

ORIGINAL PAPER

Simultaneous immunophenotypical assessment of troponin and extracellular matrix molecules in myocardium of patients with sudden cardiac death

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Abstract

In patients with sudden unexpected cardiac death, there is a relationship between the interstitial fibrosis of the myocardium and matrix molecules with a role in global remodeling of the cardiac stroma. Tissue samples of left ventricular myocardium from 17 middle-aged patients with sudden cardiac death, following acute or chronic ischemic cardio(myo)pathies, were analyzed using standard HE stain and the indirect triserial ABC peroxidase immunohistochemical method for a panel of four antibodies involved in the dynamic remodeling of extracellular matrix: matrix metalloproteinase 9 (MMP9), tenascin X (Tn-X), TGF- β , CD54 (ICAM-1), together with simultaneously assessment of troponin in myocardic fibers. The most sensitive reaction was noticed for ICAM-1 in 71% of cases, followed by MMP9 in 59% of cases and TGF- β in 47% of cases (with great specificity for capillary vessels), in the extracellular matrix of the residual cardiomyocytes. A direct correlation, statistically significant was recorded between troponin and MMP9 ($r = 0.65$, $p = 0.01$), troponin and ICAM-1 ($r = 0.31$, $p = 0.02$), respectively ICAM-1 and tenascin ($r = 0.72$, $p = 0.01$). The extensive expression of ICAM-1 in the extracellular matrix from the perilesional area probably plays a role in the stimulation of new developing adhesion substrates between residual cells and adjacent stroma, while the over expression of troponin in the residual cardiomyocytes is accompanied by a high expression of MMP9 in the myocardic interstitium, with heterogeneous remodeling of the ventricular stroma. The simultaneous IHC expression of tenascin and ICAM-1 suggests a colocalization required for the nerve sprouting in the residual myocardium and for developing new focal cell-matrix adhesion contacts.

Keywords: sudden death, matrix molecules, damaged myocardium.

Introduction

Cardiovascular diseases are now the main cause of mortality in Romania and around the world, the cardiac failure being the main consequence of heart diseases (ischemic cardiopathy, cardiomyopathies, etc.), with an inadequate output. In the modern society, in the circumstances of an increasing mean age of the population, also increases the number of persons who survive the acute attacks of ischemia and who will develop later cardiac failure. During the evolution of heart diseases, the main phenomenon is the permanent decreasing of cardiomyocytes due to ischemia or apoptosis, the residual cells being incapable of an efficient compensatory activity.

In this context, the cardiac matrix system is less known, because the vast majority of the studies are oriented to cardiomyocytes characterization; the interrelations between different non-cardiac cell populations (such as fibroblasts and endothelial cells) and the direct influence of extracellular matrix raises a lot of questions.

In the damaged myocardium, the fibroblasts become activated and begin the cardiac remodeling due to an

increased proliferation and migration. Consequently, the ECM expands (due to its own components: collagen, proteoglycans, glycoproteins) and some tumoral factors (cytokines, growth factors and proteases) are released, with autocrine and paracrine effects to fibroblasts phenotype and activity. In patients with sudden unexpected cardiac death, there is a relationship between the interstitial fibrosis of the myocardium and several molecules involved in global remodeling of microenvironment matrix system of myocardium.

Material and Methods

Tissue samples

We have retrieved from our database, seventeen archived formalin-fixed, paraffin-embedded samples of myocardial tissue from left ventricle, of young and middle-aged adults (sex ratio M : F = 2 : 1, mean age: $m = 30$ years old, $SD = \pm 2$), with sudden cardiac death, following acute or chronic cardio(myo)pathies or scarring myocardic fibrosis. Sections were cut at $5 \mu\text{m}$ and stained using the standard Hematoxylin–Eosin stain and van Gieson.

Immunohistochemistry (IHC)

The indirect triserial ABC peroxidase immunohistochemical method was used for a panel of four antibodies involved in the dynamic remodeling of extracellular matrix: matrix metalloproteinase 9 (MMP9), tenascin X (Tn-X), TGF β , CD54 (ICAM-1), together with simultaneously assessment of troponin in myocardial fibers. The immunohistochemistry (IHC) was performed on 3 μ m thick sections from 10% formalin-fixed paraffin-embedded tissues, according to the indirect triserial Avidin–Biotin–Complex method of Hsu SM *et al.* [1], modified by Bussolati G and Gugliotta P [2]. Briefly, the procedure was: deparaffinization in xylene and alcohol series, rehydration, washing in phosphate saline buffer (PBS), incubation with normal serum, for 20 minutes, incubation with primary antibody overnight, standard labeled streptavidin-antibody biotin (LSAB) kit (DAKO), washing in carbonate buffer and development in 3,3'-DAB hydrochloride/H₂O₂; microwave antigen retrieval in M-citrate buffer pH 6.0 was performed for certain antibodies. All specimens were counterstained with Meyer's Hematoxylin, examined and photographed on a Nikon Eclipse 600 microscope. To ensure the reliability of the experimental study, internal quality control of immunohistochemical techniques was performed as part of an implemented and certified quality assurance system (ISO 9001/2001). Details of the used antibodies are shown in Table 1.

Table 1 – The antibodies used in the studied batch

Antibody	Producer	Clone	Dilution	Specificity
Troponin	Novocastra	T1/61	1:10	Muscle contraction protein
Tenascin (Tn-X)	Novocastra	49	1:100	Matrix molecule
CD54 (ICAM-1)	Novocastra	23G12	1:25	Adhesion molecule
TGF beta (CD105)	Novocastra	4G11	1:50	Extracellular matrix vessels
MMP9	Neomarkers	Poly	1:100	Matrix metalloproteinase

Statistics

For correlation between parameters, statistical analysis was performed, using the Student *t*-test, “paired two samples for mean” variant, one-group two tails, for uniform distributed data, from the Analysis Tool Pack of Microsoft Excel 2003, running under Windows XP Professional. A value of $p < 0.05$ was considered significant.

Results

The classic histopathology investigation showed various degrees of diffuse or focal interstitial and perivascular fibrosis, foci of interstitial lipomatosis in 47% of cases, associated with coronary atherosclerosis. Related to the extracellular matrix of the residual cardiomyocytes, the most sensitive reaction was noticed for ICAM-1 (CD54/endoglin) in 12 of 17 cases (71%), followed by MMP9 in 10 cases (59%) and TGF- β (CD105) in eight cases (47%) (with great specificity for capillary vessels). Tenascin (Tn-X) was positive in six

cases (35.3%) in the extracellular matrix (ECM) and troponin in five cases (29.4%) in the cytoplasm of the perilesional residual cardiomyocytes. The IHC expression of the studied markers is presented in Figure 1. ICAM-1 was focally positive in the ECM (Figure 2), near the residual cardiomyocytes and isolated, around capillary vessels. MMP is a class of proteases, secreted by fibroblasts, playing a role in matrix remodeling, through an enzymatic digestion of collagen fibers; in this study, the MMP9 was variably positive in the ECM (Figure 3), around residual cardiomyocytes. TGF- β was expressed in new formed capillary vessels, showing a marked microvascular density in the nearby of residual myocardial fibers (Figure 4). Tn-X was detected in the ECM (Figure 5), around perilesional residual cardiomyocytes, with a variable staining in the hypoxic myocardial areas. Troponin was positive in the cytoplasm of the residual myocytes (Figure 6) and in the normal adjacent myocardium (positive intern control).

A direct, statistically significant, correlation was recorded between troponin and MMP9 ($r = 0.65$, $p = 0.01$; Figure 7), troponin and ICAM-1 ($r = 0.31$, $p = 0.02$), respectively ICAM-1 and tenascin ($r = 0.72$, $p = 0.01$; Figure 8). Other correlations between different parameters were noticed, but with no statistical significance (Tn-X/TGF- β : $r = 0.71$, $p = 0.2$; Tn-X/MMP: $r = 0.53$, $p = 0.1$; CD54/TGF- β : $r = 0.8$, $p = 0.2$; CD54/MMP: $r = 0.51$, $p = 0.65$; TGF- β /MMP: $r = 0.48$, $p = 0.6$).

Discussion

According to a study of six patients with interstitial fibrosis, selected from 270 individuals with sudden cardiac death, the expression of TGF- β was significantly increased in the lesional areas *versus* controls; subsequently, this marker was thought to be a potential mediator of a heterogeneous interstitial remodeling, with a predilection for the left ventricular wall, in idiopathic myocardial fibrosis and sudden cardiac death [3]. In the patients undergoing coronary interventions for unstable angina, the presence of C-reactive protein (CRP) was associated to a significant overexpression of ICAM-1 in myocardial interstitium, the later proposed as a risk marker for a major cardiac event such as myocardium infarct, the expression of ICAM-1 being promoted by the CRP in an inflammatory context [4]. The plasma level of matrix metalloproteinase 3 (MMP3) is an independent prognostic factor in stable coronary artery disease [5], while the presence of both MMP3 and MMP9, genetic or IHC identifiable in the post-infarct residual myocardium are risk factors for coronary artery complicated plaques in ischemic heart disease [6]. An increased expression of MMP was also detected in the lung, after a sudden and violent death [7]. A new strategy in cardiac cell-based therapies is that connexin 43 (Cx43) expressing cell grafts, implanted to those who had suffered a myocardium infarct, may prevent the post-infarct arrhythmias [8]. In addition, MMP7 affects Cx43 level and the electrical conduction, with an improved survival rate after a myocardial infarct, MMP7 being involved in

post-infarct remodeling [9]. It is known that the acute coronary syndrome is accompanied by a raised troponin T-level, but according to a study done on more than 1000 patients, this event occurs also, in ~38% of hospitalized patients, without previously cardiac symptoms, forecasting a possible occurrence of an acute coronary syndrome with a worse outcome [10]. A previously study on 50 cadavers has concluded that

the IHC determination of troponin C and T expression in myocardial tissue may be used as an index of myocardium damage [11]. In canine models there was demonstrated that the Tn-X expression is detected and located near sympathetic nerve sprouts, after myocardial infarct, suggesting that Tn-X plays a role in the myocardial nerve cells growth after cellular injuries (e.g. myocardial infarct) [12].

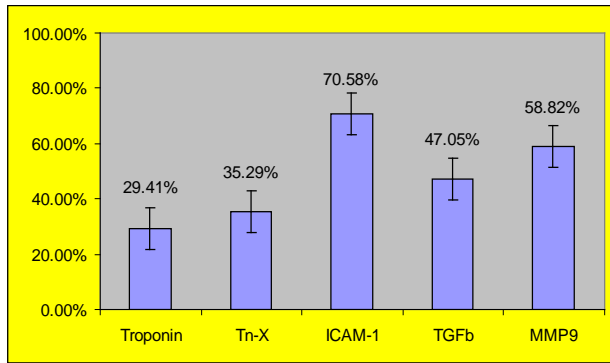


Figure 1 – IHC expression of the used markers in the studied batch.

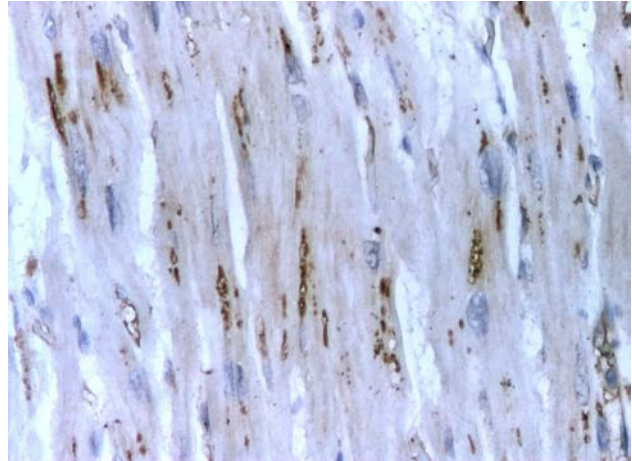


Figure 2 – CD54 (ICAM-1) expression in the ECM, among the residual myocardial fibers, IHC, ob. 20x.

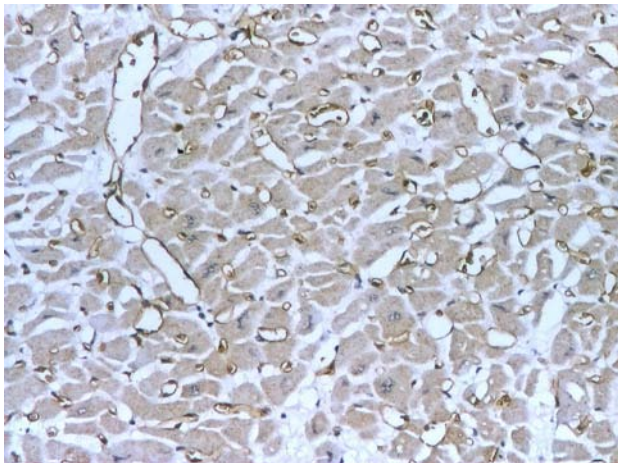


Figure 3 – IHC positive reaction for MMP9 in the ECM and vessels, ob. 10x.

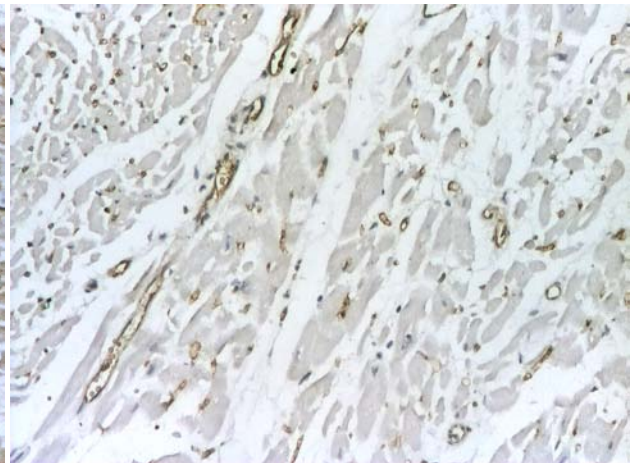


Figure 4 – TGF-b staining positive in capillary vessels, around residual cardiomyocytes, IHC, ob. 10x.

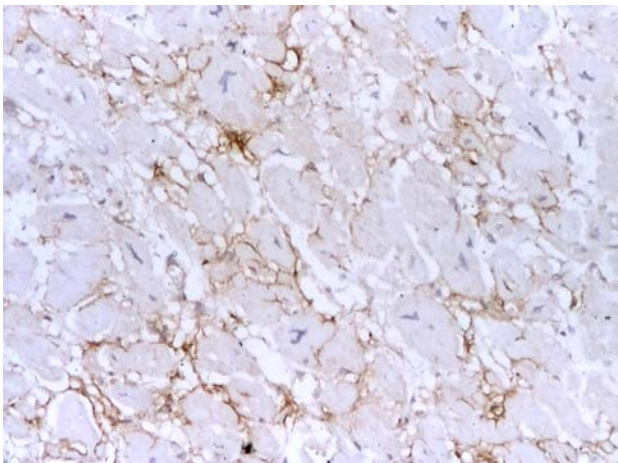


Figure 5 – IHC positive reaction for Tn-X in the ECM, ob. 10x.

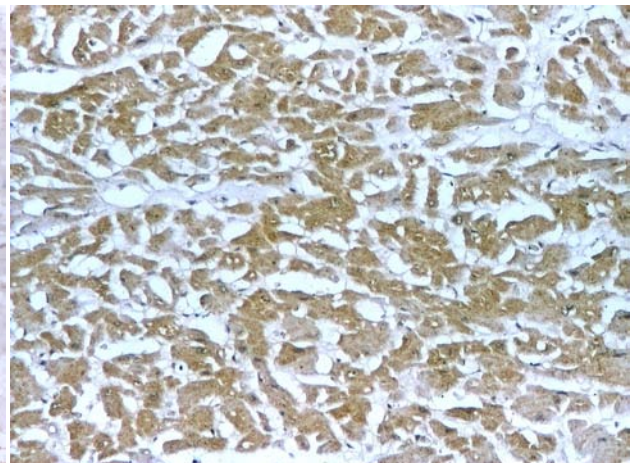


Figure 6 – IHC diffuse positive reaction for troponin in perilesional residual cardiomyocytes, ob. 10x.

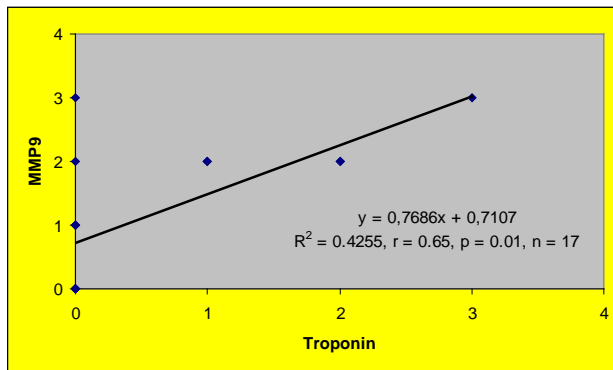


Figure 7 – Correlation between troponin and MMP9 in the studied batch.

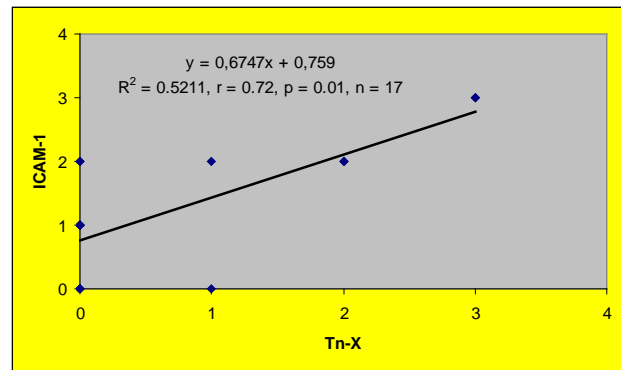


Figure 8 – Correlation between Tn-X and ICAM-1 in the studied batch.

Conclusions

The extensive expression of ICAM-1 in the extracellular matrix from the perilesional area probably plays a role in the stimulation of new developing adhesion substrates between residual cells and adjacent stroma, while the overexpression of troponin in the residual cardiomyocytes is accompanied by a high expression of MMP9 in the myocardial interstitium, with heterogeneous remodeling of the ventricular stroma. The simultaneous IHC expression of tenascin and ICAM-1 suggests a colocalization required for the nerve sprouting in the residual myocardium and for developing new focal cell-matrix adhesion contacts.

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