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Immunoexpression of MMP-8, MMP-9 and TIMP-2 in dilated cardiomyopathy

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Abstract

Alteration of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) expression has been studied for various cardiac diseases, including dilated cardiomyopathy (DCM), with the significance of surrogate markers of extracellular matrix (ECM) remodeling. In this study, we determined the MMP-8, MMP-9 and TIMP-2 immunoexpression in the heart of patients diagnosed with DCM in relation to a histological composite score (HCS). The study included 40 cases of heart fragments that were processed by the usual paraffin inclusion technique, followed by a semi-quantitative evaluation of histopathological parameters, which summed, allowed the establishment of a HCS. Subsequently, the cases were immunohistochemically processed for MMP-8, MMP-9 and TIMP-2, followed by the semi-quantitative evaluation of their expression intensity. MMP-8 was identified only in myocardiocytes, while MMP-9 and TIMP-2 were present in both myocardiocytes and stroma, but with different intensity. The increasing intensity of MMP-8 and TIMP-2 immunoreactions was significantly associated with low HCS. In case of MMP-9, the immunostaining intensity analysis in relation to the HCS level revealed insignificant differences, but we found an association of increased and moderate intensity with low HCS. The imbalance between TIMPs and MMPs disrupts the ECM architecture and contributes to the remodeling process in DCM, aspect that can be used in the development of new clinical therapies.

Keywords: dilated cardiomyopathy, MMP-8, MMP-9, TIMP-2.

Introduction

Myocardial remodeling represents a predictive factor for the appearance of dilated cardiomyopathy (DCM), in the same time matrix metalloproteinases (MMPs) being proteins involved in extracellular remodeling and strictly controlled by tissue inhibitors of metalloproteinases (TIMPs). MMPs play pivotal roles in the development of fibrosis by controlling the extracellular matrix (ECM) degradation [1].

In the normal heart, MMPs and TIMPs are essential for ensuring the physiological structure of the ECM, characterized by a collagen fluctuation [2, 3]. As a result, alteration of MMPs and TIMPs expression has been studied for various cardiac diseases, including DCM, having the significance of surrogate markers of ECM remodeling [2, 3].

In cardiac diseases, MMPs and TIMPs expression is unbalanced, which may contribute to the disintegration of collagen from myocardial tissue and the modification of intracellular signaling of cardiomyocytes [4]. Therefore, left ventricular dilatation is thought to be due to the collagen network disintegration by increasing the collagenolytic activity of MMPs and TIMPs, pharmacological inhibition of MMPs during the stimulation period preventing both ventricular dilatation and depreciation of the left ventricle pump function, claiming relevance to the MMPs inhibition concept in remodeling it [5].

In this study, we determined the expression of MMP-8, MMP-9 and TIMP-2 in the heart of patients diagnosed clinically with DCM, in relation to histopathological parameters.

Materials and Methods

The study included 40 cases, represented by the fragments taken from the left ventricle in patients clinically diagnosed by DCM, in the Department of Cardiology, Emergency County Hospital of Craiova, Romania, and autopsied in Department of Pathology of the same Hospital. We also introduced six normal heart fragments from autopsy of patients whose deaths were due to other causes than cardiac ones.

The harvested fragments were fixed in 10% buffered formalin, processed by the usual paraffin embedding technique, followed by 3–5 μm sectioning and Hematoxylin–Eosin (HE) staining. For the semi-quantitative evaluation of histopathological parameters, we used a three-degree scale (grade 1: ≤10%, grade 2: 10–50%, grade 3: ≥50%) for the assessment of myocardial atrophy, vacuolar degeneration, myocytolysis, cytoplasmic accumulation of mucin, nuclear pleomorphism, and interstitial fibrosis. Then, we computed the histological composite score (HCS) by summing the values given to each histopathological parameter, and we considered low HCS for the value between 1–6 and high HCS for values between 8–12.

Subsequently, we performed serial sections that were immunohistochemically processed using an amplification polymer-based detection system [polymer-Horseradish

peroxidase (HRP) Histophine, Nichirei, Japan, ready-to-use, code 414151F]. The visualization of the reactions was done with the 3,3'-Diaminobenzidine (DAB) chromogen (Dako, code 346), and for the validation of the reactions, we used positive and negative external (by omitting the primary antibody) controls (Table 1).

Table 1 – ***The used antibodies: clone, dilution, antigen retrieval and external control***

Antibody	Clone	Producer	Dilution	Antigen retrieval	External control
MMP-8	Polyclonal	Atlas Antibodies	1:50	Microwaving in citrate buffer, pH 6	Spleen
MMP-9	2C3	Santa Cruz Biotechnology	1:50	Microwaving in citrate buffer, pH 6	Lung
TIMP-2	3A4	Santa Cruz Biotechnology	1:50	Microwaving in citrate buffer, pH 6	Kidney

MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

We followed the evaluation of the semi-quantitative expression of MMP-8, MMP-9 and TIMP-2 through a scoring system based on the assessment of positive cell staining intensity. The intensity of the score was marked with 1 (low intensity), 2 (moderate intensity) and 3 (high intensity).

The statistical analysis was done using the χ^2 (*chi*-square) test in the Statistical Package for Social Sciences (SPSS) 12 software, the results being considered significant for p-values <0.05.

Results

The histopathological analysis of the 40 cases selected with the clinical diagnosis of DCM revealed a number of changes such as myocardial atrophy, vacuolar degeneration, myocytolysis, mucin cytoplasmic accumulation, nuclear pleomorphism, and interstitial fibrosis (Figure 1A). For all selected cases, normal and DCM, we investigated the intensity of MMP-8, MMP-9 and TIMP-2 expression (Table 2).

Table 2 – ***The distribution of cases according to the intensity of MMP-8, MMP-9 and TIMP-2 immunoreaction***

Marker		MMP-8			MMP-9			TIMP-2		
Intensity		***1***	***2***	***3***	***1***	***2***	***3***	***1***	***2***	***3***
Normal	Myocardiocyte	0	6	0	0	6	0	0	6	0
	Stroma	0	0	0	4	2	0	0	6	0
DCM with low HCS	Myocardiocyte	0	8	25	0	26	6	0	27	5
	Stroma	0	0	0	0	26	6	0	27	5
DCM with high HCS	Myocardiocyte	0	0	7	2	3	0	4	0	0
	Stroma	0	0	0	2	3	0	0	0	0
χ^2 test		p<0.001			p=0.062			p<0.001		

MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; DCM: Dilated cardiomyopathy; HCS: Histological composite score.

Immunoreaction for MMP-8 has been identified in all cases with DCM and control as well. The intensity of normal heart expression was moderate compared to DCM that was moderate or high. In the control cases, the marker was present only in myocardiocytes, with cytoplasmic pattern, diffuse, or predominantly perimembranous, with moderate intensity (Figure 1B). In DCM with low HCS, we identified eight cases with moderate immunostaining intensity and 25 cases of high intensity (Figure 1C), unlike cases of DCM with high HCS in which all seven cases revealed increased intensity (Figure 1D).

Immunostaining for MMP-9 was identified in 37 (92.5%) of the selected DCM cases and in all normal cases with different expression. For the control cases, we observed moderate positivity in myocardiocytes and stroma, in rare fibroblasts and endothelial cells (Figure 1E). In DCM with low HCS, we observed in six cases high intensity staining, in 26 cases moderate intensity (Figure 1F) and in DCM with high HCS intensity of staining was low in two cases and moderate in three cases (Figure 1G). In DCM, MMP-9 immunoexpression was identified in both myocardial and stromal levels.

Immunoreaction for TIMP-2 has been identified in 36 (90%) cases of DCM, as well as in all control cases. For control cases, we observed the presence of myocardiocytes and stromal cells with a moderate intensity (Figure 1H). In DCM with decreased HCS, expression was cytoplasmic, in myocardial and stromal cells, with moderate intensity in 27 cases or high in five cases (Figure 1I). In DCM with high HCS, we only identified cytoplasmic expression in myocardiocytes, with reduced intensity, in four cases (Figure 1J).

The statistical analysis revealed that the intensity of MMP-8 and TIMP-2 related to the HCS level indicated significant differences (p<0.001, χ^2 test), the increased intensity of the immunostaining being associated with low HCS (Figure 2, A and B). In contrast, for MMP-9, the immunostaining intensity analysis in relation to the HCS level revealed insignificant differences (p=0.062, χ^2 test), but we found an association of increased and moderate immunostaining intensity with low HCS (Figure 2C).

Discussions

Many studies dedicated to MMPs and TIMPs in cardiac pathology target DCM [6], but also congestive heart failure [7], acute myocardial infarction [8–10], diastolic dysfunction of left ventricle and fibrosis in hypertension [11]. The common feature of these pathologies is the remodeling of myocardial tissue, ECM and fibrosis [12], leading to ventricular dilatation and contractile dysfunction. In this context, the balance between MMPs that degrades the ECM and TIMPs appears to be responsible for remodeling the ECM.

MMPs represent proteases which are Ca^{2+}/Zn^{2+} dependent, released in the inactive form as pro-MMPs, which are activated either by cleavage of a terminal amino-

propeptide or by autoproteolysis by another MMPs [13].

MMP-8 (neutrophil collagenase, polymorphonuclear leukocyte collagenase) represents an enzyme that cleaves collagen from connective tissue. MMP-8 is usually found in neutrophil-specific granules, but is also expressed by epithelial, endothelial, fibroblast and macrophage cells [14].

In this study, immunoreaction for MMP-8 was identified in all cases with DCM. In DCM, the positivity of the reaction was identified cytoplasmically only in myocardiocytes, with moderate or increased intensity in DCM with low HCS, respectively, increased in DCM with high HCS, the increased intensity being associated with low HCS.

MMP-8, as well as MMP-9, is most often associated with inflammatory processes and has been shown to increase rapidly and at high levels in adult patients after myocardial infarction and in cardiac surgery [8, 15, 16], and in addition, the presence of expression was reported in the samples taken from the myocardium of patients in the terminal stage of cardiomyopathic disease [17]. Studies of children with post-transplant DCM reported an increase in MMP-8 and MMP-9 and a decrease in TIMP-1 and TIMP-2 [18]. In another study in patients diagnosed with idiopathic DCM, the authors reported that the activity of MMP-8 was increased approximately 30 times in comparison with the activity of gelatinase [19].

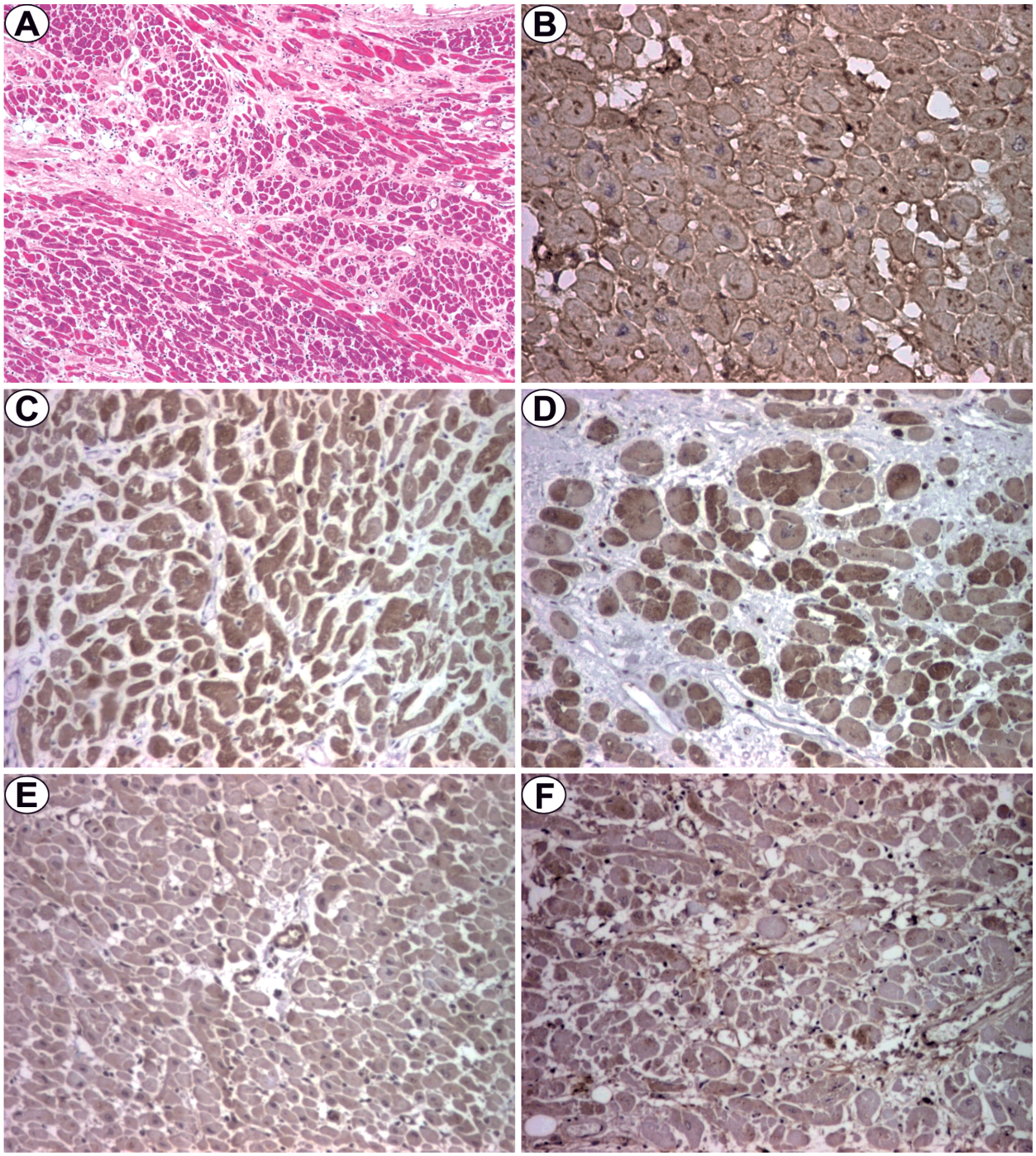

Figure 1 – *(A) DCM; (B) Normal heart; (C) DCM with low HCS; (D) DCM with high HCS; (E) Normal heart; (F) DCM with low HCS. HE staining: (A) ×40. Anti-MMP-8 antibody immunostaining: (B–D) ×100. Anti-MMP-9 antibody immunostaining: (E and F) ×100. DCM: Dilated cardiomyopathy; HCS: Histological composite score; HE: Hematoxylin–Eosin; MMP: Matrix metalloproteinase.*

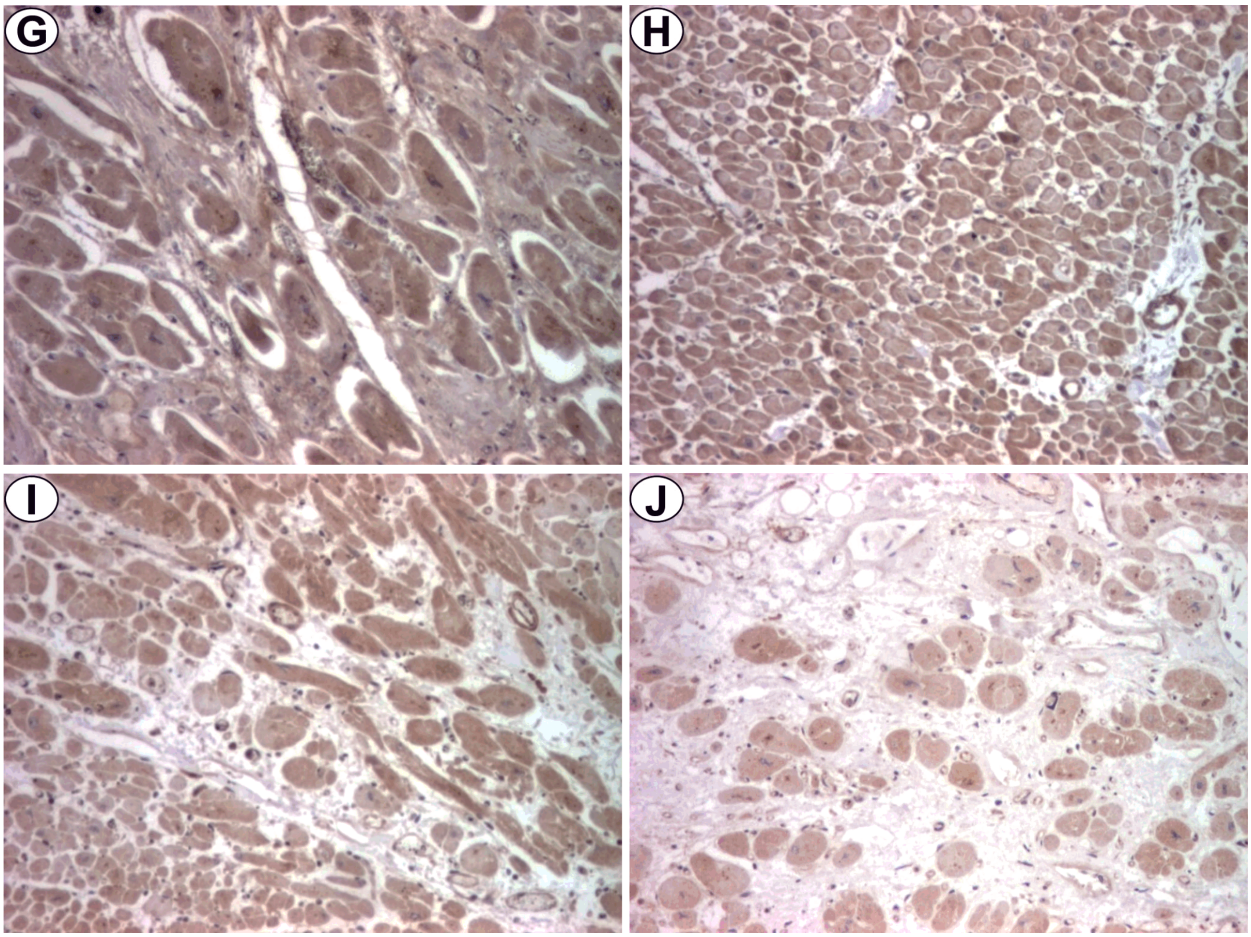

Figure 1 (continued) – ***(G) DCM with high HCS; (H) Normal heart; (I) DCM with low HCS; (J) DCM with high HCS. Anti-MMP-9 antibody immunostaining: (G) ×100. Anti-TIMP-2 antibody immunostaining: (H–J) ×100. DCM: Dilated cardiomyopathy; HCS: Histological composite score; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.***

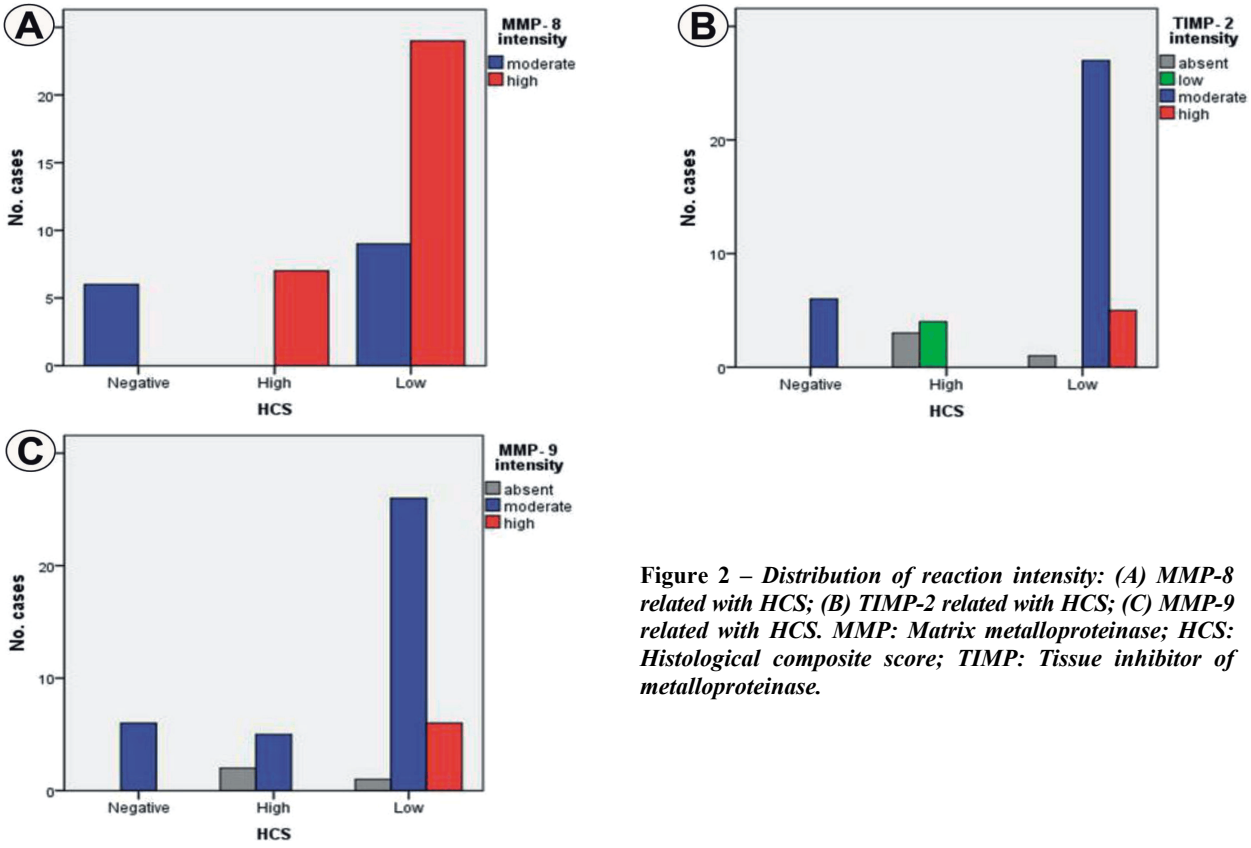

Figure 2 – ***Distribution of reaction intensity: (A) MMP-8 related with HCS; (B) TIMP-2 related with HCS; (C) MMP-9 related with HCS. MMP: Matrix metalloproteinase; HCS: Histological composite score; TIMP: Tissue inhibitor of metalloproteinase.***

MMP-9, also known as the 92-kDa type IV collagenase, B gelatinase or 92-kDa gelatinase, is a matrixin involved in the ECM degradation. Rouet-Benzineb *et al.* have located MMP-9 in cardiomyocytes and identified myosin heavy chain cleavage by MMP-9, indicating a directly damage to the contractile system by MMP-9 [20].

In this study, immunostaining for MMP-9 was present in 37 (92.5%) cases of DCM and in all control cases. For normal heart cases, the intensity of the reaction was moderate, while in DCM with low HCS, we noticed high or moderate intensity staining, and DCM with high HCS the intensity was poor or moderate. This staining pattern suggests the presence of MMP-9 both in the early and advanced stages of the disease, in which case its expression decreases.

MMP-9 is a key enzyme in the degradation of the components of the cardiac matrix, suggesting a role in remodeling it, resulting in enlargement of left ventricle and systolic activity depression. Overexpression of MMP-9 is a frequent aspect for activation of matrix in terminal cardiac failure, regardless of baseline disease [21, 22]. In patients with DCM, increased levels of MMP-9 were reported using the enzyme-linked immunosorbent assay (ELISA) method [23]. Additionally, the heart of patients with DCM has been reported to have the immunoreactivity of both gelatinases, MMP-2 and MMP-9, localized exclusively in cardiomyocytes and closely associated with the striated structure, association supported by the usual gelatinase periodicity, suggesting a close association between gelatinases and myosin [20].

Another study reports the modification of MMP-9 expression only in myocardial fibroblasts, not in cardiac cells [24]. Significantly higher MMP-2, MMP-9 and TIMP-1 values were reported in DCM compared to control cases without differences in serum concentrations in patients with DCM, regardless of the disease duration or presence/absence of fibrosis [25].

TIMP-2 works both as an MMP inhibitor and as an activator. Several studies support the role of TIMP-2 from cardiac tissues in the proteolysis of ECM [26–28].

In the study, immunoreaction for TIMP-2 was identified in 36 (90%) cases of DCM. For normal heart cases, we observed moderate intensity in both compartments, myocardial cells and stromal cellular elements. In DCM with decreased HCS, expression was also present in myocardial and stromal cells with moderate or high intensity, while in DCM with high HCS staining was identified in myocardiocytes with low intensity.

In several studies, elevated TIMP-2 levels were associated with systolic dysfunction, acute myocardial infarction, end-stage idiopathic DCM [4], and stage 2–3 acute renal failure from decompensated heart failure [29]. Yokoseki *et al.* demonstrated on endomyocardial biopsy fragments derived from the right ventricle that the left ventricle ejection fraction is significantly correlated with TIMP-2 expression in patients with idiopathic DCM [30]. In one study, ELISA quantification of TIMP-2 indicated that protein levels decreased significantly in DCM heart *versus* control hearts, TIMP-2 immunoreactivity having similar diffuse staining patterns in normal cardiomyocytes and DCM [20]. However, other studies reported decreased TIMP-2 levels in patients with coronary artery disease [31] and systolic heart failure [32] and those who died after a mitral valve surgery [33].

Conclusions

The imbalance between TIMPs and MMPs disrupts the ECM architecture and contributes to the remodeling process in DCM. Overexpression of MMP-8 and TIMP-2 was associated with low HCS, respectively, of early DCM cases with reduced histological changes. In addition, the presence of MMP-8, MMP-9 and TIMP-2, both in low and high HCS, suggests a continuous remodeling of the ECM and increased levels of collagen in all evolutionary stages of DCM. Identification of biomolecules that adjust cardiac remodeling may be helpful in the development of new clinical therapies.

Conflict of interests

The authors declare that they have no conflict of interests.

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