J. Cell. Mol. Med. Vol 9, No 3, 2005 pp. 704-713

Early induction of matrix metalloproteinase-9 transduces signaling in human heart end stage failure

Karni S. Moshal [a], Neetu Tyagi [a], Valerie Moss [a], Brooke Henderson [a], Mesia Steed [a], Alexander Ovechkin [a], Giorgio M. Aru [b], Suresh C. Tyagi [a] *

[a] Department of Physiology and Biophysics, University of Louisville, School of Medicine, Louisville, Kentucky, USA
[b] Department of Cardiothoracic Surgery, University of Mississippi Medical Center, Jackson, Mississippi, USA

Received: June 1, 2005; Accepted: August 25, 2005

Abstract

Extracellular matrix (ECM) turnover is regulated by matrix metalloproteinases (MMPs) and plays an important role in cardiac remodeling. Previous studies from our lab demonstrated an increase in gelatinolytic-MMP-2 and -9 activities in endocardial tissue from ischemic cardiomyopathic (ICM) and idiopathic dilated cardiomyopathic (DCM) hearts. The signaling mechanism responsible for the left ventricular (LV) remodeling, however, is unclear. Administration of cardiac specific inhibitor of metalloproteinase (CIMP) prevented the activation of MMP-2 and -9 in ailing to failing myocardium. Activation of MMP-2 and -9 leads to induction of proteinase activated receptor-1 (PAR-1). We hypothesize that the early induction of MMP-9 is a key regulator for modulating intracellular signaling through activation of PAR and various downstream events which are implicated in development of cardiac fibrosis in an extracellular receptor mediated kinase-1 (ERK-1) and focal adhesion kinase (FAK) dependent manner. To test this hypothesis, explanted human heart tissues from ICM and DCM patients were obtained at the time of orthotopic cardiac transplants. Quantitative analysis of MMP-2 and -9 gelatinolytic activities was made by real-time quantitative zymography. Gel phosphorylation staining for PAR-1 showed a significant increase in ICM hearts. Western blot and RT-PCR analysis and in-situ labeling, showed significant increased expression of PAR-1, ERK-1and FAK in ICM and DCM. These observations suggest that the enhanced expression and potentially increased activity of LV myocardial MMP-9 triggers the signal cascade instigating cardiac remodeling. This early mechanism for the initiation of LV remodeling appears to have a role in end-stage human heart failure.

Keywords: real time zymography • PAR-1 • ERK-1 • FAK • capillaries • phosphorylation • kinase • endothelial myocyte coupling • cardiac synchronization

Introduction

Key pathological markers for end-stage human heart failure include myocardial collagen accumulation, disorganization of the cardiac myofibrils, extracellular matrix (ECM) remodeling, which contribute to diminish systolic performance as well as decreased compliance [1]. Increased fibrosis and ventricular dilation have been suggested to be secondary to either myocardial damage due to ischemic cardiomyopathy (ICM) [2] or inflammation in patients with idiopathic dilated cardiomyopathy (DCM).

ECM remodeling contributes to progressive LV remodeling, dilation, and heart failure [3]. Most tis-

* Correspondence to: Suresh C. TYAGI, PhD
Department of Physiology and Biophysics, School of Medicine
500 S Preston Street, 1115-A, University of Louisville,
Louisville, Kentucky-40202, USA.
Tel.: 502-852-3381; Fax:502-852-6239
E-mail: s0tyag01@louisville.edu

sue disruption of ECM is mediated by extracellular proteinase [4]. Serine, cysteinyl, carboxyl and matrix metalloproteinases (MMPs) are extracellular proteinases stored in tissue [5]. Serine and MMPs which work at neutral pH are involved in remodeling of ECM during tissue development [6–8]. MMPs are released as a latent form of proenzyme and zymogen [9, 10]. We showed that serum containing growth factors and inflammatory cell proteinase can activate MMP by proteolytic cleavage of pro-MMP in human heart fibroblast and endothelial cells [11, 12].

Previous studies clearly demonstrated a significant increase in steady state latent MMPs and disintegrin metalloproteinase in the failing human myocardium [13]. Recent studies have also demonstrated that MMP-9 content and activity are increased in the failing human heart [14–17] and MMPs are implicated in the progression of ventricular dilation and the development of heart failure [18–20]. We have shown that MMP-9 induced shedding of adhesion molecule (β-integrin), which may instigate signal transduction by activating the focal adhesion kinase (FAK) leads to extracellular remodeling induced by the heart failure.

There is an increasing evidence for physiological and patho-physiological role of protease activated receptors (PAR) in multiple tissues, including the cardiovascular system [21]. Although there have been many studies of PAR mediated intracellular signaling the signaling pathway following activation of PAR has not been elucidated. Recent data support the role of G-protein coupled PAR in the activation of downstream signaling pathways involving MAP Kinases (ERK-1) and FAK, resulting in altered pathophysiological conditions. Interestingly, we showed that administration of cardiac specific inhibitor of metalloproteinase (CIMP) ameliorated MMP-2 and -9 activities in chronic volume overloaded heart failure [22]. We further demonstrated an induction of PAR-1 in this chronic heart failure model that was prevented by the administration of CIMP [23]. We hypothesize that there is an early activation of MMP-9 in infarcted hearts which acts as an extracellular insult, inducing the phosphorylation of PAR-1 which activates the downstream MAPK-ERK-1 pathway.

Material and methods

Source of tissue

Human heart tissue was obtained at the time of orthotropic cardiac transplantation in the operating room at the University of Mississippi Medical Center. Myocardial tissue from the failing heart was examined in 10 patients with documented coronary artery disease and myocardial infarction (ischemic cardiomyopathy, ICM) and in 10 patients with idiopathic dilated cardiomyopathy (DCM). All patients were in either New York Heart Association functional class III or IV (Table 1). Five normal control samples were obtained from unused hearts. The average age of infracts was 4±1 yr. Tissue specimens from ICM, DCM and normal hearts were collected and processed within 1–2h. An Institutional Review Board waiver was obtained before collection of the tissue from explanted hearts.

Preparation of cardiac tissue homogenates

Cardiac tissue extracts were prepared as described previously [24]. Briefly, tissues were washed several times with cold PBS to get rid of blood contamination, cut into small pieces followed by incubation overnight in cacodylic acid extraction buffer (10mM cacodylic acid (pH 5.0), 0.15M NaCl, 20mM $CaCl_2$, 1.5mM NaN_3, 1μM $ZnCl_2$ and 0.01% triton X-100) in a shaker at 4°C. Homogenates were centrifuged at 12000xg and supernatant was stored at -80°C.

Real time semi-quantitative zymography

In gelatin zymography there is no need to use a serine protease inhibitor. Latent MMPs are always visualized by SDS-PAGE zymography; this is one of the advantages of the method. Others have used fluorogenic probes to determine real time MMP activity [25, 26]. We determine real time active MMP molecules by "semi-quantitative" zymography. Total protein in the tissue extract was estimated according to the method of Bradford [27]. In order to analyze the activities of MMP-2 and -9 at different time intervals, gelatin zymography was done as described previously [24] with slight modification. Briefly, 20μg protein was electrophoresed in non-reducing conditions on an 8% SDS-PAGE containing gelatin (1mg/ml) from porcine skin (Sigma Chemicals, St Louis, MO). After electrophoresis, gels were rinsed two times with renatura-

tion solution, 2.5% Triton X-100 (30 min each). The gels were then washed with water to remove excess triton X-100, followed by incubation of the appropriately cut strips in the developing buffer (50mM Tris base, 5mM $CaCl_2$ pH 7.6) at 37°C for different time intervals. After an appropriate period of time, strips were taken out, stained with coomassie blue and analyzed for MMP-2 and -9 activities. Bands were analyzed densitometrically using BioRad software (Quantity One). In zymography, active MMP is mainly visualized, although some time, due to unfolding by SDS, latent MMP can be seen. However, the goal of this study was to measure levels of active MMP-2 and -9 molecules in heart failure tissue versus normal hearts, therefore, we measured MMP activity in the time-dependent fashion.

Reverse Transcriptase-PCR

Total RNA was isolated from Human heart tissue with TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions and quantified by measuring the absorbance at 260nm. RNA revealing equally intense fluorescence bands of 18S and 28S rRNA was used. The cDNA was synthesized from 1µg total RNA in a final reaction volume of 20µl by reverse transcriptase (RT). The RNA samples were incubated (70°C, 5 min) with 1µl oligo (deoxythymidine) primers in a final volume of 5µl. Samples were then incubated (42°C, 60 min) in 15µl of a reaction buffer (1x) containing $MgCl_2$; 5mM, deoxynucleotide triphosphates (dNTPs; 10mM) and Im-Prom-II™ reverse transcriptase (1µl/15µl reaction) and rRNasin (Promega, Madison) as RNase-inhibitor (20U) 15µl. The reaction volumes were brought up to 15µl with autoclaved water. A negative control was included.

The expression level of the RNA was determined by Semi-Quantitative PCR. PCR amplification was done with 2µl of each cDNA sample. Amplification of human G_3PDH cDNA, was evaluated using the primer sequences, 5'-TGA AGG TCG GAG TCA ACG GAT TTGGT-3'(sense) and 5'-CAT GTG GGC CATGAG-GTCCACCAC-3'(anti-sense) with a predicted size of 983bp. Primer pairs for amplification of human PAR-1 cDNA were 5'-CAGTTTGGGTCTGAATTGTGTCG-3' (sense) and 5'-TGCACGAGCTTATGCTGCTGAC-3'(anti-sense) with a predicted size 505bp. Primer pairs for amplification of human FAK cDNA were 5'CAGCT-TACAGCCCAACATGA-3'(sense) 5'-GCCAGTCTTTC-CGTTCAGAG-3'(anti-sense) with a predicted size 313bp. Primer pairs for human ERK-1 cDNA were 5'-ACTATGACCCGACGGATGAG-3'(sense) 5-CTAAC-AGTCTGGCGGGAGAG-3'(anti-sense) with a predicted size of 215bp.

The denaturation step was carried out for 30s at 94°C, annealing for 2min at 60°C, and extension for 3min cycles at 72°C. G_3PDH samples were taken at 25 cycles and FAK, PAR-1; ERK-1 samples were taken at 30 cycles. The final extension step was performed at 72°C for 3min. Amplification mixtures were analyzed by 1% agarose gel electrophoresis containing ethidium bromide.

Antibodies and Western blotting

Tissue homogenates were prepared. Aliquots containing equal amount of proteins were resolved by 10% Laemmli SDS-PAGE under reducing conditions followed by electro-transfer overnight at 0.03 A in cold to immuno-blot PVDF membrane (Bio-Rad, Hercules, CA). The non-specific sites on the membrane were blocked by 5% (w/v) fat-free milk solution. Membranes were washed 3 times (10min each) with PBS-T. Immuno-detection was carried out by incubation of PVDF membrane with anti-PAR-1, ERK-1 (Zymed, San Francisco, CA) and FAK antibodies (Neomarkers, Fremont, CA) diluted 1:1000 in PBS, followed by washing four times and incubation with anti-goat alkaline-phosphatase secondary antibody (1:1000 dilution). Bands were developed using AP-conjugate substrate kit (Bio-Rad, Hercules, CA).

Phosphoprotein gel staining

To verify PAR-1 phosphorylation, phosphoprotein gel staining was performed in reducing 8% polyacrylamide gels as per manufacturer's guidelines. Briefly, tissue extract was desalted and resuspended in 2x reducing gel loading buffer. An equal amount of protein was loaded followed by standard SDS-PAGE. After electrophoresis, gels were immersed in fixing solution (50% methanol and 10% acetic acid) for 30min, followed by four washings (each for 10min) with dH_2O. After washing, the gels were incubated in the dark in Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR) with gentle agitation for 2h, followed by destaining for 3h. Immunoprecipitation was done using anti-IgG agarose for 30min at 4°C in a rocker. Supernatant was collected and used after centrifugation at 1400xg for 10min. After phospho-staining, the gel was stained for 3h with coomassie blue for the total protein. Stained gels were visualized using

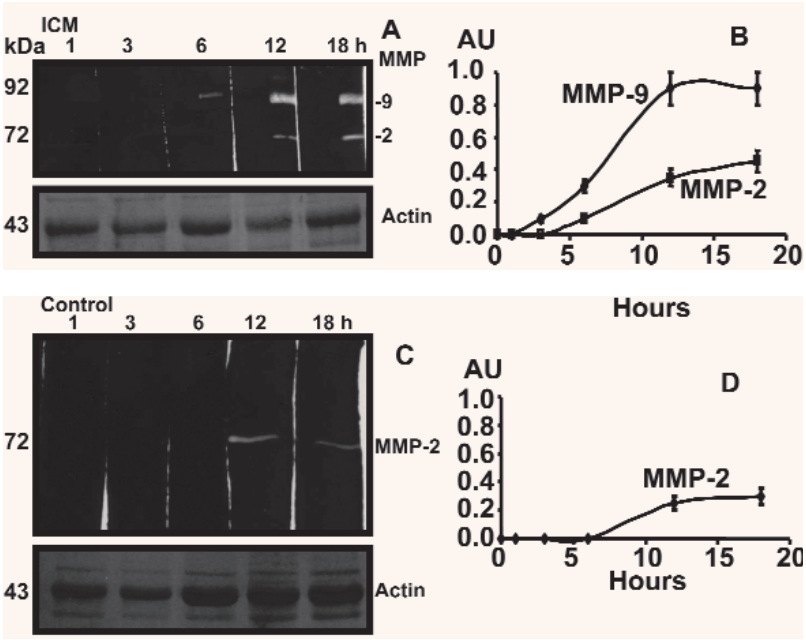

Fig. 1 Real time semi-quantitative zymographic (RT-Q-Zymography) analysis of MMP-2 and -9 activities in failing human myocardium: (A), ischemic cardiomyopathic (ICM) left ventricle (LV) tissue homogenates were loaded onto 1% gelatin zymographic gels. After electrophoresis strips were separated and incubated in activation buffer. Strips were taken out at different times 1, 3, 6, 12 and 18 hours after incubation. Corresponding actin bands are shown. Identical amounts of protein were loaded on to each lane; (C), Control hearts. (B), ICM; (D), Normal hearts; bands were scanned and normalized with actin and represented by line graphs. Each point represents an average ± SD from 5 different samples. Molecular weight markers are shown on left of the gels.

UV trans-illumination (Kodak Gel Logic 200 Imaging System).

Histology and *in situ* labeling

Tissue sections (10 μm thick) were prepared from paraffin-embedded formalin-fixed tissues. The tissue was deparaffinized by xylene, followed by labeling with van Gieson stain for vascular elastin, anti-CD-31/PECAM-1 for endothelial cells. Secondary IgG-FITC was used for fluorescence detection.

Statistical analysis

Data are presented as average ± S.D. Student's t-test was used to comparing results for ICM and DCM hearts with normal hearts. Statistical significance was considered at $p<0.05$

Results

Early induction of MMP-9

Real-time-quantitative zymography was performed, to determine number of active MMP-2 and -9 molecules per samples. In the failing human myocardium, levels of active MMP-9 molecules were apparent as early as 6h, whereas in normal hearts, there was no induction of MMP-9 activity, and the levels of constitutive MMP-2 was not apparent till 12h. The levels of active MMP-2 molecules were also induced in ICM as compared to normal hearts (Fig. 1). Similar results were obtained for DCM. The active form of MMP-2 and -9 are less than 72 and 92kD, respectively. In heart failure, we found all active form very little in latent form. In normal hearts, however, we found mostly the latent form. These observations suggest that active MMP-9 molecules appear prior to MMP-2, and there were more active MMP-2, and -9 molecules in failing myocardium at a time compared to normal hearts.

PAR-1, FAK, ERK-1

To determine whether active MMP-9 transduces *via* the activation of PAR-1, the levels of PAR-1 were measured. The levels of PAR-1 were elevated in ICM and DCM as compared to normal myocardium (Fig. 2). The phosphorylation of PAR-1 was measured using in-gel phosphorylation staining. The immuno-precipitation experiments clearly showed phosphorylation of PAR-1 (Fig. 2C). The levels of active FAK were also increased in ICM and DCM hearts as compared with normal hearts (Fig. 3). In

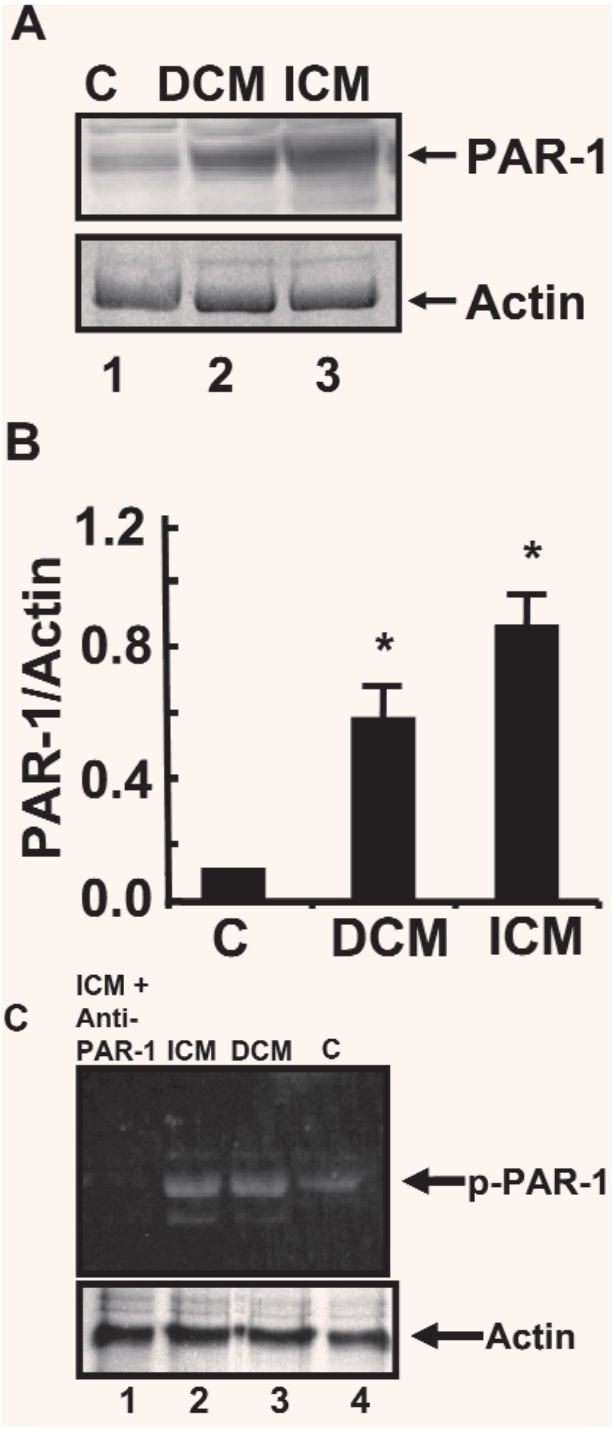

Fig. 2 Western blot analysis of proteinase activated receptor-1 (PAR-1). (A): Identical amounts of LV tissue homogenates from control, ischemic cardiomyopathic (ICM) and idiopathic dilated cardiomyopathic (DCM) hearts were loaded on to reducing SDS-PAGE. Protein was blotted and labeled with anti-PAR-1 and anti-β-actin antibodies. (B): Increase in PAR-1 expression normalized with actin levels is represented by bar graphs. Each bar represents an average ± SD from 5 different samples. (C): PAR-1 phosphorylation (p-PAR-1): Gels were incubated in phosphorylating buffer (Molecular Probes). The phosphorylated bands were detected by UV-illumination. Lane 1, ICM tissue homogenates immuno-precipitated with anti-PAR-1 antibody prior to loading on to the gels; lane 2, ICM; lane 3, DCM; lane 4, controls. (C): Arrow indicates phosphorylated bands. The corresponding actin bands are shown.

addition, the levels of active ERK-1 were also induced in ICM and DCM as compared with control hearts (Fig. 3). These results suggested that receptor phosphorylation is involved in activation of down stream signaling molecules FAK and/or ERK-1, causing cardiac remodeling.

Semi-quantitative RT-PCR analysis for PAR-1, ERK-1 and FAK genes showed that the level of mRNA expression of PAR-1, ERK-1 and FAK were significantly increased in ICM and DCM hearts as compared to normal hearts (Fig. 4).

In situ labeling

We evaluated intactness of vascular structures in normal hearts by van Gieson labeling. The labeling with anti-CD-31 suggested organized endothelial cells in normal myocardium (Fig. 5). The staining of tissue sections from ICM hearts for endothelial capillaries showed an increased expression of FAK (Fig. 6). The labeling with anti-PAR-1 and ERK-1 suggested their increased expression around capillary endothelial cells (Fig. 7).

Discussion

Increased myocardial MMP-2 and -9 activities are associated with changes in LV function and geometry in the rat model of overload [28]. Previous studies from our lab [11–15] showed that there was an increase in MMP-2 and MMP-9 activity with DCM and ICM hearts which contributes to LV remodeling. The activation kinetics for MMPs and the mechanism for LV remodeling were largely unknown. We would like to suggest, based on the present results that at a given time there are more active MMP-9 molecules in ICM or DCM than in the normal hearts. The similar case is also true for MMP-2. With the help of real

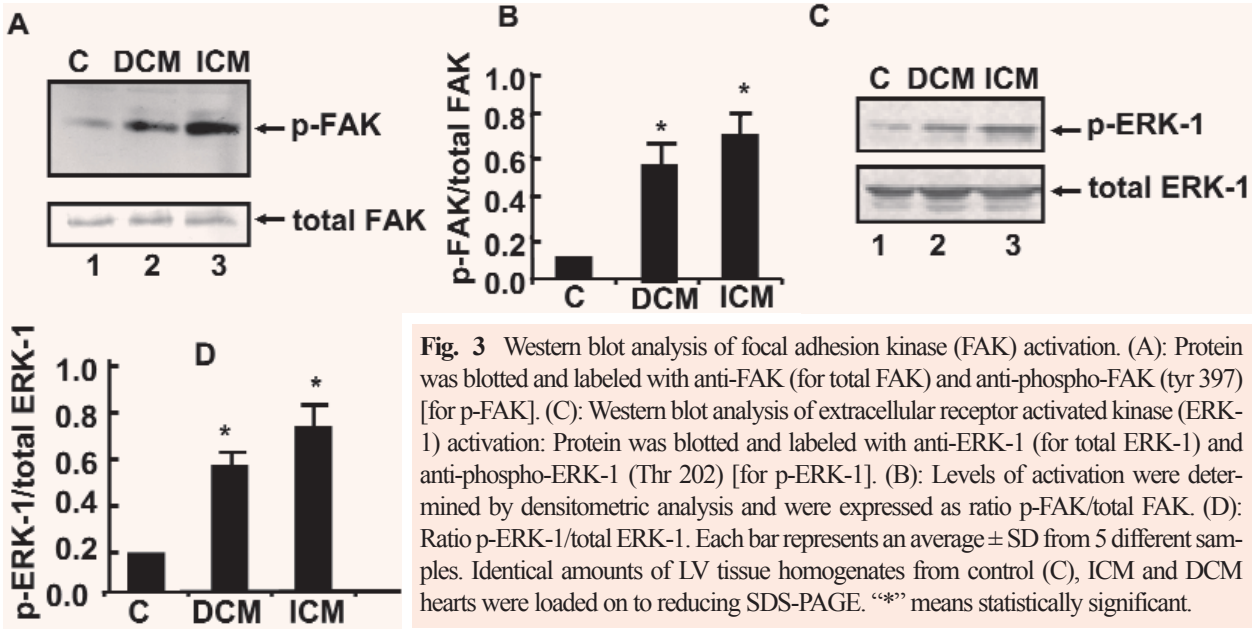

Fig. 3 Western blot analysis of focal adhesion kinase (FAK) activation. (A): Protein was blotted and labeled with anti-FAK (for total FAK) and anti-phospho-FAK (tyr 397) [for p-FAK]. (C): Western blot analysis of extracellular receptor activated kinase (ERK-1) activation: Protein was blotted and labeled with anti-ERK-1 (for total ERK-1) and anti-phospho-ERK-1 (Thr 202) [for p-ERK-1]. (B): Levels of activation were determined by densitometric analysis and were expressed as ratio p-FAK/total FAK. (D): Ratio p-ERK-1/total ERK-1. Each bar represents an average ± SD from 5 different samples. Identical amounts of LV tissue homogenates from control (C), ICM and DCM hearts were loaded on to reducing SDS-PAGE. "*" means statistically significant.

time quantitative zymography, we demonstrated for the first time that there is an early and enhanced level of active MMP-9 molecules compared to MMP-2. However, MMP-2 activity was also higher in the human end-stage failing heart compared to the normal human heart. These findings are in accordance with Bendeck *et al.* [29], which suggested that MMP-9 is expressed within 6h after injury in rat carotid arteries and continues to be expressed up to 6 days, whereas MMP-2 activity is markedly increased after 4 days of injury. MMP-9 up-regulation is a common feature of matrix activation in terminal heart failure, irrespective of the underlying disease. 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 [30].

As our previous studies have shown, serum containing growth factors and inflammatory molecules can activate MMPs by proteolytic cleavage of pro-

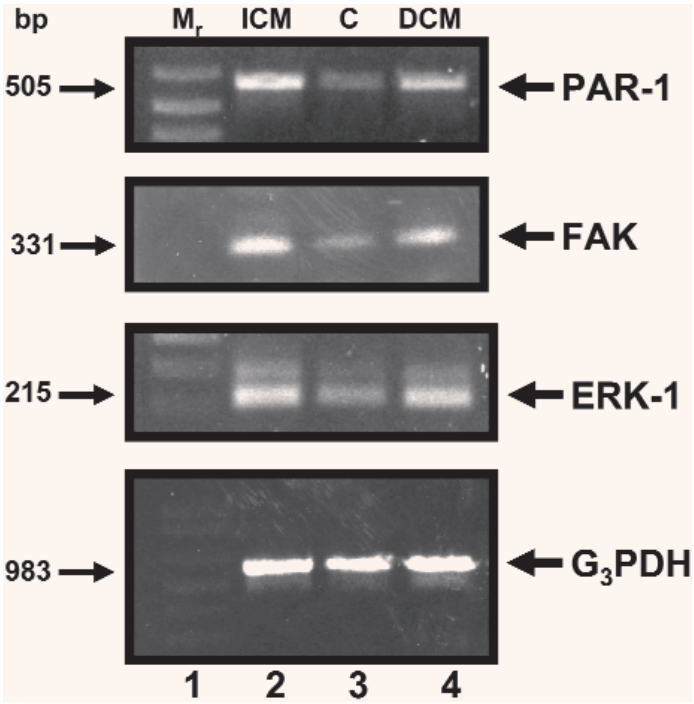

Fig. 4 Semi-quantitative RT-PCR analysis of PAR-1, FAK, and ERK-1 in LV of control (C), ICM and DCM hearts: Identical amounts of total mRNA was reverse transcribed to cDNA. PCR was performed to measure the levels of mRNA for PAR-1, FAK, and ERK-1 as compared to control G_3PDH. Lane 1, molecular weight markers; lane 2, ICM; lane 3, control; lane 4, DCM. Arrows in the left side represent corresponding molecular weights.

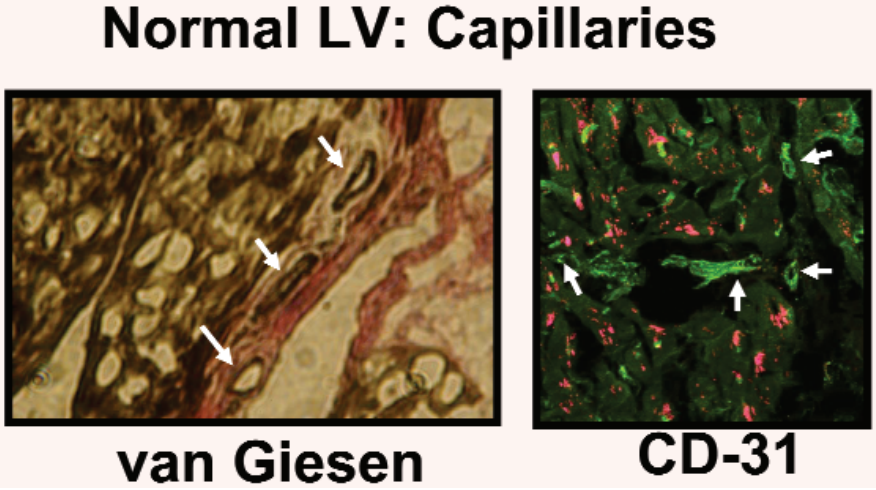

Fig. 5 Normal LV tissue was labeled with van Giesen for vascularity and anti-CD-31 antibody-FITC for endothelium. Paraffinized LV tissues were cut into 10 µm thin sections. The de-paraffinized sections were labeled. The arrows indicate vessels in van Giesen stains and capillary endothelial cells in the CD-31 stains. 40 x magnifications.

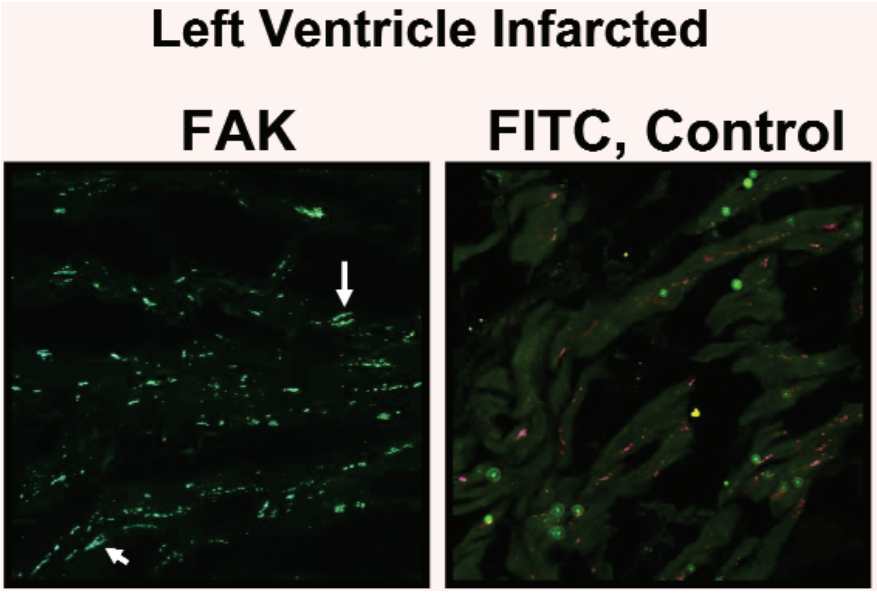

Fig. 6 ICM LV tissue was stained with primary anti-FAK antibody, and labeled with secondary IgG-FITC. Negative controls were labeled with IgG-FITC alone, shown in right panel. Paraffinized 10µm tissue sections were de-paraffinized and labeled with respective antibodies. Arrows indicate FAK labeling in capillary endothelium. 40 x magnifications.

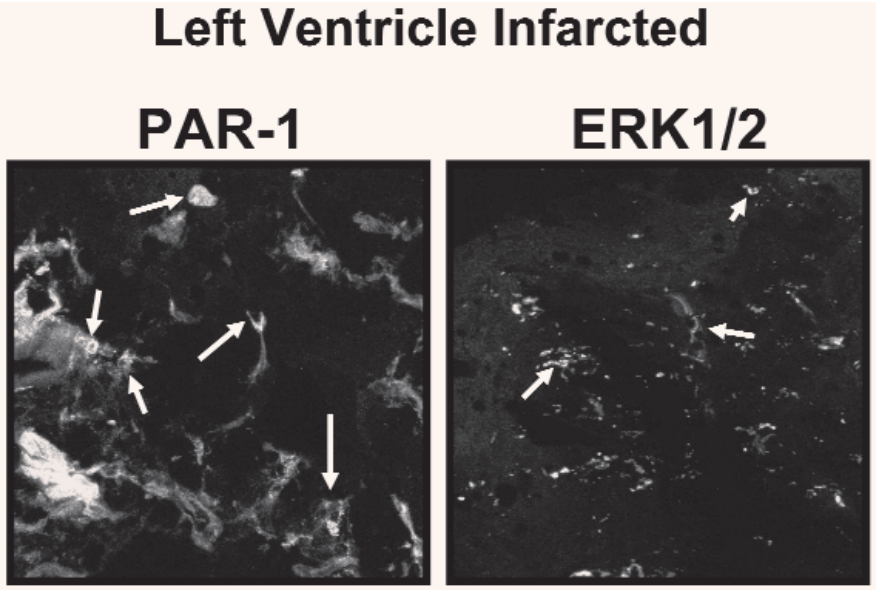

Fig. 7 ICM LV tissue was stained with primary anti-PAR-1 (left panel) and ERK-1 (right panel) antibodies, and labeled with secondary IgG-FITC. Paraffinized 10µm tissue sections were de-paraffinized and labeled with respective antibodies. Arrows indicate PAR-1 and ERK-1 labeling in capillary endothelium. 40 x magnifications.

Table 1 Clinical data of DCM and ICM heart failure patients

	Age (years)	Sex	LV volume, ml	%EF
Normal	26±10	M/F	85±11	N/A
ICM	54±7	M/F	180±20	30±5
DCM	56±8	M/F	175±15	35±7

MMP in human heart fibroblast and endothelial cells [11, 12]. It is likely that intracellular signaling transduction pathways activated by MMPs are mitogen-activated protein kinase (MAPK) pathways. Hence, regarding the mechanism responsible for LV remodeling in failing human heart tissue, we propose that MMP-9 is the key regulator for LV remodeling through activation of PAR and/or shedding of integrin molecule by instigating the downstream signaling cascade for MAP Kinase-ERK and FAK.

Damiano *et al.* [31] noticed that cardiovascular responses are mediated by protease-activated-receptor-2 and thrombin receptor (PAR-1) and are distinguished in mice deficient in PAR-1 and -2. Cho *et al.* [32] showed that p38 MAPK and ERK pathways contribute to the transcriptional regulation of MMP-9 in arterial smooth muscle cells, but they haven't mentioned any role for PAR in activation of MAPK. Our present study showed that activation of MMP-9 leads to phosphorylation of PAR-1 there by activating MAPK-ERK-1 and FAK.

Sabri *et al.* [33] found that serine protease (thrombin) activated the PAR-1, a high affinity receptor for thrombin which stimulates extracellular signal-regulated protein kinases and modulates contractile function in cardiomyocytes. Our findings are in concordance with Sabri *et al.* [33], that the phosphorylation of PAR leads to activation of signal regulated protein kinases (ERK-1 and FAK) causing cardiac dysfunction and uncoupling of cardiac synchronization.

Although the present study was carried out in human heart samples, it did not establish a "cause-and-effect" relationship between MMP-9 and PAR-1. It is equally likely that an increase in PAR-1 activation leads to increased MMP-9 expression, or those they are completely independent events. However, in an experimental study of chronic heart failure, we demonstrated that activation of MMP-9 in chronic

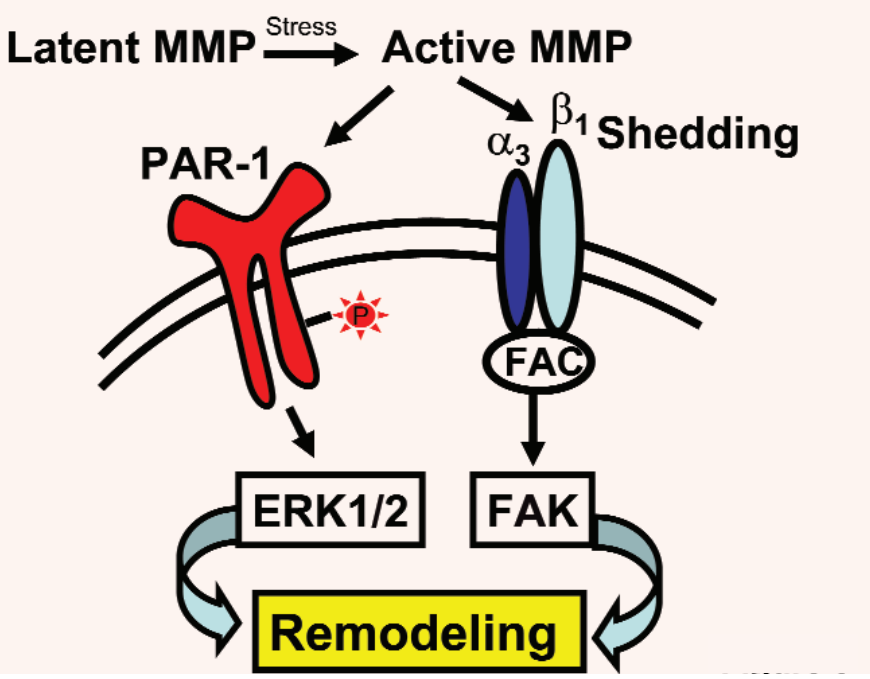

Fig. 8 Schematic presentation of plausible mechanism of activation of latent myocardial MMP and signal transduction. The MMP in the active form activates PAR-1 and sheds the beta-1 integrin. This transduces activation of FAK and ERK-1, leading to cardiac remodeling.

volume overload was ameliorated by exogenous administration of cardiac specific inhibitor of metalloproteinase that inhibited MMP-9 [22]. We also showed that PAR-1 was activated in chronic increased preload and that the administration of inhibitor suppressed the PAR-1 expression [23]. Collectively, we suggest that early induction of MMP-9 induces PAR-1.

The focus of the present study was to measure MMP and not the serine protease. Given the fact that the thrombin-mediated coagulation system is activated in dilated cardiomyopathy [34, 35], these data show the increased PAR-1 activity localized to the endothelium. Previously, we showed that MMP-9 was expressed in microvascular endothelium [11, 12], therefore, it seems likely that thrombin (a serine proteinase) and MMP-9 both are present within the microvasculature.

The limitation of our study was that although we detected MMP-9 earlier (at 6h) as compared to MMP-2 (appearing for the first time at 12h) in the developing buffer and not necessarily earlier expressed and activated in the tissue; we have not discriminated between the infarcted hearts in term of age of the infarction. To determine whether PAR-1 activation is MMP dependent, additional experiments are required using MMP inhibitors such as CIMP or phenathroline. Also to block ERK1/2 by PD98059, we will demonstrate whether ERK1/2 is involved in MMP activation. These experiments are in progress.

We conclude that in ICM, there was a significant increase in the expression of PAR-1, ERK and FAK, both at gene and protein levels followed by DCM and normal heart tissues. The results support the hypothesis that MMP-9 plays an important role in intracellular signal transduction by activating MAP Kinase-ERK and FAK, thereby modulating the transcriptional factors, important in extracellular matrix remodeling (Fig. 8).

Clinical significance of the study

The findings of the present study provide direct evidence that robust and early changes in LV myocardial MMPs occur in the progression of heart failure and provide a potential novel pharmacological target for modulating LV structure and geometry in this pathological process.

Acknowledgements

A part of this study was supported by NIH grants HL-71010 and HL-74185.

References

1. **Brilla CG, Rupp H.** Myocardial collagen matrix remodeling and congestive heart failure. *Cardiologia* 1994; 39: 389–93.
2. **Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblick EH, Olivetti G, Anversa P.** Structural basis of end-stage failure in ischemic cardiomyopathy in humans. *Circulation* 1994; 89: 151–63.
3. **Factor SM.** Role of extracellular matrix in dilated cardiomyopathy. *Heart Fail.* 1994; 9: 260–8.
4. **Alexander CM, Werb Z.** Proteinases and extracellular matrix remodeling. *Curr Opin Cell Biol.* 1989; 1: 974–82.
5. **Khohka R, Denhardt DT.** Matrix metaloproteinases and tissue inhibitors of metalloproteinases: a review of their role in tumorigenesis and tissue invasion. *Invasion Metastasis* 1989; 9: 391–405.
6. **Dano K, Andreason PA Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L.** Plasminogen activators, tissue degradation and cancer. *Adv Cancer Res* 1985; 44: 139–266.
7. **Mignatti P, Robbins E, Rifkin DB.** Tumor invasion through human amniotic membrane: requirement for a proteinase cascade. *Cell* 1986; 47: 487–98.
8. **Liotta LA, Steeg PS, Stetler-Stevenson WG.** Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; 64:327–36.
9. **Harper E, Blotch KJ, Gross J.** The zymogen of tadpole collagenase. *Biochemistry* 1971; 10: 3035–41.
10. **Vaes G.** A latent collagenase by bone and skin explant in culture. *Biochem J* 1971; 123: 23–4.
11. **Tyagi SC, Ratzaska A, Weber KT.** Myocardial matrix metalloproteinase: activation and localization. *Mol Cell Biochem.* 1993; 126: 49–59.
12. **Tyagi SC, Kumar SG, Glover G.** Induction of tissue inhibitor and matrix metalloproteinases by serum in human-heart derived fibroblast and endomyocardial endothelial cells. *J Cell Biochem* 1995; 58: 360–72.
13. **Hunt MJ, Aru GM, Hayden MR, Moore CK, Hoit BD, Tyagi SC.** Induction of oxidative stress and disintegrin metallo proteinase in human heart end stage failure. *Am J Lung Cell Cell Mol Physiol* 2002; 283: L239–45.
14. **Tyagi SC, Campbell SE, Reddy HK, Tjahja E, Voelker DJ.** Matrix metalloproteinase activity expression in

infracted, non-infracted and dilated cardiomyopathic human hearts. *Mol Cell Biochem* 1996; 155: 13–21.

15. **Tyagi SC, Kumar SG, Haas SJ, Reddy HK, Voelker DJ, Hayden MR, Demmy TL, Schmaltz RA, Curtis JJ.** Post-transcriptional regulation of extracellular matrix metalloproteinase in human heart end stage failure secondary to ischemic cardiomyopathy. *J Mol Cell Cardiol.* 1996; 28: 1415–28.
16. **Thomas CV, Coker ML, Zellner JL, Handy JR, Crumbley AJ III, Spinale FG.** Increased matrix metalloproteinase activity and selective up-regulation in LV myocardium from patients with end-stage dilated cardiomyopathy. *Circulation* 1998; 97: 1708–15.
17. **Li YY, Feldman AM, Sun Y, McTiernan CF.** Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. *Circulation* 1998; 98: 1728–34.
18. **Mann DL, Spinale FG.** Activation of matrix metallo proteinases in the failing human heart: breaking the tie that binds. *Circulation* 1998; 98: 1699–1702.
19. **Coker ML, Thomas CV, Clair MJ, Hendrick JW, Krombach RS, Galis ZS, Spinale FG.** Myocardial matrix metallo proteinase activity and abundance with congestive heart failure. *Am J Physiol.* 1998; 274: H1516–23.
20. **Spinale FG, Coker ML, Thomas CV, Walker JD, Mukherjee R, Hebbar L.** Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: relation to ventricular and myocyte function. *Circ Res.* 1998; 82: 482–95.
21. **Kawabata A, Kuroda R.** Protease-activated receptor (PAR), novel family of G protein-coupled seven transmembrane domain receptors: activation mechanisms and physiological roles. *Jpn J Pharmacol.* 2000; 82: 171–4.
22. **Cox MJ, Hawkins UA, Hoit BD, Tyagi SC.** Attenuation of oxidative stress and remodeling by cardiac inhibitor of metalloproteinase protein transfer. *Circulation* 2004; 109: 2123–8.
23. **Moshal KS, Tyagi N, Henderson B, Ovechkin AV, Tyagi SC.** Protease activated receptor and endothelial-myocyte uncoupling in chronic heart failure. *Am J Physiol Heart Circ Physiol.* 2005; 288: H2770–7.
24. **Tyagi SC, Matsubara L, Weber KT.** Direct extraction and estimation of collagenase(s) activity by zymography in microquantities of the rat myocardium and uterus. *Clin Biochem.* 1993; 26: 191–8.
25. **Hattori S, Fujisaki H, Kiriyama T, Yokoyama T, Irie S.** Real-time zymography and reverse zymography: a method for decting activities of MMP and their inhibitors using FITC-labeled collagen and casein as substrates. *Anal Biochem*. 2002; 301: 27–34.
26. **Deschamps AM, Apple KA, Leonardi AH, McLean JE, Yarbrough WM, Stroud RE, Clark LL, Sample JA, Spinale FG.** Myocardial interstitial MMP activity is altered by mechanical changes in LV load interaction with the angiotensin type 1 receptor. *Cir Res*. 2005; 96: 1110–8.
27. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248–54.
28. **Brower GL, Janicki JS.** Contribution of ventricular remodeling to pathogenesis of heart failure in rats. *Am J Physiol.* 2001; 280: H674–83.
29. **Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA.** Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Cir Res.* 1994; 75: 539–45.
30. **Ducharme A, Frantz S, Aikawa M, Rabkin E, Lindsey M, Rohde LE, Schoen FJ, Kelly RA, Werb Z, Libby P, Lee RT.** Targeted deletion of MMP-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infraction. *J Clin Invest.* 2000; 106: 55–62.
31. **Damiano BP, Cheung WM, Santulli RJ, Fung-Leung WP, Ngo K, Ye RD, Darrow AL, Derian CK, de Garavilla L, Andrade-Gordon P.** Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. *J Pharmacol Exp Ther.* 1999; 288: 671–8.
32. **Cho A, Graves J, Reidy AM.** Mitogen-activated protein kinases mediated matrix metalloproteinase-9 expression in vascular smooth muscle cells. *Arterioscler Thromb Vas Biol.* 2000; 20: 2527–32.
33. **Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, Steinberg SF.** Signaling properties and functions of two distinct cardiomyocyte protease activated receptors. *Circ Res*. 2000; 86: 1054–61.
34. **Riza Erbay A, Turhan H, Aksoy Y, Senen K, Yetkin E.** Activation of coagulation system in dilated cardiomyopathy: comparison of patients with and without left ventricular thrombus. *Coron Artery Dis*. 2004; 15: 265–8.
35. **Yamamoto K, Ikeda U, Furuhashi K, Irokawa M, Nakayama T, Shimada K.** The coagulation system is activated in idiopathic cardiomyopathy. *J Am Coll Cardiol*. 1995; 26: 1757–8.