Collagen I and III, MMP-1 and TIMP-1 immunoexpression in dilated cardiomyopathy

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Abstract
The extracellular matrix (ECM) remodeling represents the pathological substrate of dilated cardiomyopathy (DCM). In this study, we statistically analyzed the immunoexpression of collagen I and III, matrix metalloproteinase-1 (MMP-1) and its tissue inhibitor-1 (TIMP-1) in the myocardial tissue in 18 cases of DCM compared to a control group. We observed a significant increase in the immunoexpression of collagen I and III in patients with DCM and a significant reduction in the immunoexpression of MMP-1 compared with the control group. Also, the collagen I and TIMP-1 expression indicated a positive linear correlation and respectively a negative linear relationship with collagen III and MMP-1. The analyzed markers in this study can be used to quantify the degree of collagen sclerosis from the ECM of DCM.

Keywords: dilated cardiomyopathy, extracellular matrix, collagen.

Introduction
Dilated cardiomyopathy (DCM) is defined by the dilatation of the left ventricle (LV) with thin ventricular walls and alteration of the LV systolic function. Despite of the new therapeutic concepts and drugs, the prognosis in this disease is still unfavorable. Etiologically, DCM can be idiopathic or caused by various infectious, toxic or metabolic factors [1].

Myocardial fibrosis is an important factor in the development and progression of pathological changes of DCM, extracellular matrix (ECM) playing a central role. The collagen I and II are the main types found in the myocardial ECM, and they are in an equilibrium caused by the activity of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) [2]. Detailed understanding of the changes in the ECM has not only a theoretical importance for shaping the exact pathogenesis of the disease, but it also has major implications in treatment. Thus, several studies have shown that treatment with angiotensin converting enzyme inhibitors [3], angiotensin II receptor blockers [4] or mineralocorticoid receptors antagonists [5] cause regression of the collagen in the ECM and other specific therapies that target MMP/TIMP system could have beneficial in the future.

In this study, we analyzed the immunoexpression of some proteins involved in ECM modeling in patients with DCM.

Materials and Methods
The study included a total of 18 cases from patients diagnosed with DCM in the Clinic of Cardiology, Emergency County Hospital, Craiova (Romania), who died between 2015–2016. The patients’ autopsies were performed in the Laboratory of Pathology of the same Hospital, and myocardial tissue fragments were harvested for the histopathological and immunohistochemical analysis. For the control group, we harvested normal fragments from 10 deceased patients without associated cardiac pathology. From the study were excluded the patients who had previous ischemic events or inflammatory cardiac processes.

For the immunohistochemical reactions, dewaxing and hydration of the sections was followed by the blocking of endogenous peroxidase, the antigen retrieval and the non-RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/
specific blocking with bovine serum albumin. Subsequently, the sections were incubated with primary antibody, overnight, at 4°C. The next day, the sections were incubated with secondary biotinylated antibody and streptavidin–HRP (horseradish peroxidase) within the working system LSAB2 (Dako, Redox, Romania, code K0675). For viewing the positive signals, we used the chromogenic detection with 3,3’-diaminobenzidine tetrahydrochloride (DAB, Dako, Redox, Romania, code K3468). To validate the results, we used positive and negative external controls, by omitting the primary antibodies.

The quantification of the immunohistochemical reaction was done by integrated optical density (IOD) using Image ProPlus 7 AMS software (Media Cybernetics, Inc., Buckinghamshire, UK). IOD is the multiplying result of pixel density from a region and the region area, for each captured image summing the values of IOD. Image acquisition was performed using Nikon Eclipse E600 microscope equipped with Lucia 5 software. At the same time, for the immunohistochemical reactions, we used descriptive analysis, indicating the location and signal intensity (low, moderate, high).

The results were collected into a computerized database, which was statistically analyzed in SPSS10 automated platform, using Student’s $t$-test. The study was approved by the Ethics Committee and in each case we obtained an informed consent from the patient family.

## Results

The histopathological study of the 18 deceased patients with the diagnosis of DCM revealed, concerning the cardiomyocytes, the cellular and nuclear size variation, the presence of cytoplasmic spaces filled with basophilic, PAS-positive amorphous material (Figure 1A). In the ECM, we observed variable collagen fibrosis, distributed both around the bundles of muscle and individually, around cardiomyocytes (Figure 1, B and C).

Fibrosis was diffuse or focal, with interstitial or peri-vascular topography. In interstitial fibrosis, we have observed the presence of fibrillar collagen in intermuscular spaces that are normally free of collagen. The distribution pattern was quite variable, from a fine perimyocardial fibrosis to massive scars, similar to those in ischemic cardiopathy. Collagen has individually surrounded the myocardial fibers that have a “shrunken” appearance due to the wavy cell membrane. This aspect was subsequently confirmed in the Masson’s trichrome staining (Figure 1D).

In the case of perivascular fibrosis, the collagen has accumulated in the adventitia of intramyocardial coronary arteries and arterioles, otherwise free. The increase in fibrous tissue considerably increased interstitial collagen or substituted on small areas the necrotic myocardial fibers.

### Collagen I immunoexpression

The collagen I immunoexpression was identified in all cases of DCM and the immunostaining was observed in cytoplasm, predominantly around the perimysium and endomysium. At this level, we observed signals in the collagen fibers, vascular walls and cellular elements represented by fibroblasts, the intensity of the reactions being moderate/high. In the case of vessels, the immunostaining was uniform and continuous, regardless of the structures caliber. Also, the immunostaining was observed in the cardiomyocytes sarcolemma basement membrane, the reactions being continuous or discontinuous depending on the sectioning direction of the tissue. In these cases, we found at the sarcolemma level, a low/moderate intensity reaction in the case of the hypertrophic cardiomyocytes and a high intensity reaction in the case of the atrophic cardiomyocytes (Figure 2A).

We have also found the existence of collagen I signals in the cardiomyocytes cytoplasm, mostly near the intra-cytoplasmic or attached to the sarcolemma vacuoles.

### Collagen III immunoexpression

The collagen III immunoexpression had a similar distribution with collagen I, the immunostaining being observed in all cases. However, the immunostaining of the perimysium, endomysium and sarcolemma had a high intensity in all cases. Also, the immunostaining was more intense and more numerous in the cardiomyocytes cytoplasm, regardless of their status (hypertrophic or atrophic) (Figure 2B).

In the case of the control group, for both collagen-analyzed types, the signal intensity was similar to the one from the DCM at perimysium, endomysium and cardiomyocytes sarcolemma levels. However, the number of positive elements of the ECM and the intracytoplasmic level was lower. This aspect was statistically significant for both collagen I ($p<0.0001$, Student’s $t$-test), and in the case of collagen III ($p<0.001$, Student’s $t$-test) (Table 2, Figure 3).

### Table 2 – IOD mean values ($\pm$SD) related to the analyzed markers

<table>
<thead>
<tr>
<th>Marker / Standard deviation (SD)</th>
<th>DCM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>$33 \times 10^6$</td>
<td>$13 \times 10^6$</td>
</tr>
<tr>
<td>Collagen III</td>
<td>$43 \times 10^6$</td>
<td>$18 \times 10^6$</td>
</tr>
<tr>
<td>MMP-1</td>
<td>$8 \times 10^6$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>$19 \times 10^6$</td>
<td>$10 \times 10^6$</td>
</tr>
<tr>
<td>SD Collagen I</td>
<td>$8 \times 10^6$</td>
<td>$9 \times 10^6$</td>
</tr>
<tr>
<td>SD Collagen III</td>
<td>$10 \times 10^6$</td>
<td>$10 \times 10^6$</td>
</tr>
<tr>
<td>SD MMP-1</td>
<td>$4 \times 10^6$</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>SD TIMP-1</td>
<td>$2 \times 10^6$</td>
<td>$2 \times 10^6$</td>
</tr>
</tbody>
</table>

**IOD:** Integrated optical density; **DCM:** Dilated cardiomyopathy; **MMP-1:** Matrix metalloproteinase-1; **TIMP-1:** Tissue inhibitor of metalloproteinase-1.

### MMP-1 and TIMP-1 immunoreactions

MMP-1 and TIMP-1 immunoreactions were observed in all investigated cases (DCM and control group) in the cytoplasm of stromal elements, represented by endothelial cells, fibroblasts and rare lymphocytes (Figure 2, C and D). The intensity of the reaction was moderate/intense and uniform. In the case of MMP-1, some discontinuous signals of variable intensity were observed at the level of cardiomyocytes sarcolemma or cytoplasm of the control group. In case of DCM, the expression of MMP-1 and TIMP-1 of cardiomyocytes was absent. The statistical analysis indicated significant lower mean IOD values in DCM when compared to the control group ($p<0.01$, Student’s $t$-test).

In the case of TIMP-1, the values were higher in DCM compared to the control group, but this aspect was not statistically significant ($p>0.05$, Student’s $t$-test) (Figure 3).
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Figure 1 – Dilated cardiomyopathy, ×100: (A) Cardiomyocytes with PAS-positive vacuoles; (B and C) Cardiomyocytes and myocardial extracellular matrix, HE staining; (D) Fibrosis, Masson’s trichrome staining.

Figure 2 – Dilated cardiomyopathy, ×100: (A) Collagen I immunostaining; (B) Collagen III immunostaining; (C) MMP-1 immunostaining; (D) TIMP-1 immunostaining.
The statistical analysis of the mean IOD value distribution for the analyzed markers revealed a positive linear correlation between collagen I and TIMP-1 ($p<0.01$, Pearson’s test). Also, negative linear relations were observed between collagen I/TIMP-1 with collagen III and MMP-1, but these aspects were insignificant ($p>0.05$, Pearson’s test) (Figures 4 and 5).

**Discussion**

The myocardial ECM plays an important role in the pathogenesis of LV dilation. It is mainly composed of collagen type I and III, which form a three-dimensional network, thereby providing stability to the ventricular geometry and alignment of the cardiomyocytes, all playing an important role in the normal functioning of the left ventricle [6]. Topographically, the cardiomyocytes are surrounded by a network of collagen lining the external surface of cells. This allows efficient contraction in systole. LV wall thinning in DCM is partly explained by the rupture and dehiscence of type I collagen fibers leading to disruption of cardiomyocytes [7–9].

MMPs are a group of zinc-dependent endogenous enzymes that are responsible for the degradation of extracellular collagen and ECM remodeling in various types of heart failure [10, 11]. Up to now, there are known at least 16 different subtypes of MMPs [12]. MMPs are regulated at different levels and the endogenous extracellular signals play an important role, such as specific inhibition and regulation carried out by tissue inhibitors of matrix metalloproteinases (TIMPs) [13].

Pathophysiologically, MMP-1 and TIMP-1 have a role in maintaining the ECM architecture [14]. There is a balance in normal myocardial tissue in the synthesis and degradation of collagen. It is assumed that any imbalance in their proteinases or proteinase inhibitor system may disrupt the architecture of the myocardial tissue. It has been reported, in DCM patients, an increase in MMP [15] and the activation of the intramyocardial system [16].

In the present study, we observed a significant increase in the expression of collagen I and III in patients with DCM and a significant reduction in MMP-1 expression compared with controls. These suggest the presence of an active process of myocardial remodeling manifested by fibrosis.

Similar to our results Thomas et al. [17] demonstrated, using immunoblotting technique, a relative decrease of MMP-1 activity in patients with idiopathic DCM compared to normals, and a 5-fold increased activity of TIMP-1, the TIMP-1/MMP-1 ratio being over 60 times higher in the group with DCM. Similar results were obtained in patients with ischemic DCM [18].

In a study, which used endomyocardial biopsies from patients with DCM, Picard et al. observed a significant increase in mRNA levels of MMP-1 and TIMP-1 compared with controls [19]. In another study, Polyakova et al. [20] analyzed the degree of myocardial fibrosis, collagen metabolism, MMP/TIMP expression and expression of some cytokines in patients with severe heart failure of various etiologies (dilated cardiomyopathy, ischemic cardiomyopathy, myocarditis). Compared with controls, in patients with heart failure, they observed different degrees of alteration in collagen metabolism, with modified expression of MMP, TIMP and cytokines, suggesting different mechanisms of fibrosis progression. In our study, the IOD value analysis indicated a negative linear relation of collagen I and III and also of MMP-1 and TIMP-1.

Other studies that have examined the relation of collagen I and III and MMP/TIMP indicated the alteration in patients with DCM, compared to the control group [20]. MMPs and TIMPs analysis in DCM also revealed elevation of other MMPs such as MMP-2, -8, -19, and TIMP-2, -3, -4, respectively [20].

In our study, we found immunoreactions at the level of cytoplasmic PAS-positive vacuoles. The vacuoles could be invagination of the plasma membrane or expansion of T tubules, as was previously described in the border area of myocardial infarction in an animal model [21]. This aspect could be a marker of cellular remodeling in chronic heart disease.
Conclusions

The markers analyzed in this study can be used to quantify the degree of collagen sclerosis from the ECM. Further studies are needed on larger groups to also analyze the nature of the vacuoles from the cardiomyocytes cytoplasm. MMP-1 and TIMP-1 immunoexpressions support the nature of the vacuoles from the cardiomyocytes cytoplasm. Further studies are needed on larger groups to also analyze quantify the degree of collagen sclerosis from the ECM.

Conflict of interests

The authors declare that they have no conflict of interests.

References


