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Fibrosis in heart failure subtypes

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Abstract. – BACKGROUND: The differences in concentrations of biomarkers between heart failure patients with dilated cardiomyopathy (HF-D) and with ischemic cardiomyopathy (HF-I) have yet to be defined. The objectives of this study were to compare the concentrations and correlation of biomarkers of inflammation, extracellular matrix (ECM) turnover and oxidative stress parameters between these populations.

PATIENTS AND METHODS: Our study consisted of 36 subjects with HF-D (LVSD = 47.2 ± 7.3 mm, LVDD = 65.1 ± 6.3 mm), 44 subjects with HF-I (LVSD = 38.0 ± 4.4 mm, LVDD = 58.5 ± 6.0 mm) and 38 controls without heart failure. Concentrations of matrix metalloproteinase (MMP)-1, MMP-2, MMP-9, MMP-13, Galectin-3, prolidase, TNF-alpha, and oxidative stress index (OSI) were measured.

RESULTS: Serum levels of MMP-2, MMP-9, and prolidase were significantly increased in HF-I group compared to healthy controls (p = 0.039, 0.019, 0.012 respectively), whereas the increases in MMP-1 and MMP-13 were not significant. This significance was stronger in the HF-D group than the HF-I group (*p* = 0.004, 0.001, 0.002 respectively). TNF-α, a marker of inflammation, was significantly increased in heart failure (*p* = 0.004) but there was no difference between HF-D and HF-I groups; however, Galectin-3 was significantly increased in the HF-D group compared to the HF-I group (*p* = 0.005). OSI showed the same response pattern as TNF-α (*p* = 0.019, 0.002 respectively). There was a positive correlation of MMP-9 levels with prolidase activity (r = 0.612, *p*: 0.003).

CONCLUSIONS: MMPs and Galectin-3 are important in cardiac remodeling; prolidase may share an undefined role in fibrosis in heart failure and may have a role in the diffuse fibrosis of heart failure.

Key Words:
Heart failure, Dilated cardiomyopathy, MMP, Prolidase.

Introduction

Heart failure (HF) is a major medical and epidemiological problem. Recent studies, both in acute and chronic HF, indicate that it remains associated with high morbidity and mortality. Cardiac remodeling is an important determinant of the clinical outcome of HF, as it is linked to disease progression and poor prognosis[1]. It has been found that, regardless of etiology, the remodeling process is a common mechanism for the progression of HF[2]. Remodeling takes place in the ECM, which is the structural component of the myocardium, and is continuously being synthesized and degraded[3].

In our study, we aimed to investigate the relationships between biologic markers of ECM with echocardiographic LV geometry in order to correlate the biomarkers to clinical HF. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade all components of the ECM[4]. However, during various cardiovascular diseases or following a myocardial infarction, a shift in the natural balance can result in myocardial remodeling. While myocardial remodeling may initially be compensatory, long-term effects on the myocardial structure and function may promulgate HF[5]. The MMP family includes soluble enzymes including collagenases (MMP-1, -13) that digest structural or fibrillar collagens (types I to III) and gelatinases (MMP-2, -9) that digest denatured collagen (gelatin) and types IV and V collagen[6]. In our study, we measured collagenases (MMP-1 and -13) and gelatinases (MMP-2 and -9). Prolidase is an exopeptidase that is important in collagen turnover, being the main regulatory enzyme in the metabolism of proline and hydroxyproline, which constitutes 20% of all collagen in the human body[7,8]. An increase in collagen turnover leads to enhanced prolidase activity, presumably because, as the final step of collagen degradation, it is a rate-limiting factor in collagen turnover[9,10]. Measuring prolidase activity may be useful in determining the relationship with other collagen destruction biomarkers. Galectin-3 is a member of a large family of β-galactoside-

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binding endogenous lectins. This lectin is a multifunctional factor that binds to distinct glycan and protein ligands[11] and triggers production of MMP; as such, it plays an important regulatory role in cardiac fibrosis and remodeling, which are key contributing mechanisms to the development and progression of HF. The involvement of Galectin-3 in the development of fibrosis has been demonstrated in the heart[12,13]. Because of the role of Galectin-3 in inflammatory and proliferation responses, Galectin-3 and TNF-α level have been studied together[14,15]. Over the past several decades, investigations in both humans and animal models of HF have provided substantial evidence that oxidative stress is increased in HF and contributes to disease progression[16]. There is substantial evidence that oxidative stress can activate many cellular responses that are characteristic of what occurs in HF, including cellular hypertrophy, changes in gene expression and cell death[17], and alterations in the turnover and properties of the ECM[18]. Reactive oxygen and nitrogen species (ROS, RNS) arising from oxidative stress include superoxide, hydroxyl radicals, hydrogen peroxide, and peroxynitrite, which in turn modify myocardial cellular and extracellular protein structure and function[19]. ROS can alter myocardial MMPs' activation state through both transcriptional and post-translational mechanisms[20] and, therefore, may have a role in remodeling of the heart[21]. The pathways for activation of hypertrophic and apoptotic cellular phenotypes appear to involve stress responsive protein kinases, many of which are activated by ROS[17]. The measurement of the serum concentrations of specific oxidants and antioxidants is not practical; rather, the total oxidant and antioxidant responses of a sample are measured and denoted as total oxidant status (TOS) and total antioxidant capacity (TAC). The oxidative stress index (OSI) is calculated as the ratio of TOS to TAC. Oxidative stress balance is determined with OSI. We investigated the relationships of biomarker levels to echocardiographic LV geometry and systolic and diastolic function, as well as to clinical cardiovascular disease risk factors.

Patients and Methods

Patients

We retrospectively enrolled 38 healthy control and 80 patients with chronic HF who were diagnosed based on previously described criteria using coronary angiographic examination[22,23]. Patients were Stage III (moderate) HF according to the New York Heart Association functional classification system. Patients with chronic HF secondary to dilated cardiomyopathy had no history of ischemic heart disease. Table I shows the descriptive and echocardiographic features of the study subjects. Clinical, electrocardiographic and echocardiographic characteristics, and laboratory parameters were determined in all patients while they were clinically stable. Patients with renal failure, diabetes mellitus, atrial fibrillation, thyroid disorder, inflammatory disease or malignancy were excluded from the study. The Ethics Committee on Medical Research at our institution approved the study protocol and all patients provided written informed consent to participate.

Echocardiography

Standard imaging was performed in the left lateral decubitus position using a commercially available system (Vivid S5 GE ultrasound, Horten, Norway). Images were obtained using a 2.5-3.5 MHz transducer in the parasternal and apical views. Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters were determined with M-mode echocardiography under two-dimensional guidance in the parasternal long-axis

Table I. Descriptive and echocardiographic features of study.

	Control (n = 38)	HF-D (n = 36)	HF-I (n = 44)	p*
Mean age (Year) mean ± SD	57.2 ± 6.4	64.8 ± 7.1	59.6 ± 7.0	0.751
Gender (F/M)	20/18	22/14	28/16	0.442
LVEF (%) mean ± SD	61.4 ± 7.5	29.2 ± 10.1	35.7 ± 8.4	0.013
LVEDD (mm) mean ± SD	48.4 ± 4.7	65.1 ± 6.3	58.5 ± 6.0	0.003
LVESD (mm) mean ± SD	31.2 ± 3.4	47.2 ± 7.3	38. ± 4.4	0.002

LVEF: left ventricular ejection fraction; LVEDD: left ventricular end diastolic dimensions; LVESD: left ventricular end systolic dimensions; HF-D: HF patients with dilated cardiomyopathy; HF-I: HF patients with ischemic cardiomyopathy. *Significance between HF-D and HF-I groups,

view, according to the recommendations of the American Society of Echocardiography (ASE)[24]. Left ventricular ejection fraction (LVEF) was calculated from apical four-chamber views, according to the modified Simpson's rule[25].

Hematological Measurements

Peripheral venous blood samples were collected from the antecubital vein after patients had remained supine for at least 15 min without discontinuing drug treatment. Whole blood was centrifuged for 15 min at 3500 g to obtain plasma. Plasma aliquots were stored at -80°C until later analysis. We measured plasma levels of MMP-1, MMP-2, MMP-9, MMP-13 (RayBiotech, Parkway Lane, Suite 200, Norcross, GA, USA), Galectin-3 (eBioscience, Bender MedSysytems, Vienna, Austria) and TNF-α (Invitrogen, Camarillo, CA, USA) using commercially available ELISA kits[26]. Plasma prolidase levels were measured by a spectrophotometric method. Briefly, prolidase activity was determined by a method that determines proline levels produced by prolidase. The supernatant was diluted twofold with serum physiologic. Twenty-five μL of this mixture was preincubated with 75 μL of the preincubation solution (50 mmol/L Tris HCl buffer, pH 7.0, containing 1 mmol/L glutathione and 50 mmol/L $MnCl_2$) at 37ºC for 30 min. The reaction mixture, which contained 144 mmol/L gly–pro, pH 7.8 (100 μL), was incubated with 100 μL of preincubated sample at 37ºC for 5 min. To stop the reaction, 1 mL glacial acetic acid was added. After adding 300 μL Tris HCl buffer, pH 7.8, and 1 mL ninhydrin solution (3 g/dL ninhydrin was dissolved in 0.5 mol/L orthophosphoric acid), the mixture was incubated at 90ºC for 20 min and then cooled on ice. Absorbance was then measured at a wavelength of 515 nm to determine proline by the method proposed by Myara et al[8]. This method is a modification of Chinard's method[27]. Intra-and inter-assay coefficient of variations (CV) of the assay were lower than 7%. TAC (total antioxidant capacity) was evaluated using a novel automated colorimetric measurement method developed by Erel. Hydroxyl radicals, the most potent biological radicals, are produced in this method. In the assay, the ferrous ion solution present in reagent 1 is mixed with hydrogen peroxide, which is present in reagent 2. The subsequently produced radicals, such as the brown-colored dianisidinyl radical cations produced by the hydroxyl radicals, are also potent radicals. Using this method, the antioxidant effect of the sample is compared to the potent free radical reactions initiated by the produced hydroxyl radicals. The assay has excellent precision values, % CV was lower than 3%. The TAC results are expressed as nmol Trolox equivalent/mg protein[28]. The TOS (total oxidant status) of supernatant fractions was also evaluated using a novel automated colorimetric measurement method developed by Erel[29]. Oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is increased by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of nanomoles of H_2O_2 equivalent/milligram of protein[30]. The ratio of the total peroxide to the total antioxidant potential gives the OSI, a marker of the degree of oxidative stress[31]. OSI value was calculated according to the following formula: OSI (arbitrary unit) = TOS (mmol H_2O_2 equiv/l) / TAC (mmol Trolox equiv/l).

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS v12.0 (SPSS Inc. Chicago, IL, USA). For continuous variables, normality was checked. The appropriate nonparametric test was chosen for variables not normally distributed. Comparisons of continuous variables between two groups were performed using Student's *t*-test or the Mann-Whitney U test. One-way analysis of variance and Kruskal-Wallis tests were used for comparing multiple groups. Pearson correlation test was used to assess relationships between the parameters. Categorical variables between groups were analyzed using the chi-square test. Results are presented as n, percent, mean±SD (standard deviation), and median (minimum-maximum). $p < 0.05$ was considered significant.

Results

Descriptive and echocardiographic features of the groups are given in Table I and biochemical parameters of groups are summarized in Table II. Serum levels of biomarkers that show ECM turnover, such as MMP-2, MMP-9, and prolidase, were significantly increased in the HF group,

Table II. Serum biochemical parameters of groups.

	Control (n = 38) mean ± SD	HF-I (n = 44) mean ± SD	HF-D (n = 36) mean ± SD	*p**	*p***	*p****
MMP-1 ng/mL	3.78 ± 0.24	3.98 ± 0.29	4.18 ± 0.29	0.184	0.245	0.321
MMP-2 ng/mL	239 ± 33	269 ± 39	327 ± 52	0.039	0.012	0.004
MMP-9 ng/mL	405 ± 81	495 ± 81	625 ± 81	0.019	0.001	0.001
MMP-13 ng/mL	0.29 ± 0.012	0.35 ± 0.017	0.33 ± 0.026	0.286	0.327	0.571
Galectin-3 ng/mL	14.2 ± 2.4	19.1 ± 2.8	29.3 ± 5.7	0.029	0.002	0.005
TNF-α ng/mL	1.57 ± 0.18	2.24 ± 0.27	2.48 ± 0.27	0.004	0.003	0.097
OSI	8.81 ± 0.21	12.6 ± 0.97	13. 1 ± 0.85	0.019	0.002	0.108
Prolidase U/L	625 ± 154	711 ± 207	1025 ± 289	0.012	0.001	0.002

*Significance between control group and HF-I groups; **Significance between control group and HF-D groups; ***Significance between HF-D and HF-I groups.

whereas the increases in MMP-1 and MMP-13 were not significant. This significance was stronger in the HF-D group than in the HF-I group. The inflammation marker TNF-α was significantly increased in HF but there was no difference between HF-D and HF-I groups but Galectin-3 was significantly increased in the HF-D group compared to the HF-I group. The oxidative stress index showed the same characteristic response features as TNF-α. There was a positive correlation between MMP-9 levels and prolidase activity (Figure 1). There were no correlations between the levels of the circulating biomarkers with echo measures of ventricular hypertrophy or systolic function in any groups ($r < 0.5, p > 0.05$).

Discussion

HF is a frequent and life-threatening syndrome that is not only the result of myocardial injury or hemodynamic overload as commonly perceived, but is also a progressive physiological and anatomical transformation of the ventricle. Progressive left ventricular dilation and eccentric hypertrophy, infarct scar thinning and, ultimately, an alteration of the left ventricular geometry from a prolate ellipse to a spherical globe characterizes this transformation. These changes are collectively referred to as ventricular remodeling and denote a worse prognosis for a patient[32]. Ventricular remodeling is a progressive process,

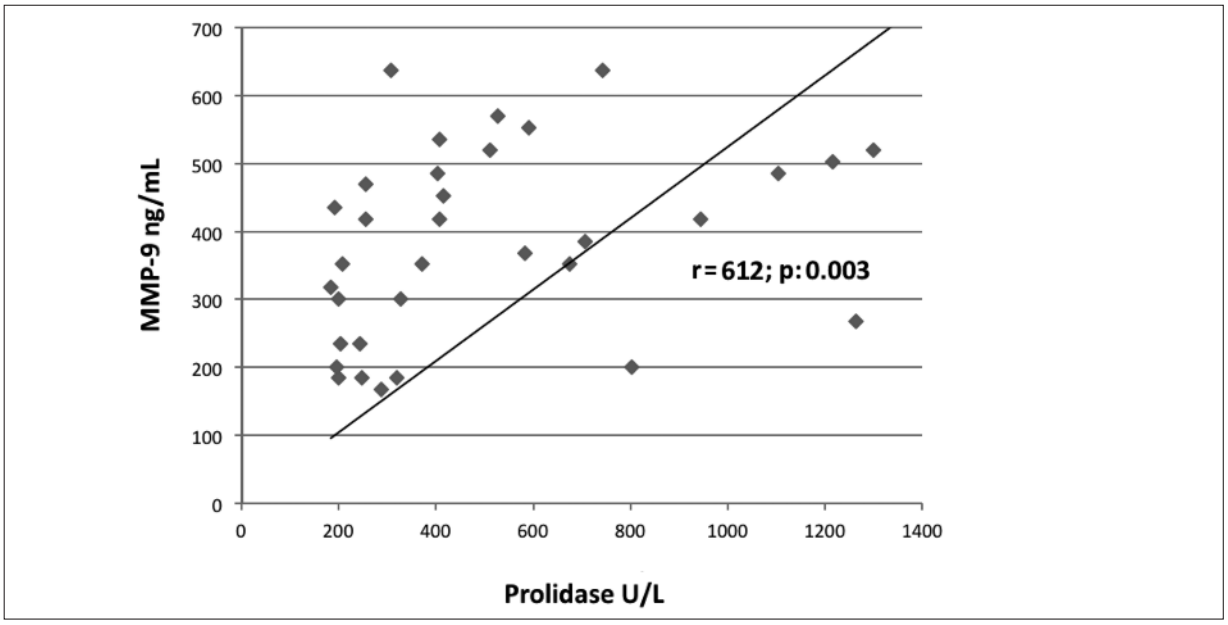

Figure 1. Correlation of serum prolidase activity with MMP-9 levels in heart failure patients with dilated cardiomyopathy.

although its clinical symptoms may not be demonstrated for years. A consensus statement defined remodeling as ''the genomic expression resulting in molecular, cellular, and interstitial changes that are manifested clinically as changes in size, shape, and function of the heart after cardiac injury"[33]. Most knowledge of ventricular remodeling stems from studies performed on patients after myocardial infarction, although remodeling can occur after any type of cardiac event[34]. Cytokines are a family of bioactive signaling molecules that regulate the inflammatory response and are involved in various cardiovascular diseases, including HF[35]. The prototypical proinflammatory cytokine, TNF, is expressed as a pro-TNF form and is subsequently inserted into the cellular membrane in various cell types[34]. TNF can depress myocardial contractility by uncoupling regulated calcium handling through both sphingosine and nitric oxide pathways and can trigger oxidative stress[37,38]. ROS reactions in the injured myocardium have been reported to play an important role in the process of remodeling after an ischemic episode[21].

In our study, MMP-2, MMP-9 and prolidase were significantly increased in heart failure (p = 0.039, 0.019, 0.012 respectively), whereas the increases in MMP-1 and MMP-13 were not significant. This significance was stronger in HF-D group than HF-I (p = 0.004, 0.001, 0.002 respectively). The inflammation marker TNF-α was significantly increased in heart failure (p = 0.004) but there were no difference between HF-D and HF-I groups. The cytokine Galectin-3 was increased significantly in HF-D group compared to the HF-I group (p = 0.005). OSI showed the same characteristic response features as TNF-α (p = 0.019, 0.002 respectively). The increase of OSI in HF showed increased oxidative stress in HF, independent of HF type (HF-D and HF-I). Also, the increased TNF in the study is consistent with previously studies; the difference was not significant ($p > 0.05$) between HF-D group and HF-I group, showing that the general inflammatory and ROS reactions were observed in HF independent of etiology[39]. The other proinflammatory cytokine, the lectin Galectin-3 (MAC-2 antigen), was increased significantly in HF-D group compared to HF-I group (p = 0.005). Galectin-3 activates macrophages[40], which are involved in organ fibrosis[41]. Researchers stated that Galectin-3 also led to an increase in myocardial collagen expression, interstitial fibrosis, and subsequent LV dysfunction[42,43]. The increase in HF-D group shows that collagen deposition is more prevalent than in HF-I because the fibrotic areas in HF-I group are local, while they are diffuse in the HF-D group. We can say that Galectin-3 may be regarded as a biomarker of fibrosis in heart failure patients with dilated cardiomyopathy.

After an initial insult to the myocardium, cardiac remodeling occurs as a compensatory mechanism; this will ultimately lead to left ventricular dysfunction and heart failure[44]. Galectin-3 is likely to play a role in this process, and has been shown to interact with various ligands located in the ECM[45] and play a central role in fibrosis and tissue remodeling[46]. In our study, the increase of Galectin-3 shows a possible role in dilatation of cardiomyocytes, as has been previously reported[47,48].

Some studies have shown that cardiac specific overexpression of TNF-α led to progressive LV dilation and remodeling within 4-12 weeks, partly due to the activation of the MMP family[49], especially MMP-2 and -9[50]; Galectin-3 also triggers activation of MMPs[51]. The enzymes primarily responsible for ECM turnover are MMPs. In our study MMP-2 and MMP-9 levels were increased in the HF group relative to the control group and levels were significantly higher in the HF-D subgroup compared to the HF-I group[52]. Recently it has been demonstrated that elevated levels of MMPs are correlated with LV systolic impairment in chronic HF[53]. Measurement of MMP levels has been important for risk stratification in patients with HF[54]. Several lines of evidence, both from various experimental models of HF and from patients with different types of HF, have indicated that elevated MMP activity is responsible for cardiac remodeling[55,56]. Our findings of MMP-9 and MMP-2 are consisted with these works, and support that MMP-9 has a main role in fibrosis.

This study is the first to investigate serum prolidase activity in HF and its subgroups. Prolidase, a cytosolic exopeptidase cleaving carboxyterminal proline and hydroxyproline of iminodipeptides, is recognized as an important regulator of endogenous protein synthesis, especially collagen, by providing endogenous proline. It is especially important in collagen turnover, being the main regulatory enzyme in the metabolism of proline and hydroxyproline, which constitute 20% of all collagen in the human body[7]. Monitoring of plasma prolidase activity might be useful in evaluating fibrotic processes[57] because increased serum prolidase activity was evidence of increased collagen turnover[58]. Previously, cardiac hypertrophy caused by loss of prolidase function was reported[59]. Taken together, these observations suggest that measur-

ing prolidase activity may potentially be useful in a wide range of HF patients in determining their risk in general and to determine the value of this new biomarker in combination with conventional risk markers. At the biochemical level, prolidase is a matrix metalloproteinase[60], and serum prolidase activity was positively correlated with oxidative stress markers, such as increased TOS and OSI previously stated[61,62]. As we found a positive correlation between MMP-9 levels with prolidase activity, it can be concluded that prolidase may be one of the bridge factors between oxidative stress and fibrosis.

Conclusions

While MMPs and Galectin-3 are important in cardiac remodeling, prolidase may share an undefined role in fibrosis in heart failure and may have a role in diffuse fibrosis heart failure. This suggests a role as a biomarker in heart failure patients with dilated cardiomyopathy.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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