartan in the elderly) study, which was designed to show the superiority of losartan over captopril, failed even to give an assurance that these two agents are of equivalent benefit in heart failure treatment. This result has led to a re-examination of the factors that determine the actions of angiotensin converting enzyme (ACE) inhibitors and sartans in the failing heart. ACE inhibitors inhibit angiotensin converting enzyme, which catalyses the formation of angiotensin II from angiotensin I; sartans are thought to inhibit the ACE system more effectively by angiotensin II receptor antagonism. On the other hand, the haemodynamic consequences of ACE inhibition and angiotensin II receptor antagonism only partially explain their beneficial effects on cardiac remodelling in heart failure. Local mechanisms have been suggested—for example, inhibition of the tissue ACE system and a decrease in the breakdown of bradykinin. Sorbi and colleagues suggested that reduced proteinuria in hypertensive patients treated with captopril reflected a direct effect of captopril on matrix metalloproteinase-2 (MMP-2) and MMP-9 activity, mediated by the sulphydryl group on the ACE inhibitor.

Comparing the effect of ACE and MMP inhibition on cardiac remodelling in a rapid pacing induced heart failure model in pigs, McElmurry and colleagues showed that both MMP and ACE inhibitors significantly reduced the degree of left ventricular dilatation. However, MMP activity in the left ventricular myocardium did not differ between animals treated with MMP inhibitors, ACE inhibitors, or combined treatment. So the question arises as to whether ACE inhibitors have a direct influence on myocardial MMP activity.
Preparation of MMP
Approximately 25 mg of frozen adult left ventricle was washed three or four times with cold saline. Cardiac tissue was then incubated in 1000 µl of extraction buffer (10 mmol/l cacodylic acid at pH 5, 0.15 mol/l NaCl, 1 mmol/l ZnCl₂, 20 mmol NaCl, 1.5 mmol/l NaN₃, and 0.01% (vol/vol) Triton X 100) at 4°C for 72 hours. The extraction buffer was collected and the pH was raised by the addition of 1.5 mol/l Tris HCl buffer (pH 8.8). Aliquots from these samples were loaded directly onto substrate gels.

Total protein in extracts
A Bio-Rad dye binding assay (Palo Alto, California, USA) was used to estimate the total protein concentration in tissue extracts according to Bradford.⁷

Visualisation of gelatinolytic activities on SDS substrate gels (zymography)
Sodium dodecyl sulfate (SDS) substrate gels were prepared as published elsewhere, with modification. Gelatine (porcine skin, 300 bloom from Sigma, St Louis, Missouri, USA) was added to standard Laemmli acrylamide polymerisation mixture at a final concentration of 1 mg/ml. Tissue extract was mixed with substrate gel sample buffer (10% SDS, 0.25 mol/l Tris HCl pH 6.8, and 0.1% wt/vol bromphenol blue) to a final mixture at a final concentration of 1000 µg/ml, and 20 µl were loaded under non-reducing conditions immediately without boiling into wells of a 4% (wt/vol) acrylamide Laemmli stacking gel on a cast vertical gel. Gels were run at 15 mA/gel while stacking, and at 20 mA/Gel during the separation phase at room temperature. Following electrophoresis the gels were soaked in 2.5% (wt/vol) Triton X 100 with gentle shaking for 30 minutes at room temperature with one change of detergent solution. The gels were rinsed and incubated overnight at 37°C in substrate buffer (50 mmol/l Tris HCl pH 8, 5 mmol/l CaCl₂, and 0.02% wt/vol NaN₃).

After incubation, the gels were stained for three minutes in Coomassie blue R250 in acetic acid:isopropyl alcohol:water (1:3:6 by volume), and destained in water overnight with or without ACE inhibitors (captopril, lisinopril: Sigma; ramiprilate: Aventis, Bad Soden, Germany). Gels were scanned and analysed by densitometry for lysis band intensity (Herolab Imaging system, Wiesloch, Germany). The MMP gelatinolytic activity results are expressed in arbitrary units expressing the videodensitometrically determined ratio of the MMP to the GAPDH RT-PCR products.

Data analysis
Statistical analysis was done using Microsoft Excel data analysis software and SPSS (SPSS Inc, Chicago, Illinois, USA). Differences in the mean value were considered significant at a probability value of p < 0.05. Values are presented as mean ± SD. Student’s t test was used to compare baseline characteristics between groups.

RESULTS
MMP-9 activity and mass in failing myocardium
Human cardiac MMPs showed gelatinolytic activity in several bands on the overnight incubated, Coomassie stained, gelatine enriched SDS-PAGE gel. From the known molecular weights of the active forms of MMP-2 and MMP-9, the gelatinolytic bands of the purified proteins MMP-2 and MMP-9, and protein detection using Western blot analysis, active MMP-2 could be identified as a 66 kDa band and MMP-9 as an 86 kDa band at zymography (fig 1). Bands observed in some cases at 92 and 72 kDa should be regarded as gelatinolytic active enzymes, fragments, and proenzymes, partially activated by interaction with SDS-PAGE components.

Concentrations of active forms of MMP-9 measured by ELISA, as well as the gelatinolytic activity of MMP-9, were found to be increased in myocardium from patients with IDC myocardium and coronary artery disease in comparison with the activity in non-failing hearts (table 2). The active form of MMP-2 and its gelatinolytic activity were increased in IDC hearts, but there were no significant differences in MMP-2 determinations between non-failing and coronary diseased myocardium.

Table 1 Primer used to detect mRNA expression by reverse transcriptase polymerase chain reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>PCR product</th>
<th>Tₚ</th>
<th>Accession code</th>
</tr>
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<tbody>
<tr>
<td>MMP-1</td>
<td>AgCAAAACATCgtACCTAC</td>
<td>TAAaAgACATCAGCCCTCC</td>
<td>564</td>
<td>55.3 X 54925</td>
<td></td>
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<tr>
<td>MMP-2</td>
<td>AgTCtGCtCCTCTCCTC</td>
<td>AACCCCATGACtGTCCCT</td>
<td>833</td>
<td>59.8 NM00453</td>
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</tr>
<tr>
<td>MMP-3</td>
<td>ACCCACCTCTACACTACAC</td>
<td>CtgTGATCCTCTCAAgTgTgTC</td>
<td>536</td>
<td>56.9 J 03209</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>CCAGTTCCCAAACCTCT</td>
<td>AcAgTagggCCgtAgAg</td>
<td>479</td>
<td>55.0 NM004994</td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>gAAAACgCAATTTAggAAC</td>
<td>gAgTACCCGGTAgACC</td>
<td>303</td>
<td>60.0 NM004994</td>
<td></td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase; PCR, polymerase chain reaction; Tₚ, annealing temperature; TIMP, tissue inhibitor of metalloproteinases.

RNA isolation: cDNA synthesis and quantitative RT-PCR
Total RNA from myocardial tissue was isolated according to the RNaseasy protocol from Qiagen (Hilden, Germany) and converted to cDNA using a commercially available kit from Promega (Madison, Wisconsin, USA). The polymerase chain reaction (PCR) MIMIC construction kit from Clontech (Palo Alto, California, USA) was used to produce a DNA fragment of known size and amount, with primer binding sites for the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as previously reported. To normalise cDNA samples, a competitive PCR from the housekeeping gene GAPDH was used at a constant concentration, under conditions reported before. Separate reverse transcriptase polymerase chain reaction assays (RT-PCR) from 2 µl of the equilibrated cDNA were done using the GAPDH primer pair (forward: Y AgAC AAC ATC GCT CAC AAG AC; reverse: 5' GAG GCA TTG CTT ATG ATC TTG, patent pending) and the primer specified in table 1. The resulting PCR products were separated on 2.0% agarose gel. The MMP mRNA results are given in arbitrary units expressing the videodensitometrically determined ratio of the MMP to the GAPDH RT-PCR products.

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Source of increased MMP activity

Expression of MMP-2 mRNA measured by quantitative RT-PCR showed no significant differences between coronary artery disease, IDCM, and non-failing myocardium (table 3). With respect to MMP-9 expression, no increase could be detected in coronary artery disease and IDCM myocardium in comparison with non-failing myocardium. In coronary artery disease, MMP-9 mRNA expression was detectable only by a highly sensitive nested PCR.

Except for the expression of MMP-1 in one patient with IDCM, we could not detect MMP-1 or MMP-3 expression in any of the diseased myocardium samples or in non-failing myocardium. TIMP-2 was highly expressed in all groups, with a tendency to increased expression in the diseased hearts. This did not reach significance because of the highly scattered data in both the IDCM and the coronary artery disease groups.

Effects of captopril, lisinopril, and ramiprilate on human MMP-9 in vitro

The inhibitory capacity of each ACE inhibitor on the MMPs was titrated. Figure 2 shows a representative example of dose dependent inhibition of zymographic activity by captopril. To compare the inhibitory effects of different ACE inhibitors on MMP-2 and MMP-9 in vitro, we titrated MMP inhibition of each ACE inhibitor until the gelatinolytic activity in zymography gel had disappeared. Untreated controls were regarded as
having 100% activity. Table 4 shows the ACE inhibitor concentration that inhibits 50% of the gelatinolytic activity (IC50). Owing to the methodological characteristics of zymography and the fact that we used heart extracts containing several gelatinolytic enzymes, fragments, and activated proenzymes rather than purified enzymes, the IC50 value is only approximate. Captopril and ramiprilate significantly inhibited MMP-2 and MMP-9 activity in vitro, while lisinopril inhibited MMP-9 but not MMP-2 (table 4). Inhibition of MMP activity was blunted by excess zinc.

**DISCUSSION**

The extracellular matrix (ECM) is responsible for cardiac cell alignment and myocardial structural integrity. Synthesis and degradation of ECM are balanced and tightly controlled in normal myocardium. In pathophysiological processes, an imbalance of protease/antiprotease systems occurs, resulting in qualitative and quantitative alterations in matrix composition. All four categories of proteases (serine, cysteine, and aspartic proteinases, and metalloproteinases) having 100% activity. Table 4 shows the ACE inhibitor concentration that inhibits 50% of the gelatinolytic activity (IC50). Owing to the methodological characteristics of zymography and the fact that we used heart extracts containing several gelatinolytic enzymes, fragments, and activated proenzymes rather than purified enzymes, the IC50 value is only approximate. Captopril and ramiprilate significantly inhibited MMP-2 and MMP-9 activity in vitro, while lisinopril inhibited MMP-9 but not MMP-2 (table 4). Inhibition of MMP activity was blunted by excess zinc.

**DISCUSSION**

The extracellular matrix (ECM) is responsible for cardiac cell alignment and myocardial structural integrity. Synthesis and degradation of ECM are balanced and tightly controlled in normal myocardium. In pathophysiological processes, an imbalance of protease/antiprotease systems occurs, resulting in quantitative and qualitative alterations in matrix composition. All four categories of proteases (serine, cysteine, and aspartic proteinases, and metalloproteinases)
have been implicated in the proteolytic process. As collagen represents the major structural protein of ECM, it has long been postulated that collagenolytic MMP plays a pivotal role in cardiac remodelling. Inhibition of the proteolytic activity of MMP has been suggested as a potential therapeutic target in various diseases, including heart failure.10–21

The data we present here show different regulatory patterns of MMP depending on the underlying aetiology of heart failure. MMP-9 upregulation is a common feature of matrix activation in terminal heart failure, irrespective of the underlying disease, whereas in our study MMP-2 was upregulated only in IDCM and remained unchanged in coronary artery disease—in line with a previous report.22

The fact that we could not detect increased MMP mRNA expression in failing myocardium suggests the presence of additional regulatory mechanisms. In particular, the strikingly low mRNA expression of MMP-9 in coronary diseased myocardium compared with normal myocardium contrasts with the large amount of activated MMP-9 measured by ELISA. A possible explanation for this phenomenon is the circadian synthesis of MMP, with inactive proenzymes kept in stock, implying a chronological imbalance between MMP synthesis and activation.13,23

Sources of MMP are fibroblasts, myocytes, endothelial cells, and infiltrating inflammatory cells.13,23 Monocytes stimulated by tumour necrosis factor α, which have been found to be increased in heart failure, are capable of expressing MMP-9.14,24 It is possible that these cells may serve as carriers of MMP-9 synthesised before they infiltrate the myocardium. This hypothesis is supported by the observation that in reperfusion of infarcted myocardium, infiltrating neutrophils are the predominant source of MMP-9 and activating enzymes.23

Another interesting question arises: what is the result of the abundant MMP-9 in the myocardium in coronary artery disease and IDCM? Heart failure is a progressive disease. MMP-9 is a key enzyme in matrix component degradation, suggesting a role in matrix remodelling that results in left ventricular enlargement and depression of systolic function. Some of the known substrates of MMP-9 are collagen fragments, gelatin, elastin, aggrecan, versican, and fibronectin. Some of these have important roles in the remodelling process. MMP-9 has been implicated in angiogenesis and apoptosis.15–20 Rouet-Benzein and colleagues localised MMP-9 immunohistochemically in cardiomyocytes and showed myosin heavy chain cleavage by MMP-9 in vitro, suggesting a direct impairment of the contractile apparatus by MMP-9.11 There is increasing evidence in studies from transgenic animals that MMP mediates crucial steps in the development of heart failure. The fact that MMP-9 knockout mice survive with no apparent defect has been related to redundancy—expression of related proteins covering for the same function is assumed.21–23 As the disease progresses, the knockout animals show a decreased ability to adapt or compensate. After experimental myocardial infarction in an MMP-9 knockout model, it was convincingly shown that the targeted deletion of MMP-9 attenuated left ventricular enlargement and collagen accumulation.24 In another mouse myocardial infarction model it was found that MMP-9 deficiency protected against cardiac rupture.25

Except for ACE inhibitors, no other drugs used in the care of heart failure patients have been shown to inhibit MMP activity directly. However, the MMP inhibitory capacity of ACE inhibitors determined by zymographic assay in vitro is not easily transferable to the situation in vivo. There are no data on tissue ACE inhibitor concentrations in human heart, but selective enrichment of ACE inhibitors has been assumed.17

Concentrations of ACE inhibitors in zymography are necessarily higher than in blood owing to the need to permeate the SDS-PAGE gel. Other technical points should be mentioned, such as different solubility, different pH, and the application of active metabolites of prodrugs (such as ramiprilate in place of ramipril). However, a non-specific effect of ACE inhibitors on MMP can be excluded by its reversibility by an excess of zinc.

The finding of a direct inhibitory effect of ACE inhibitors on MMP in vitro is of uncertain clinical relevance. The course of the proteolytic activity in the human disease process—from starter event to end stage heart failure—remains unknown. The question of timing arises; do ACE inhibitors affect remodelling more in the earlier stages or in the end stage of heart failure? Do patients who do not respond to ACE inhibition predominantly develop end stage heart failure requiring transplantation?

Conclusions
No definitive statement on the relevance of the interaction of ACE inhibitors and the MMP system is possible. In considering the assumed influence of ACE inhibitors on MMP, we have emphasised the differences in MMP inhibiting capacity between the various different ACE inhibitors.

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