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Evaluation of cardiac microvasculature in patients with diffuse myocardial fibrosis

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Abstract

Myocardial fibrosis is one of the most common histopathological lesions found in chronic heart diseases. Progressive development of myocardial fibrosis will cause heart failure, an extremely debilitating and life threatening condition. The correlation between the severity of fibrosis and myocardial microcirculation is an important prognostic factor in this disease entity. In our study, myocardial microvascular density evaluation of the patients with high blood pressure (hypertension), atrial fibrillation (AF), coronary heart disease, and heart failure showed a significant decrease of the values of this parameter, which means that myocardial fibrosis is the direct result of stimulation of myocardial fibroblasts induced by local hypoxia.

Keywords: myocardial fibrosis, endothelial cells, fibroblasts, myofibroblasts, cardiomyocyte, extracellular matrix.

Introduction

Myocardial fibrosis (MF) is defined as a significant increase of the amount of fibrillar collagen in myocardial tissue [1]. From a histopathological point of view, most of the heart diseases (valvular disease, ischemic heart disease, hypertension, dilated cardiomyopathy, diabetic cardiomyopathy, myocardial infarction, heart failure of different etiologies, etc.) have been associated with larger or smaller changes of the extracellular matrix (ECM), including myocardial fibrosis [2–5].

The myocardium is a complex structure, being composed of several cell types; some of them are proper cells (cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells), while others are migrated cells (lymphocytes, plasma cells, mast cells and macrophages).

Migrated cells interact with permanent cells in both pathological and physiological conditions to support cardiac functions [6, 7]. From all of these cells, cardiac fibroblasts represent the largest population of myocardial cells, representing approximately two-thirds of these cells, while cardiomyocytes are about two-thirds of the myocardial tissue [8]. In pathological conditions, in the myocardial interstitium there is a population of cells called myofibroblasts that are extensively involved, together with fibroblasts, in synthesis and excessive deposition of ECM [9–11].

Myocardial fibrosis causes a high rigidity of the cardiac wall, leading to a continuous decrease in the cardiac function, and leading, eventually, to the occurrence of progressive heart failure [12–15]. Also, the excessive ECM affects the electro-mechanical coupling of the cardiomyocytes and increases the risk of developing arrhythmias.

Although it is known that coronary microvascular ischemia is involved in the pathogenesis of MF [16], recent data show that there always is a correlation between small vessels from within the myocardium and fibrosis [17].

In our study, we aimed to assess microvessel density from areas with myocardial fibrosis, compared with the microvasculature from areas without fibrosis, in several types of chronic cardiovascular diseases.

Materials and Methods

The study was performed on human myocardial tissue fragments from the entire thickness of the left ventricle (from endocardium to epicardium) sized at around 2/2 cm, being centered on the base of anterior interventricular artery (in the immediate vicinity of detachment of the anterior circumflex artery from left coronary artery), harvested during autopsy from 23 patients which died in the Emergency County Hospital of Craiova, Romania, during the years 2014 and 2015.

We selected a unique anatomical infarction area for sampling of the biological material in order to prevent any discrepancies resulted from the different distribution of myocardial microvascular depending on the topographical area of the myocardium.

Among them, 20 patients with ages between 56 and 80 years old and died due to cardiovascular diseases (hypertension, atrial fibrillation, coronary heart disease, heart failure) constituted the investigated group (there were selected five patients for each of these main causes of death: five with hypertension, five with atrial fibrillation, five with coronary heart disease and five with heart failure), and three patients, with ages between 24 and 31 years, which died as a result of serious injuries through accidents and constituted the control group.

We selected for the control group only adults aged less than 35 years without any cardiovascular disease in order to eliminate any false positive results due to myocardial senescence.

The tissue was fixed in neutral buffered formalin, at room temperature, followed by paraffin embedding according to routine protocols. Sectioning was performed on a Microm HM350 rotary microtome equipped with a water bath sections transfer system (Thermo Scientific Inc., Walldorf, Germany). For the histological study, we have utilized 4-μm thick sections stained with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichrome (for selective highlighting of collagen fibers). For immunohistochemistry, we utilized tissue sections collected on poly-L-lysine coated slides, dried overnight at 45^0C, in a thermostat. All sections have been deparaffinized, rehydrated in graded alcohol series and subjected to further histological or immunohistochemical protocols. For immunohistochemistry, sections were subjected to antigen retrieval by microwaving in citrate buffer pH 6 for 20 minutes, incubated in 1% hydrogen peroxide in distilled water for further 30 minutes in order to block the endogenous peroxidase activity, and then kept for another 30 minutes in a 3% phosphate-buffered saline (PBS) skimmed milk solution. The sections were next incubated at 4^0C for 18 hours with mouse anti-human primary antibodies [anti-α-SMA for detecting myofibroblasts (clone 1A4, diluted as 1/100, Dako, Glostrup, Denmark), anti-CD34 for detecting endothelial cells (clone QBEnd 10, diluted as 1/50, Dako), and anti-CD68 for detecting macrophages (clone KP1, diluted as 1/200, Dako)], and the next day the signal was amplified for 30 minutes with an anti-mouse peroxidase polymer detection system adsorbed for human immunoglobulins (Nichirei Bioscience, Tokyo, Japan). The signal was finally visualized with 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark) and the slides were coverslipped in a xylene-based mounting medium after a Mayer's Hematoxylin staining step. All slides were processed in the same time during each protocol for consistency, together with negative control slides, which were obtained by omitting the primary antibodies.

Light microscopy images were captured on a Nikon Eclipse 55*i* microscope equipped with a 5 Mp color cooled CCD (charge-coupled device) camera, under the Image ProPlus 7 AMS image analysis software (Media Cybernetics Inc., Buckinghamshire, UK). For quantification purposes, the slides have been photographed randomly in regions of interest with a 20× objective, collecting 10 images for each tissue block. The vessels have been directly tagged in the images and their average density has been reported on the objective's area. For quantifying the expression of α-SMA (alpha-smooth muscle actin) and CD68, an RGB (red, green and blue) profile has been set-up for each staining, and then for each image, the integrated optical density (IOD) of the signal was calculated. Values in each category of data were averaged first for each slide and then for each pathological entity. Finally, representative values for each pathological group were represented graphically and analyzed utilizing Microsoft Excel 2013 and SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). A one-way ANOVA (ANOVA – analysis of variance) test was utilized for multiple comparisons. In all cases, $p<0.05$ was used to indicate statistical significance.

Results

Our study has aimed to evaluate myocardial microvasculature in patients with chronic cardiovascular diseases to which histopathology and a trichrome staining revealed the presence of myocardial interstitial fibrosis. Pathologies were examined by comparing histopathological samples from the left ventricle from patients deceased with high blood pressure, atrial fibrillation, ischemic heart disease, and cardiomyopathy. Microvascular density in patients with chronic cardiovascular diseases was compared to a group of middle-aged people without cardiovascular disease (deaths by traffic accidents).

In order to evaluate microvascular densities, we used an antibody directed against endothelial cells, CD34. Also, we have intended to correlate myocardial fibrosis intensity with the presence of myofibroblasts in myocardial interstitium and the presence of macrophages, cells that are involved in myocardial wall remodeling processes by phagocytosis and by production of matrix metalloproteinases.

Classical histopathological HE and GS trichrome stainings showed that the interstitial myocardial fibrosis areas had different spreads and, most often, they showed continuity to perivascular fibrosis (Figures 1 and 2). Most often, the myocardiocytes occurred totally or partially damaged, with myocardium architecture being modified by replacing the muscle areas with fibrous connective tissue. Among collagen fibers, one could identify elongated cells, with fibroblast-like morphology.

In some patients with atrial fibrillation, we identified a widened interstitium, with few collagen fibers (Figures 3 and 4), which showed that in some cases the interstitium can accumulate other strongly hydrated elements of the intercellular matrix. Microvasculature assessment using anti-CD34 antibodies showed that the average number of vessels was much lower for the pathology group than the control group [$F(4, 15)=10.91$, $p<0.001$].

Thus, while in the control group the mean vascular density was of 1.615 vessels/mm^2, in patients with hypertension the density was of 848 vessels/mm^2, for those with atrial fibrillation of 934 vessels/mm^2, for those with dilated cardiomyopathy it was of 917 vessels/mm^2, and for those with heart failure of 605 vessels/mm^2. In other words, there was a significant decrease of microvascular density to nearly half the value of the control group in all chronic cardiovascular diseases. In heart failure, decreasing of the microvascular density was more accentuated, exceeding by more than half the value of the control group (Figure 5).

Also, the microvascular density was different from one area to another for the same histopathological sample, in areas with extensive fibrosis the number of blood vessels being reduced by 50–70% compared to areas without fibrosis (Figures 6 and 7).

Given the fact that the interstitial fibrillogenesis is a type of repair which involves fibroblasts and myofibroblasts, we have further investigated the reaction of myofibroblasts using an anti-α-SMA antibody. The resulting data have shown that in chronic cardiovascular diseases myofibroblasts are not present in areas of interstitial fibrosis (Figure 8), so they do not participate substantially in the synthesis of collagen fibers, thus this being the task of resident fibroblasts.

Regarding the reaction of macrophages, as indicated by the use of the specific anti-CD68 antibody, in chronic cardiovascular diseases there was a significant increase in their number compared with controls [$F(4, 15)=7.51$, $p<0.01$] (Figure 9). If the average number of macrophages in control group was of 53 cells/mm^2, in arterial hypertension they reached a density of 105 cells/mm^2, in atrial fibrillation of 149 macrophages/mm^2, in dilated cardiomyopathy and heart failure there were of 81 cells/mm^2 (Figure 10). Thus, the increasing densities of macrophages would support a heart remodeling process, especially in the intercellular matrix.

Discussion

In physiological conditions, there is a tight relationship between myocardiocytes and myocardial fibroblasts, and this allows the heart to maintain its shape together with the optimal performance of the myocardium and if the whole heart. The fibroblasts are responsible for the local homeostasis maintenance, as well as for changes in the ECM during pathological conditions. These cells can be considered as having "sensory" qualities, having the ability to recognize changes in the local mechanical and chemical microenvironment [18–20] ECM, and to adapt to the demands to which the myocardium undergoes. The structure and composition of ECM ensures an equilibrate microscopic structure of the heart, mediates intercellular relations as well as the intercellular matrix and cells relationship, allowing the myocardiocytes to perform their functions [21].

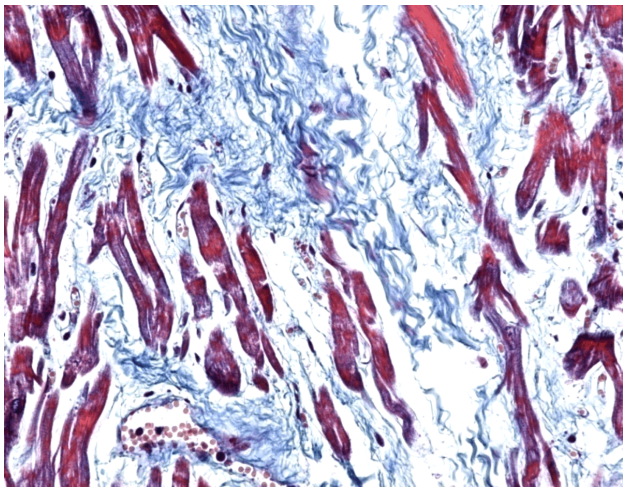

Figure 1 – ***Area of diffuse interstitial fibrosis characterized by the accumulation of collagen fibers with uneven disposition in the myocardium interstitium. GS trichrome staining, ×100.***

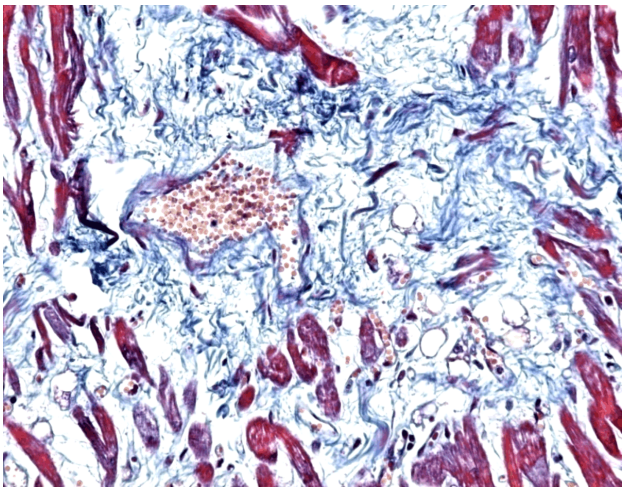

Figure 2 – ***Perivascular and interstitial fibrosis. GS trichrome staining, ×100.***

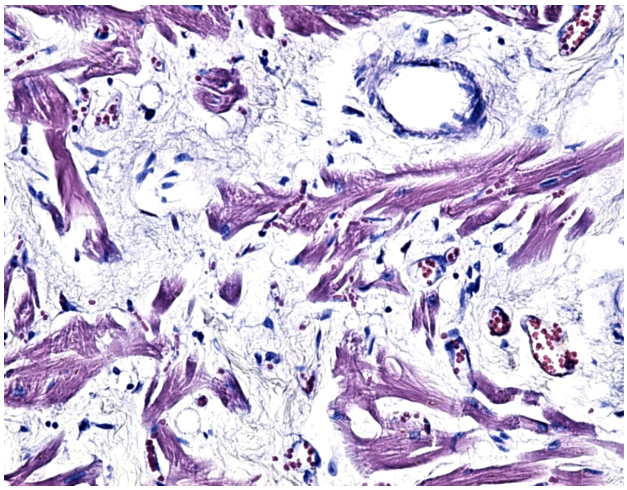

Figure 3 – ***Interstitial fibrosis associated with myocardiocytes remodeling. GS trichrome staining, ×100.***

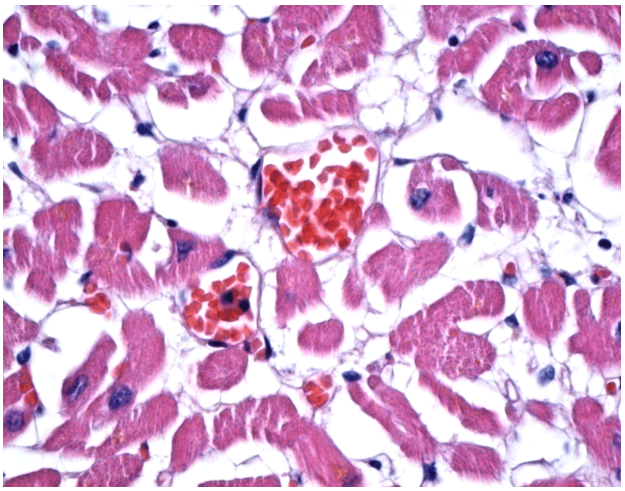

Figure 4 – ***Myocardium area from a patient with atrial fibrillation, with enlarged interstitial spaces. HE staining, ×200.***

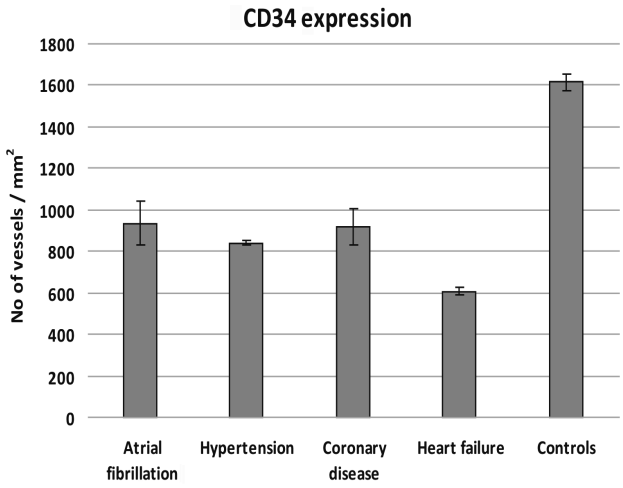

Figure 5 – *Compared microvascular density, as it results from the use of the anti-CD34 antibody. Data are presented as average ± standard deviation of the means.*

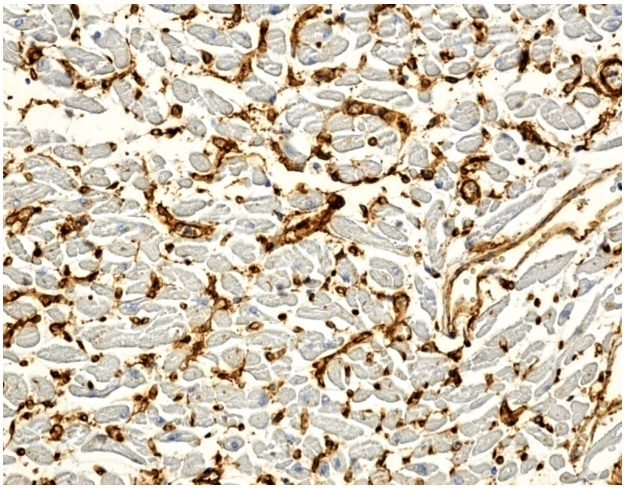

Figure 6 – *Myocardium area from a patient with atrial fibrillation, at distance from fibrotic area, in which there is a relative homogenous distribution of the micro-vessels. Immunostaining for anti-CD34, ×100.*

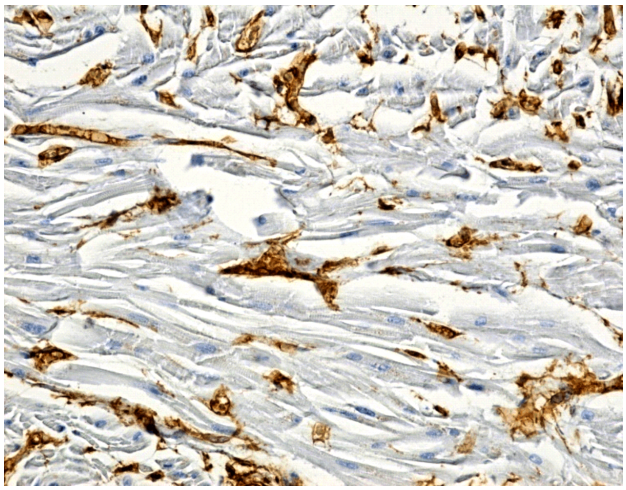

Figure 7 – *Area of myocardium fibrosis with a reduced number of vessels, unevenly distributed. Immunostaining for anti-CD34, ×100.*

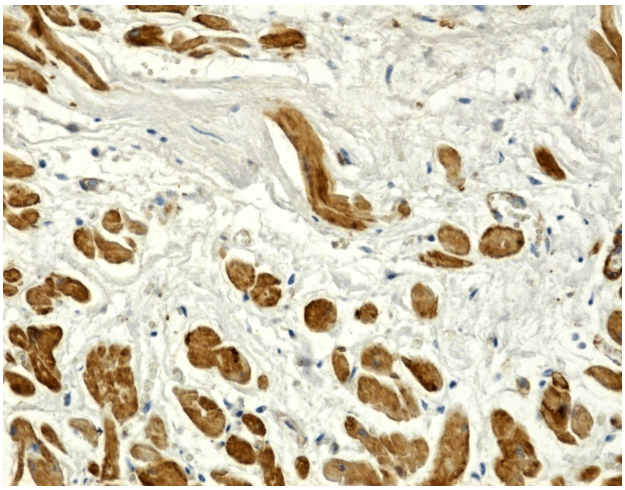

Figure 8 – *Area of myocardium fibrosis without the presence of interstitial myofibroblasts. Immunostaining for anti-α-SMA, ×100.*

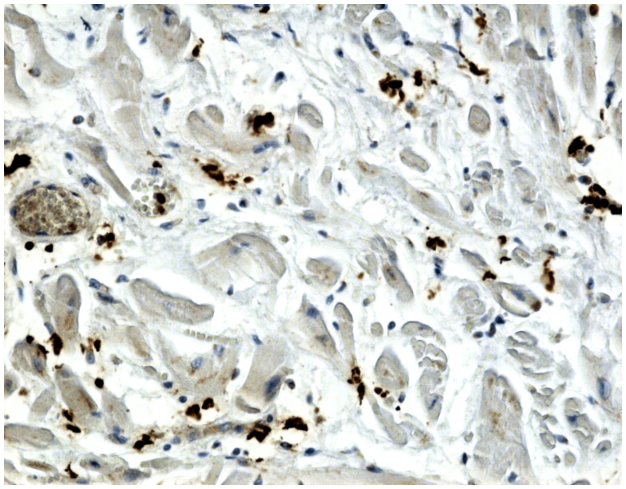

Figure 9 – *Area of myocardium fibrosis with macro-phages present in the interstitium. Immunostaining for anti-CD68, ×100.*

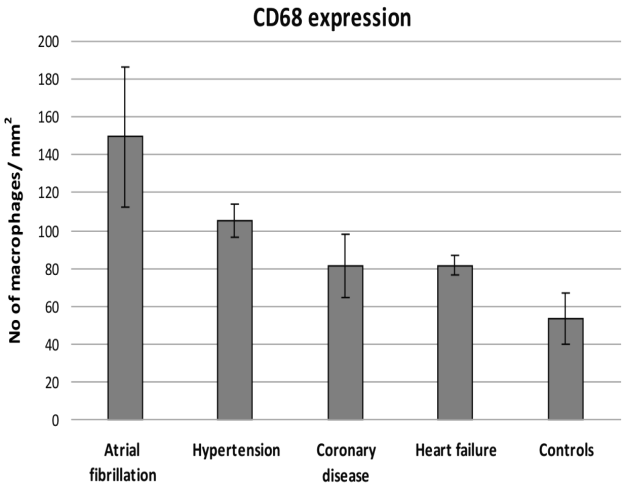

Figure 10 – *Macrophage infiltration in different heart conditions. Data are presented as average ± standard deviation of the means.*

ECM is a complex structure consisting of collagen (mainly collagen type I and III), proteoglycans, glycoproteins, cytokines, growth factors, proteases, etc. [22]. Overall, ECM forms a network surrounding the cells and provides mechanical support for different types of heart cells, blood vessels and nerves, distributes mechanical forces throughout the myocardial tissue, transmits mechanical/chemical signals towards the cells and separates electrically the atria from the ventricles allowing for different contraction rates for each heart chambers [15].

ECM undergoes a continuously remodeling process even under physiological conditions, from the intrauterine life until to the end of life. Changing ECM is an essential process for maintaining the structural integrity of the myocardium tissue, for repair and remodeling under mechanical needs of the heart, but in pathological conditions can cause myocardial dysfunction [23–25]. As shown in this study, often ECM changing is accomplished by increased synthesis of collagen by interstitial myocardial fibroblasts, which accumulate as fibrillary collagen in myocardial interstitium, leading to myocardial fibrosis [26]. Myocardial fibrosis leads in turn to an increase in passive stiffness of the heart wall causing diastolic dysfunction, as an overall hallmark of heart failure [27].

Depending on the anatomical site where this pathophysiological mechanisms initiates, this might lead to the genesis of myocardial fibrosis, and according to some authors, there are three types of fibrosis: perivascular fibrosis, interstitial fibrosis and replacement fibrosis [25, 28]. Perivascular fibrosis is characterized by the deposition of collagen fibers in adventitia of the intramural arteries and occurs predominantly in hypertension; interstitial fibrosis or reactive consists of deposition of fibrillar collagen around cardiomyocytes without affecting the latter, while replacement fibrosis is a connective repair process of the myocardium after myocardiocytes are destroyed, occurring mainly in ischemic heart disease and especially heart attacks [29–31].

In our study, we have identified the presence of three types of myocardial fibrosis in all studied cardiovascular diseases, which suggests that the pathophysiological mechanisms of myocardial fibrosis are complex and non-specific, so that it cannot be said that for example in hypertension appears only perivascular fibrosis and in ischemic cardiomyopathy it is present only replacement fibrosis. Therefore, if both the perivascular and interstitial fibrosis are excessively developed, they interfere with the function of the myocardiocytes and can damage their structure, ultimately generating a replacement fibrosis.

Evaluating the microvascular density in patients with chronic cardiovascular diseases, we have noticed a significant decreasing in microvascular densities for all patients, regardless of the primary heart condition. We consider thus that the occurrence of myocardial fibrosis is caused by local hypoxia due to reduced number of microvessels. In these conditions of hypoxia, fibroblasts proliferate and synthesize increased amounts of ECM, especially collagen, which can lead to even more hypoxia and myocardiocyte dysfunction. Our immunohistochemical study using an anti-α-SMA antibody revealed that myofibroblasts are absent in areas of myocardial fibrosis in patients with chronic cardiovascular disease.

Numerous studies have shown that changes in myocardial microcirculation and microvascular dysfunction can be a source of chronic ischemia underlying ECM changes, including the occurrence of myocardial fibrosis [32, 33]. The most common morphological changes observed in myocardial microvessels in chronic cardiovascular disease are the uneven changes of myocardial pre-arterioles and arterioles due to the development of deposits of collagen or amyloid, intimal hyperplasia or hypertrophy of the tunica media, underling certain rheological disorders of the blood manifested by increasing of the viscosity of the blood or the appearance of microemboli [34]. In patients with morphological changes of myocardial microvessels, the ischemia can be exacerbated by other factors: smoking, stress, high level of lipids, diabetes, etc. [35, 36].

In our study, assessing of the reaction of macrophages showed an increase in their number (even doubling in some cardiovascular conditions) and an intensification of immunohistochemistry reaction, which indicates the presence of larger quantities of lysosomes and thus increasing the local phagocytosis process. For us, it is clear that in the myocardial interstitium remodeling processes occur involving macrophages.

We believe that the interrelations between myocardiocytes, endothelial cells and fibroblasts are still far from being fully understood. Deciphering the molecular mechanisms of intercellular communication will enable the development of therapeutic measures to prevent myocardial fibrosis and chronic heart diseases progress to heart failure.

Conclusions

Interstitial myocardial fibrosis affects uneven the wall of the left ventricular myocardium. It is often associated with perivascular fibrosis and replacement fibrosis. Cardiac microvascular density in chronic diseases (hypertension, atrial fibrillation, dilated cardiomyopathy and heart failure) was significantly reduced compared to microvascular density in patients without heart disease. Interstitial myocardial fibrosis is not accompanied by increasing of myofibroblasts-like cells, extracellular matrix and particularly the collagen, being synthesized mainly by the local fibroblasts. In contrast, in chronic cardiovascular diseases, it was noticed an increasing in the number of macrophages that participate in the remodeling of the myocardium wall.

Conflict of interests

The authors declare that they have no conflict of interests.

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