Myocardial interstitial fibrosis – histological and immunohistochemical aspects

Background and Aim: The process of myocardial fibrosis is based on the remodeling of the extracellular matrix (ECM), which leads to increased myocardium stiffness and, in turn, severe alterations of the cardiac function. This phenomenon is consecutive to various types of cardiac disease, primarily infarction, due to inflammatory reactions involving various types of cellular mechanisms. We aimed in this paper to assess both microscopically and immunohistochemically the main aspects of ischemic myocardium found in areas of fibrosis and compare the findings with normal images from unaffected cardiac tissue of the same type.

Materials and Methods: Our study included infarction fragments of 2/2/2 cm harvested after autopsy from 26 patients who died during 2014 at the Emergency County Hospital of Craiova, Romania, following the diagnostic of ischemic cardiomyopathy. After usual histological preparations, we performed both classical staining with Hematoxylin-Eosin and trichromic Goldner-Szekely, as well as immunohistochemical assessment using anti-alpha-SMA, anti-desmin, anti-CD34 and anti-CD68 antibodies.

Results: We have evaluated myocardial fibrosis in all areas of the myocardium and noted that it primarily develops around the arterioles and metarterioles. The area of myocardial fibrosis was more extensive, myocardiocytes being surrounded by collagen fibers. Collagen interstitial fibrosis, gave a “brindle” look to the myocardium. Myocardial fibrosis areas did not reveal myofibroblasts. We found lower numbers of blood vessels with an uneven distribution in areas of myocardial fibrosis. In the areas of normal myocardium, the macrophage numbers were two to three times lower than in areas of fibrosis, also displaying larger volume, with intense reaction to CD68.

Conclusions: Myocardial interstitial fibrosis occurs in outbreaks, mainly perivascular, but it affects larger areas of the myocardium interstitial space. We believe that interstitial fibrosis is a progressive process that can become permanent, thus constituting a promising therapeutic target for a large variety of cardiac conditions.

Keywords: cardiac fibrosis, cardiomyocyte, endothelial cell, fibroblasts, myofibroblasts, extracellular matrix.

Introduction

Myocardial fibrosis is a pathological process of remodeling of the extracellular matrix (ECM), often leading to increased myocardial wall stiffness, which can cause significant changes in cardiac function [1–3]. This process is similar to a healing process that takes place in all organs after several assaults and is characterized by the accumulation of fibroblasts who produce excessive EMC, which leads to changes in body architecture. Myocardial fibrosis is most commonly encountered in ischemic heart disease, but it has been highlighted in other diseases in the absence of myocardial ischemia, such as hypertension [4–6], valvular heart disease [7], diabetic cardiomyopathy [8, 9], hypertrophic cardiomyopathy [10–13] and idiopathic dilated cardiomyopathy [14–17]. The most extensive fibrosis occurs in myocardial infarction, where ECM changes are secondary to local inflammatory reactions, followed only by myocyte death and their replacement with connective tissue rich in collagen fibers [18].

The pathophysiological mechanisms that lead to this myocardial fibrosis are varied, some acute (as in myocardial infarction), others are progressive and potentially reversible (as in hypertensive cardiomyopathy). Myocardial fibrosis is associated with heart rhythm disorders, worsening ventricular systolic function, abnormal cardiac remodeling and increased ventricular wall stiffness [19, 20].

We believe that ischemia plays an essential role in the development of myocardial fibrosis process.

Aim

Therefore, in this study, we aimed to evaluate some microscopic and immunohistochemical aspects of ischemic myocardium from fibrosis areas compared to areas without fibrosis.

Materials and Methods

In our study, we evaluated infarction fragments of around 2/2/2 cm harvested when conducting autopsy from 26 patients who died at the Emergency County Hospital of Craiova, Romania, during 2014 and were diagnosed with ischemic cardiomyopathy. Fixating the biological material was carried out in neutral formaldehyde solution at room temperature, followed by inclusion in paraffin according to the histopathology protocol. Cutting the biological material was performed in the rotating microtome (HM350 Microtome) equipped with a system to transfer water bath sections (STS microM). For the histological study, were performed 4 μm thick sections.
that were stained with Hematoxylin–Eosin (HE) and trichromic Goldner–Székely (GS). For the immunohistochemical study, histological sections were collected on slides coated with poly-L-lysine and dried in a thermostat at 37°C for 24 hours. Then, we followed the classical protocol for sections: dewaxing followed by hydration. For antigen unmasking, the slides were boiled in a solution of sodium citrate, pH 6, for 21 minutes (seven cycles of three minutes) in a microwave oven. After boiling and cooling, the slides were rinsed in tap water and washed in distilled water for 15 minutes. Block endogenous peroxidase was performed by incubating the slides in 3% hydrogen peroxide for 30 minutes at room temperature followed by washing in distilled water for 10 minutes and a wash in a solution of phosphate-buffered saline (PBS) 1% for five minutes. Afterwards, blocking the non-specific sites followed by using 2% skim milk for 30 minutes. Sections were then incubated with the primary antibody for 18 hours (overnight) in a refrigerator at 4°C. Then, we followed the classical protocol for sections: dewaxing followed by hydration. For antigen unmasking, the slides were boiled in a solution of sodium citrate, pH 6, for 21 minutes (seven cycles of three minutes) in a microwave oven. After boiling and cooling, the slides were rinsed in tap water and washed in distilled water for 15 minutes. Block endogenous peroxidase was performed by incubating the slides in 3% hydrogen peroxide for 30 minutes at room temperature followed by washing in distilled water for 10 minutes and a wash in a solution of phosphate-buffered saline (PBS) 1% for five minutes. Afterwards, blocking the non-specific sites followed by using 2% skim milk for 30 minutes. Sections were then incubated with the primary antibody for 18 hours (overnight) in a refrigerator at 4°C. The next day, we applied biotinylated secondary antibody for 30 minutes at room temperature and then performed a washing in 1% PBS (three baths of five minutes), and then we applied Streptavidin-HRP (Horse-radish peroxidase) for 30 minutes at room temperature, followed by another wash in 1% PBS 3×5 minutes. The signal was detected using 3,3′-Diaminobenzidine (DAB) (Dako) and the reaction was quenched in 1% PBS. Afterwards, we performed contrasting with Mayer’s Hematoxylin, dehydration in alcohol, xylene and mounting using DPX (Fluka).

For the immunohistochemical study, we used the following antibodies:

- Anti-alpha-smooth muscle actin (α-SMA) to highlight myofibroblasts (clone 1A4, sodium citrate, pH 6, 1/100 dilution, Dako);
- Anti-desmin to show myocardial fibers (clone D33, sodium citrate, pH 6, 1/50 dilution, Dako);
- Anti-CD34 to evidence the myocardial microvascularization (clone QBEnd 10, sodium citrate, pH 6, 1/50 dilution, Dako);
- Anti-CD68 to highlight macrophages (clone KP1, sodium citrate buffer, pH 6, 1/200 dilution, Dako).

## Results

For our study, we selected cases that showed moderate clinical forms of ischemic heart disease, to capture the changes that occur in the extracellular matrix during the early stages of myocardial ischemia. In addition, for comparison of the results, we retained only the myocardium from the left ventricle, the sterno-costal area, taken from the immediate vicinity of the interventricular artery, knowing that myocardial histology varies from one heart chamber to the other.

As shown in our pictures, normal ventricular myocardium consists primarily of myocardiocytes and little connective extracellular matrix (ECM) (Figure 1, A and B). We did not highlight the ECM in stains for collagen fibers, other than perivascular around blood vessels with a caliber greater than 50 μm.

In our study, we found that the process of fibrosis, characterized by the appearance of new collagen fibers, uniformly affect the myocardial mass; on the same histological piece (with an area of 2/2 cm), we found areas of normal myocardium and areas of myocardial fibrosis. We noted that myocardial fibrosis develops primarily perivascular, particularly around the arterioles and metarterioles (Figure 2, A and B). Further studies with more powerful magnifications, in trichromic GS staining (specific to collagen fibers), allowed us to note that the area of myocardial fibrosis is more extensive, thin collagen fibers being placed between myocardiocytes. In most cases, the main aspect was that of collagen interstitial fibrosis, giving a "brindle" look to the myocardium, through alternation of areas with fibrosis and areas of myocardial cells (Figure 3A); however, in around a quarter of cases, fibrosis appeared expanded being similar to granulation tissue, giving the appearance of replacement fibrosis (Figure 3B). We should mention that replacement fibrosis was always accompanied by interstitial fibrosis, fibrosis replacement on the edge of the highlighted aspects of interstitial fibrosis. These aspects make us believe that microscopic replacement fibrosis is a more serious stage of development of interstitial fibrosis unless the pathophysiologic mechanisms that lead to myocardial fibrosis are interrupted.

In areas of fibrosis, we found a greater number of fibroblasts compared to areas without fibrosis. Image analysis revealed that the vast majority of fibroblasts showed a large nucleus, hypochromic, nucleoli and abundant cytoplasm, which corresponds to the stage of ‘activated fibroblasts’ (Figure 4, A and B). These microscopic aspects confirm that the main cells involved in interstitial myocardial fibrosis are fibroblasts.

Starting from the fact that the EMC can be produced by several types of cells, including myofibroblasts, in our study, we used anti-α-SMA antibody to highlight the nature of these cells. In our study, myocardial fibrosis areas did not reveal myofibroblasts (Figure 5, A and B).

In order to assess the relationship between myocardiocytes and interstitial fibrosis, we used the anti-desmin antibody, which marks the intermediate filaments of the cytoskeleton structure of myocardiocytes. In areas of normal myocardium (Figure 6A), we could observe a uniform and intense immunostaining with desmin of myocardiocytes; in areas of myocardial fibrosis, they were weak and unevenly colored (Figure 6B), which betrays the reduced amount of intermediate filaments in the structure of these cells.

For the assessment of myocardial microvascularity, we performed anti-CD34 antibody specific marking of endothelial cells. In areas free of infarction, we noted the presence of a well-developed network of blood vessels with numerous capillaries, relatively evenly distributed myocardial interstitial connective tissue (Figure 7A). In areas of myocardial fibrosis, the number of blood vessels was much lower and the distribution was uneven (Figure 7B). Numerical evaluation of blood microvessels in the two areas showed that in areas of normal myocardium their number ranged between 1500 and 2200 capillaries/mm², whereas in the areas of diffuse myocardial fibrosis, the number of blood capillaries ranged from 600 to 1100/mm². These data show that interstitial myocardial fibrosis in areas of myocardial vasculature is reduced by 2–3 times compared to areas without fibrosis.
Given that the myocardial fibrosis process involves not only the synthesis of EMC but also its reshuffle, we have decided to evaluate the response of macrophages by using anti-CD68 antibody in myocardial fibrosis areas. Macrophages are cells involved in phagocytosis and reshuffle connective tissue disorders. In our study, we identified a large number of macrophages in the areas of myocardial fibrosis compared with the surrounding normal myocardium (Figure 8, A and B). Thus, in the areas of normal myocardium, the macrophage numbers varied from 28 to 52 cells/mm², while the areas of fibrosis their number varied from 110 to 140 cells/mm². Besides the much higher number in areas of fibrosis, macrophages showed a much higher volume, with intense reaction to CD68, which shows an increase in the number of lysosomes and intensified phagocytic activity.

Figure 1 – (A) Microscopic aspect of a normal myocardium area (longitudinal section). We could observe myocardiocytes, which occupy the larger part of the myocardium mass, while the interstitial connective tissue is weakly represented, being formed by lax connective tissue with numerous fibroblasts and blood capillaries (HE staining, ×400); (B) Normal myocardium, transversal section (HE staining, ×400).

Figure 2 – (A) Image of interstitial myocardial fibrosis: “brindle” aspect (Trichromic GS staining, ×200); (B) Adipocytes present in an area of perivascular myocardial fibrosis (Trichromic GS staining, ×200).

Figure 3 – (A) Perivascular myocardial fibrosis (HE staining, ×200); (B) Extended myocardic replacement fibrosis with the appearance of granulation tissue (Trichromic GS staining, ×100).
Figure 4 – (A) Area of fibrosis with numerous fibroblasts (HE staining, ×200); (B) Detailed picture in which we could observe the presence of activated fibroblasts, with round, large, hypochromic nucleus and abundant cytoplasm (HE staining, ×400).

Figure 5 – (A) Area of interstitial fibrosis in which we found no myofibroblasts (Anti-α-SMA immunostaining, ×200); (B) Image of extended perivascular myocardial fibrosis, in which we found no myofibroblasts. Additionally, we could observe the atherosclerosis process, with the enlargement of arteriolar wall, reduction and deformity of the vascular lumen and disarrangement of the muscular tunic (Anti-α-SMA immunostaining, ×200).

Figure 6 – (A) Immunohistochemical image of normal myocardioocytes (Immunostaining with anti-desmin antibody, ×400); (B) Area of myocardic fibrosis, with weak and uneven reaction of myocardioocytes to desmin (Immunostaining with anti-desmin antibody, ×400).
Discussion

Myocardial fibrosis is a pathological process, a response of the myocardium to abuse or assault to which it is subjected. The presence of pathological entities such as hypertension, ischemic heart disease, valvular heart disease, cardiac arrhythmias, dilated cardiomyopathy, diabetes and so on causes changes in the entire myocardium and primarily the ventricular section.

It is known that in the structure of myocardium, about 70–80% of the cell mass is represented by cardiomyocytes, which ensure the pump function of the heart, while the remaining 20–30% is represented by fibroblasts, endothelial cells, smooth muscle cells and cells of the immune system [21, 22]. While cardiomyocytes are more than two-thirds of the myocardial tissue, cardiac fibroblast cells are the most abundant [23, 24]. After some studies, fibroblasts are essential cells within the myocardium, being considered as “sensory cells” that sense mechanical and chemical changes in myocardial microenvironment and synthesizes all biological components that make up the ECM necessary for the proper functioning of the heart [25, 26]. In addition, fibroblasts ensure homeostasis and remodeling of the ECM, providing communication with cardiomyocytes, endothelial cells and smooth muscle cells and are involved in the processes of angiogenesis, cell proliferation and apoptosis [24].

As we mentioned in this study, myocardial fibrosis is similar to a healing process, characterized by the emergence of an increased number of fibroblasts in the myocardium and in excess deposit of ECM, especially collagen fibers [27, 28]. The emergence and development of the myocardial fibrosis process involves significant changes both to the ECM and to the cardiomyocytes.

Depending on etiopathogenic mechanisms, myocardial fibrosis can be divided into two types: reactive or interstitial fibrosis and fibrosis replacement or remodeling. Reactive fibrosis following a prolonged cardiac stress and occurs in cardiovascular disease with an increased pressure and volume, including cardiomyopathy [29–31], while replacement fibrosis occurs in myocardial infarction [32–34].

In our study, in patients with ischemic heart disease, reactive fibrosis appeared in diffuse perivascular foci, mainly around arterioles and metarterioles. Other authors, working on experimental animals have shown that reactive
interstitial fibrosis had a diffuse distribution in the interstitium, predominantly perivascular [35, 36]. The size of these areas of fibrosis was variable, depending on the severity of myocardial ischemia and was accompanied by arteriosclerotic changes. Foci of fibrosis have been characterized by the accumulation of collagen (likely type I and III), sometimes organized in bundles and an increased number of reactive fibroblasts. In some foci of fibrosis, we identified adipocytes. It must be noted that thin collagen fibers were identified in the interstitium in much larger areas, which shows that reactive fibrosis affects large areas of myocardium. The majority of the interstitial spaces appeared enlarged, even if there was no evidence of collagen fibers. It is possible that these spaces contain an excess of other components of the ECM (procollagen, proteoglycans, fibronectin, tenascin, elastin, laminin, etc.), also synthesized by fibroblasts [28, 37, 38].

The presence of large quantities of fibroblasts show that they are the main cells involved in myocardial fibrosis. According to some authors, under physiological conditions, ECM fibroblasts maintain homeostasis [39–41]. In pathological conditions (heart disease, hypertension, cardiomyopathy, diabetes mellitus, myocardial infarction) fibroblasts present in the interstitial myocardium (resident fibroblasts) proliferate and intensify synthesis activity developing and excreting all components of biochemical ECM in the interstitial space [42–45].

Numerous studies on animal models showed that under pathological conditions, cardiac fibroblasts turn into myofibroblasts, cells that actively participate in the synthesis of ECM components [33, 46, 47]. In our study, using a specific marker (α-SMA) we have not identified myofibroblasts in areas of interstitial fibrosis. It is possible that phenotypic changes that occur in cardiac fibroblasts differ from species to species, and the reaction of human fibroblasts is not the same as the experimental animal.

Evaluating microvascular density in areas of fibrosis, we observed a significant reduction in blood capillaries (about 3–4 times) compared to other areas without fibrosis. These microscopic aspects reiterate the importance of myocardial ischemia in the process of ECM remodeling. To date, there is insufficient data to explain the vascular resuffle in these areas. According to some authors [28, 48], angiogenesis depends on the extracellular signals that modulate the behavior of endothelial cells and fibroblasts that would significantly contribute to the homeostasis of cardiac vessels. Some studies have shown that fibroblasts, the synthesis of paracrine-acting factors such as the fibroblast growth factor (FGF) or the vascular endothelial growth factor (VEGF) interact with angiogenesis by potently stimulating endothelial cells [49–51].

Although several studies support the idea that the interstitial fibrosis reaction is an adaptive response in order to maintain the capacity of the contractile heart [28], which does not alter the myocardiocytes, we have found that around the foci of fibrosis, myocardiocytes are disorganized, and by using the desmin I myocardiocyte marker, we have shown that the suffering is significant, at least in terms of the desmin composition of these cells and thus the morphology of the myocardiocyte cytoskeleton.

We also noticed, using the anti-CD68 antibody, that there is a very active phagocytosis in the myocardium interstitium affected by fibrosis, which suggests that the process of interstitial fibrosis is in a constant state of reshuffle.

We believe that there are multiple complex relationships between myocardiocytes, interstitial fibroblasts and endothelial cells, incompletely known and that could become a therapeutic target, for that the process of fibrosis can extend in the absence of therapeutic measures to clear etiological factors leading to heart failure.

Conclusions

Myocardial interstitial fibrosis occurs in outbreaks, mainly perivascular, but it affects larger areas of the myocardium interstitial space. In areas of fibrosis, we detected a large number of collagen fibers sometimes organized in bundles, and reactive fibroblasts, but could not reveal myofibroblasts. The number of capillaries in areas of fibrosis and adjacent areas was significantly reduced compared to areas without fibrosis. Myocardiocytes within adjacent areas of fibrosis appeared disorganized and use anti-desmin showed a profound alteration of their cytoskeleton. The presence of increased numbers of macrophage phagocytosis demonstrates intense activity and a significant process of remodeling the ECM. We believe that interstitial fibrosis is a progressive process that can extend becoming replacement fibrosis and heart failure may result in a lack of therapeutic measures that suppress the etiological factors of heart disease.

Conflict of interests

The authors declare that they have no conflict of interests.

References


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