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Cellular immunophenotypes in human embryonic, fetal and adult heart

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Abstract

The cellular immunoprofile of cardiac dysfunctions and lesions of ischemic etiology are insufficiently studied to date, especially regarding the contribution of non-cardiomyocytic structures. Aiming to explore this immunoprofile, we used immunohistochemistry applied on embryonic, fetal and adult normal or ischemic myocardium. We observed a decrease of smooth muscle alpha-actin expression in fetal *vs.* embryonic cardiomyocytes, its absence in normal adult myocardium and its intense expression in the fibrotic scars of ischemic myocardium. DDR2 and vimentin, which are present in the interstitial cells and cardiomyocytes of the embryo, fetus and normal adult heart, are absent in the fibrotic scar tissue and cicatricial infarction, the latter expressing smooth muscle alpha-actin and CD34. This suggested that myofibroblasts and not local fibroblasts that participate in ischemic remodeling. An EGFR-positive vascular network was better represented in the ischemic heart than in the adult normal one, a fact possibly related to EGFR implication in cardiac ischemic pre- and post-conditioning. Therefore, cardiomyocytes and non-cardiomyocytic cells have an undulating immunoprofile according to the intrauterine life stage or age after birth, and a variable contribution in cardiac lesions, mostly in ischemic ones.

***Keywords*:** heart, immunohistochemistry, cardiac development, ischemic disease.

Introduction

Currently, cardiovascular diseases are the main cause of death worldwide, including our country [1]. Paralleling life expectancy increase, the number of persons surviving acute myocardial infarctions, but developing a cardiac failure, is incrementing continuously [2]. The intimate cellular and molecular mechanisms, as a substrate of cardiac dysfunctions and lesions, are insufficiently studied, especially the role of non-cardiomyocytic components.

The study of these elements is difficult because a good part of literature results derives from animal experimental models and these ones usually cannot be extrapolated to humans; also, the cell populations do not evolve linearly during cardiac development or in different pathological situations [3].

The identification of non-cardiomyocytic cells and the extracellular matrix (ECM) components is based on immunohistochemical markers like actin, desmin, vimentin, type IV collagen, fibronectin, laminin, sustaining their mesodermal origin. Cardiac valves were considered to originate in the endothelial cells and not in the primitive mesenchyme [4].

Cardiac fibroblasts organized in a myocardial three-dimensional network [5] became recently a fashionable research topic because of their heterogeneity, activation capacity, and *immunophenotypic switch* possibilities, with contractile or secretory properties acquirement [6–8]. The immunohistochemical characterization of myocardial fibroblasts is difficult; these cells do not express markers with absolute specificity. Although the myocardial mass is mostly represented by cardiomyocytes (75%), these are much less numerous (only 25%) as compared to fibroblasts [9].

The morphologic and immunohistochemical characterization of cardiac fibroblasts can provide useful data on the pathologic processes in which they are involved. As an example, myocardial remodeling, produced by fibrosis associated with microvascularisation reduction and alteration of normal myocardial structure, is considered to be a consequence of excessive accumulation of ECM by some authors, process based on fibroblasts recruitment [3].

Cardiac fibroblasts form gap junctions between them or with cardiomyocytes, which allow their intervention in global cardiac function regulation, therefore being proposed as potential therapeutic targets [10, 11]. They have few and not entirely specific identification markers (vimentin, DDR2 – collagen receptor of the discoidin domain, cadherin 11) [12]. They present multiple possibilities of phenotype modulation and, once activated, they express smooth muscle alpha-actin and can be misinterpreted as myofibroblasts [3]. Their transformation to myofibroblasts is recognized only by some authors [13]. Other observations sustain that cells involved in fibrosis are myofibroblasts resulted from an endothelial-mesenchymal transition and not from the fibroblastic switch [14]. A recent study concerning the detection of specific micro-RNA from immature and adult heart [15] suggests that myofibroblasts identified

in fibrotic areas do not originate from stromal fibroblasts.

Animal models using different cell types in cardiomyoplastic purpose (fetal cardiomyocytes, fibroblasts, skeletal myoblasts and endothelial precursor cells) served for the study of the pathological remodeling phenomena, but their use in therapy has not produced durable results in clinical trials [2, 11]. This fact was explained by the insufficient knowledge about signaling molecules and soluble factors contribution used or produced during stem cells therapy and by the incapacity of regenerated cardiomyocytes to reintegrate the microenvironmental matrix system of the myocardium, in order to provide a synchronized contractile activity with the rest of the heart [16, 17].

Smooth muscle alpha-actin (Act) is temporarily positive early in cardiac development only in muscular structures and not in primitive valvular ones [18], which have probably an endothelial origin [19]. Experiments on cell lines originating from stem cells distinguished that Act expression is associated with a pacemaker phenotype in cultured cardiomyocytes [20]. In adults, Act is expressed in subendocardial small smooth muscle fascicles and, according to certain authors, in excito-conductor structures [21].

In atrial fibroblasts cultures, Act is more intensely expressed than in ventricular ones, suggesting a higher capacity of response to irritating stimulation through fibrosis in atria as compared to ventricles [22]. These fibroblasts would be of hematopoietic origin, suffering a transformation in Act-positive myofibroblasts and producing fibrosis. They are different from local fibroblasts, which generate matrix collagen [23]. In patients with atrial fibrillation, the extensive atrial fibrosis was associated with overexpression of both smooth muscle alpha actin and bFGF genes; bFGF gene is codifying a protein with angiogenic and regenerative properties, characteristic for myofibroblasts, and not for stromal fibroblasts [24].

Desmin is one of the markers early expressed during cardiac embryogenesis [25], the intensity of cardiomyocytes staining for desmin increases progressively with age. Desmin is essential in maintaining the structure of cardiomyocytes [26] and their mitochondrial function [27], and is localized in the cytoplasm, along the cardiomyocytic membrane, in the first weeks of embryogenesis. It becomes an integrated structural filament, with an incomplete network, in the fetal period, and a complete network at the age of one year [28]. In the fetal heart, desmin expression increases firstly in the atrial wall and proximal zone of the pulmonary and cava veins [29]. A decrease in desmin-positive myocytes was reported in the myocardial tissue from patients with advanced ischemic disorders, compared with healthy people. The deficiency of this intermediary filament in the cytoskeleton was associated with a reduction in cardiac function [30] and a poor prognosis [31] and is used by some authors to mark acute ischemia zones on autopsy pieces [32].

Vimentin (57 kD), the most frequent protein subunit of the mesenchymal cells intermediary filaments, appears during cell differentiation and is expressed by all connective tissue cells, including fibroblasts [28]. All primitive mesenchymal cells express vimentin. During cardiac differentiation, vimentin is replaced especially by desmin; however, some authors consider that vimentin is expressed in adults during post-ischemic regenerative processes [33]. In some sterile pericarditis in adults, an increased epicardial expression of vimentin, suggesting fibroblastic proliferation, was reported [34], but a coexpression of smooth muscle alpha-actin in this epicardium suggests a myofibroblast induced fibrosis [23].

It is known that connective tissue and fibroblasts form a structural network sustaining myocytes and blood vessels, otherwise defined as the cytoarchitecture of the cardiac tissue. If fibroblasts ensure extracellular matrix (ECM) homeostasis in normal myocardium, in case of a lesion (i.e. myocardial infarction), fibroblasts are activated and contribute to cardiac remodeling through increased proliferation and migration. These processes determine secondary ECM expansion (by its components: collagen, proteoglycans, glycoproteins) and humoral factors secretion (cytokines, growth factors, proteases) [11], with autocrine and paracrine effects on myocytes and fibroblasts activity and phenotype [16], ending in fibrosis [35]. If (and in what way) the fibroblasts interfere with mast cells or with endothelial cells, which also produce different active factors [17], is still unknown.

The fibroblasts distribution is relatively uniform in the normal myocardium (they surround every myocyte, presenting a small total volume), but they represent 50% of its volume in the sinoatrial node [9]. In this localization, the fibroblastic cells are small and have many processes, expressing connexin (Cx) 40 in rabbits and Cx43 [9] in human sinoatrial node [36, 37].

Connexin 43, both a fibroblastic and a cardiomyocytic marker, is a transmembrane protein which forms gap junctions in vertebrates, acting in normal embryonic development or cardiac muscular depolarization; its expression reflects the relationship between cardiac development and the neural crest [38]. Connexin gene mutations can determine development or functional abnormalities, also involving the heart, its absence in mice experimental models generating sudden death [39]. The majority of gap junctions formed by Cx43 is localized in the intercalary discs and rarely on the lateral surfaces of myocardiocytes [40]. A decrease in Cx43 expression generates a dysfunction of gap junctions, determining microstructural changes [41, 42].

DDR2 is a tyrosine-kinase receptor, having fibrillar collagen I and III as specific ligands [9, 43], being an important non-integrin regulator of cell – extracellular matrix communication. Its specific activation determines matrix metalloproteinases stimulation, inducing ECM modeling [44]. The distribution of DDR2 positive cardiac cells is changing during heart development: DDR2 is first expressed in epicardial cells and in mesenchymal and endothelial cells of the cardiac cushions [45], which express both DDR2 mRNA and protein [5]. Its expression appears initially on the apex and the atrio-ventricular groove and is much more intense subepicardially; it becomes uniform in post-natal myocardium and remains that way in the adult heart [45].

CD34 is a cell-surface glycoprotein, which functions as an intercellular adhesion factor. It is a well-known marker of progenitor blood cells and connective tissue, with a large distribution, both in the fetal and adult periods. A recent study about fetal tissues notes a rich fetal CD34 positive vascular network, associated with CD34+ connective structures disposed in bundles, which delimitates myocardial lamellar segments [46]. Experiments proved that CD34+ cells purified from peripheral blood could maintain the myocardial integrity and function following a myocardial infarction [47], since the number of CD34+ cells increases in the myocardium located near the infarction zone. The application of bone-marrow CD34+ stem cells for the induction of a therapeutic angiogenesis in myocardial ischemia is promising, although the mechanism used by these cells to induce neovascularisation is still unknown [48]. Also at experimental level, precursors of CD34+ cells have been identified in mouse epicardium as having the ability to transform in cardiomyocytes, endothelial cells or smooth muscle cells [49].

EGFR is a cell-surface receptor of the epidermal growth factors family, with roles in proliferation, migration, cell adhesion and apparently even in ischemic lesions [50] and preconditioning [51]. Through the ERK-MAPK-AKT signal pathway, it stimulates DNA synthesis in cardiac fibroblasts, and cardiomyocyte differentiation [52]. Angiotensin II or I induced EGFR trans-activation can produce cardiac hypertrophy, followed by cardiac failure [53, 54].

CD117 represents a type III tyrosine-kinase receptor, linked to stem cells factors (SCF), with intracellular transmission of survival, proliferation and differentiation signals, being present even in cardiovascular precursors from the embryonic heart [55]. CD117+ cells have a particularly important role in cardiac remodeling, some of them originating in epicardial mesothelium, through epithelial-mesenchymal transition [56]. A CD117+ stem cell population was isolated from both murine and human adult heart, proved able to generate endothelial cells, smooth muscle cells and functional cardiomyocytes [49]. It was discovered that hematopoietic CD117+ cells have the capacity to function as cardiac progenitors since they are able to differentiate in cardiomyocytes [57]. It seems that some CD117+ but CD45- cells would be endothelial progenitor cells [58].

Our study aimed to identify the histological particularities and the immunohistochemical expression of cardiac cell subpopulations in different life periods.

Materials and Methods

We tested four study series of human cardiac tissue: adult hearts – four cases without cardiac symptoms (one woman and three men, aged between 50 and 53 years), 14 cases of adult hearts with chronic ischemic myocardial lesions with ages between 55 and 70-year-old (six women and eight men), fetal hearts (30 cases) with gestational ages between 16 and 28 weeks, and four embryonic hearts (six and seven weeks), all selected from the "Victor Babeş" Institute archive.

The tissues harvested from all cases included: myocardium from the atrioventricular septum, atrio-caval regions, right and left ventricular walls.

The embryos were sectioned cranio-caudally and serial sections involving the regions mentioned above were obtained.

Histopathologic analysis was performed on tissue fragments fixed in 10% neutral buffered formalin (pH 7) for 24–48 hours, paraffin embedded, sectioned at 3 μm, standard HE stained. In addition, special stains for connective tissue were used: Trichromic Masson and Elastic van Gieson.

Immunohistochemical analysis (IHC) was made using sections displayed on slides treated first with poly-L-lysine. The IHC method used was an indirect bistadial technique with hydrosoluble dextran polymeric skeleton (DAKO EnVision Systems, Carpinteria, CA, USA), according to the producer's specifications. The antibodies used were: vimentin (V9 clone, 1:50); smooth muscle alpha-actin (1 A4 clone, 1:50); CD34 (QBEnd10 clone, 1:50); CD117 (polyclonal, 1:250); muscle actin (HHF35 clone, 1:50); CD31 (JC70A clone, 1:50); desmin (D33 Clone, 1:100), DAKO, Carpinteria, CA, USA; connexin 43 (CXN-6, 1:50), Santa Cruz, CA, USA; DDR2 (3B11E4 clone, 1:500), Abcam, UK; EGFR (29.1 clone, 1:1000), SIGMA, USA. We used the antigen retrieval techniques, thermal or enzymatic pretreatment for some antibodies, according to the producer's specifications. Both positive and negative controls were used.

We used Analysis Tool Pak program from the MS–Excel 2003, under license of Windows XP Professional (Microsoft Inc., CA, USA), for the descriptive and comparative statistics and to determine correlations between variables. We used the analytic functions of the Student *t*-test for averages. A predictive value of $p<0.05$ was considered as statistically significant.

Results

Histopathological and immunohistochemical aspects

Immunohistochemically, there is no marker with absolute specificity for cardiomyocytes or non-cardiomyocytic cells. However, the corroboration of some protein expressions, proteins involved in myocardial or interstitial cells structure, allows the formation of immunoprofiles for various structures of embryonic, fetal or adult heart.

The **embryonic** heart structures were well highlighted on HE staining, without autolytic or artifactual changes. Cardiomyocytes presented a round-polygonal shape, clear, abundant cytoplasm and small nuclei, located centrally/eccentrically, with frequent mitoses (Figure 1a). The internal atrial zone had cardiac cushions of young mesenchymal tissue with myxoid aspect. The implantation zone of the large vessels presented an ordered fibrillar structure, with a thick wall of the emerging arteries and a thin wall of the large veins, lumina full of nucleated red cells. Fine arcuate structures composed of rounded cells with a rich cytoplasm penetrated from the epicardium through the myocardium, especially in the ventricular wall; they were single cell layered and formed small spaces containing nucleated red cells (Figure 1b).

Immunohistochemically, smooth muscle alpha-actin (Act) was well represented in the large vessels wall and the atrial cardiomyocytes (Figure 1c). In ventricles, it was expressed only in the subendocardial and subepicardial cell layer, in the last location the muscle cells delimitating small arcuate structures (Figure 1d); the rest of the ventricular cardiomyocytes were negative. A negative reaction was also observed in the myxoid structures of the cardiac cushions at the separation zones of the atria from the ventricles (Figure 2a).

Vimentin was diffusely and well expressed in the atrial wall and the large vessels and focally in the ventricular wall, where it appeared in elongated fibroblastic cells and fine connective tissue bundles disposed around bicellular cardiomyocytic lamellae (Figure 2b). CD34 was expressed only in endocardium (Figure 2c), while completely negative in all other structures of the heart, as well as were EGFR and CD31.

DDR2 was expressed in the atrial muscle but not in the mesenchymal area of the endocardial cushions (Figure 2d). In the ventricles, it was expressed only in the subepicardicardial layer of myocardiocytes.

Desmin marked myocardiocytes sometimes intensely, but was negative in cardiac cushions (Figure 3a). Usually, the ventricular muscle was less stained (Figure 3b) with desmin, while vimentin stained it strongly and diffusely (Figure 3c).

CD117 was positive in rare epicardial cells. connexin 43 was poorly expressed in muscle cells, as small granular cytoplasmic deposits with an irregular disposition, and isolated in epicardial cells.

On **HE** staining, the **fetal hearts** presented capillary vasodilatation, with focal disposition in the myocardium. Unlike embryonic cardiomyocytes, fetal cardiomyocytes showed elongated forms (Figure 4a), with a pale, eosinophilic cytoplasm and centrally located nuclei, with oval or, rarely, rod shape. Mitoses were absent in the fetal hearts, regardless of gestational age. The interstitial tissue was apparently scarce in all cases, but the capillary network was very rich. Fibroblasts were difficult to identify in HE staining because of their vicinity with capillary endothelial cells.

Immunohistochemically, Act had various appearances, at different fetal ages. In the 16–20 weeks fetal hearts, Act was distributed largely in atrial cardiomyocytes, while ventricular cardiomyocytes were less stained (Figure 4b). After 20 weeks, Act was expressed in vessels and only isolated in subendocardial atrial cardiomyocytes, while being negative in ventricular cardiomyocytes (Figure 4c).

Muscle actin (clone HHF35) was expressed diffusely in fetal cardiomyocytes, but with less intensity as compared to vascular walls.

Desmin was expressed in all cases, regardless of the fetal age, the reaction being more intense in atrial cardiomyocytes than in ventricular ones (Figure 4d).

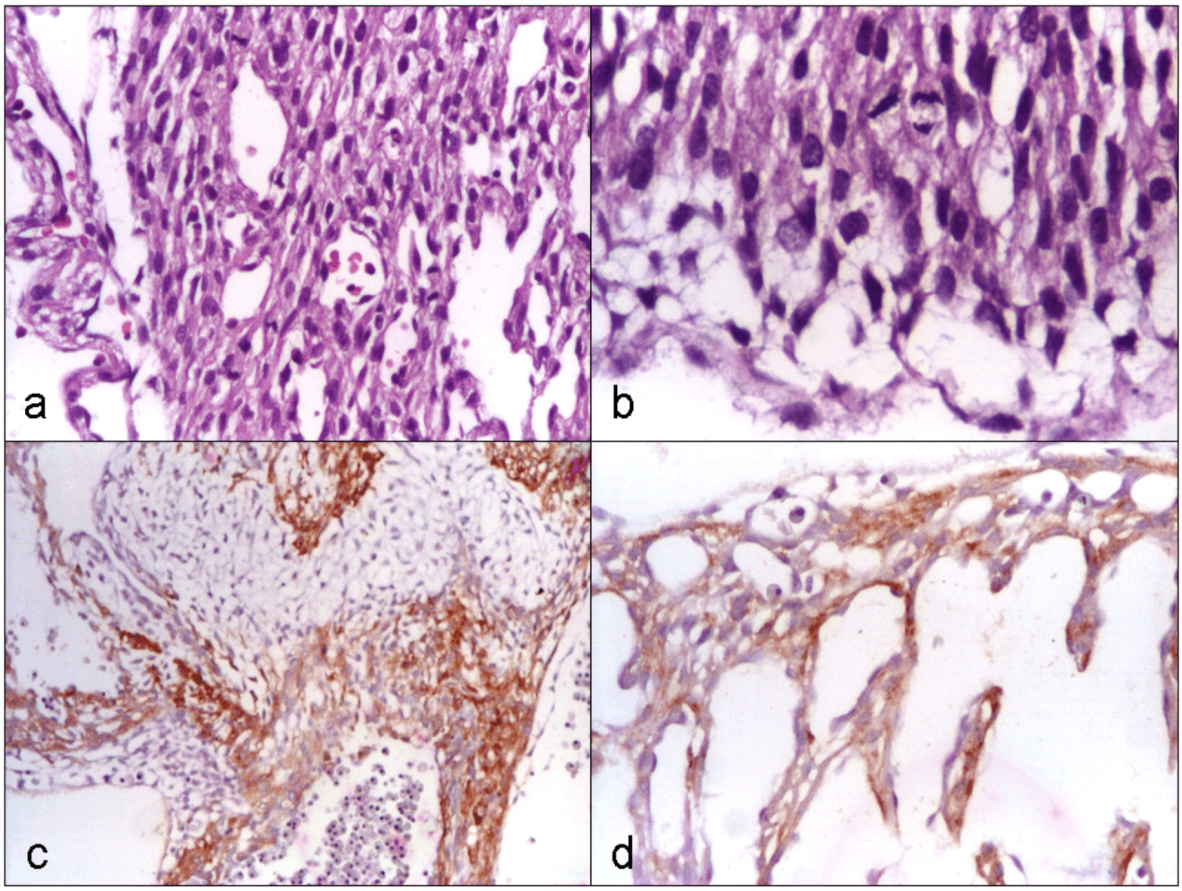

Figure 1 – ***Embryonic myocardium: (a) ventricular wall with mitoses (HE stain, ob. 20×); (b) ventricular wall with subepicardial arcuate structures (HE stain, ob. 40×); (c) IHC staining for smooth muscle alpha actin (Act) – positive reaction in atrial, ventricular muscle and large vessel walls (ob. 10×); (d) IHC staining for Act – positive in the ventricular wall (ob. 20×).***

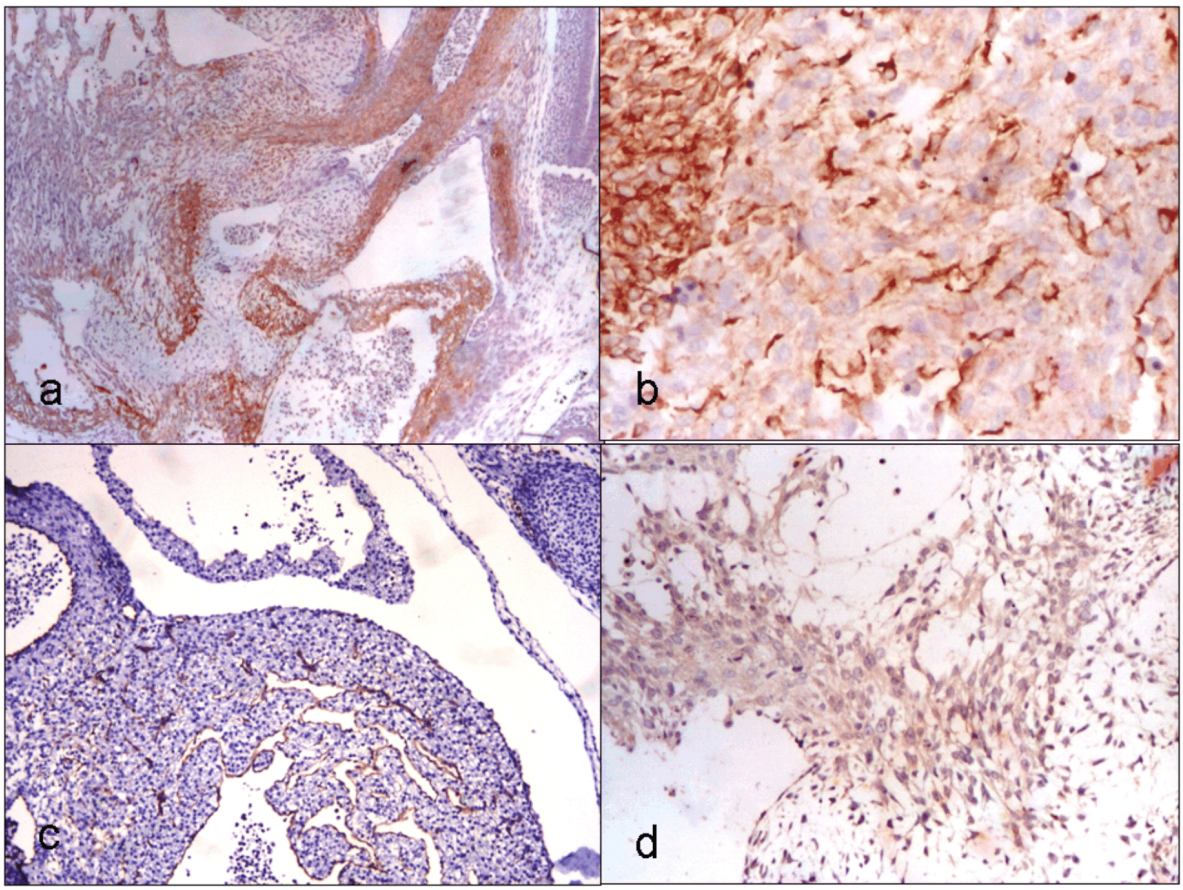

Figure 2 – ***Embryonic myocardium: (a) IHC staining for Act – positive in atrial, ventricular myocardium and large vessel walls; negative in the endocardial cushions (ob. 4×); (b) IHC staining for vimentin – positive in fine connective bundles delimitating bilamellar cardiomyocyte layers (ob. 20×); (c) IHC staining for CD34 – positive in embryonic endocardium (ob. 10×); (d) IHC staining for DDR2 – positive in myocardiocytes (ob. 20×).***

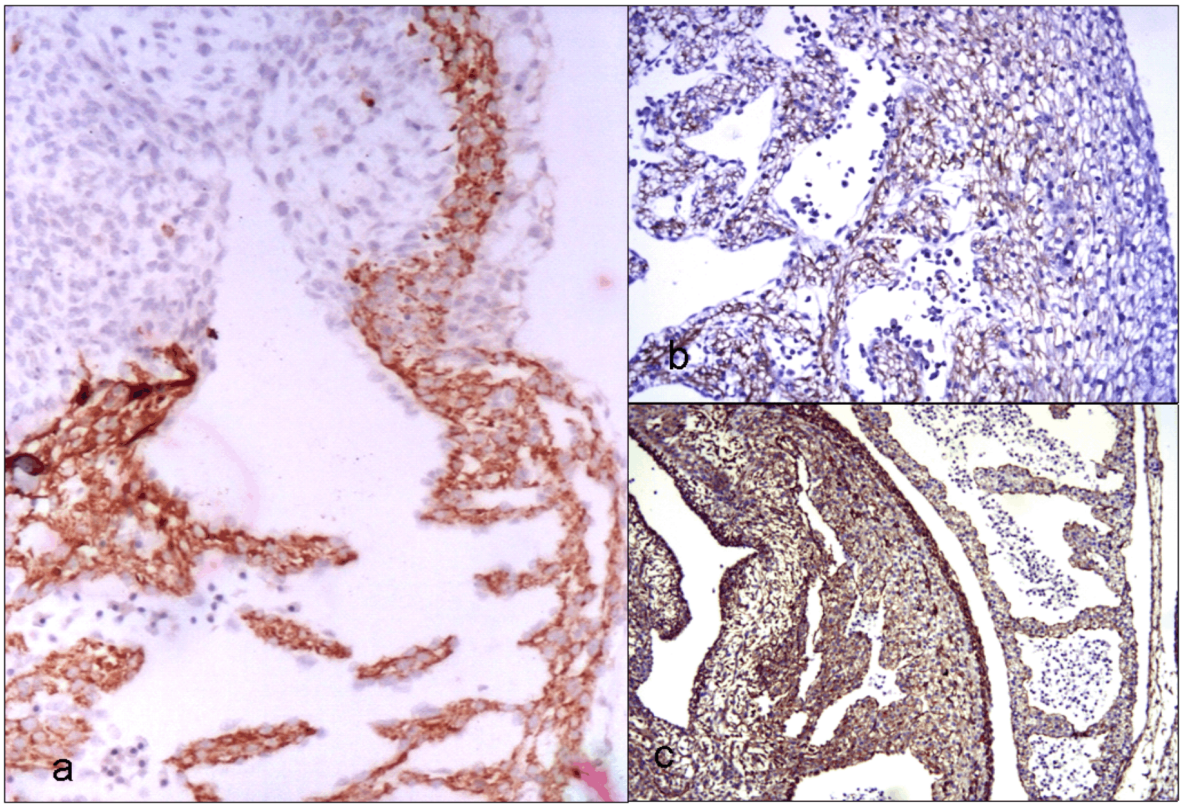

Figure 3 – ***Embryonic myocardium: (a) IHC staining for desmin – positive in cardiac walls and large vessels, negative in endocardial cushions (ob. 20×); (b) IHC staining for desmin – positive in ventricular myocardium (ob. 20×); (c) IHC staining for vimentin – positive diffuse reaction (ob. 10×).***

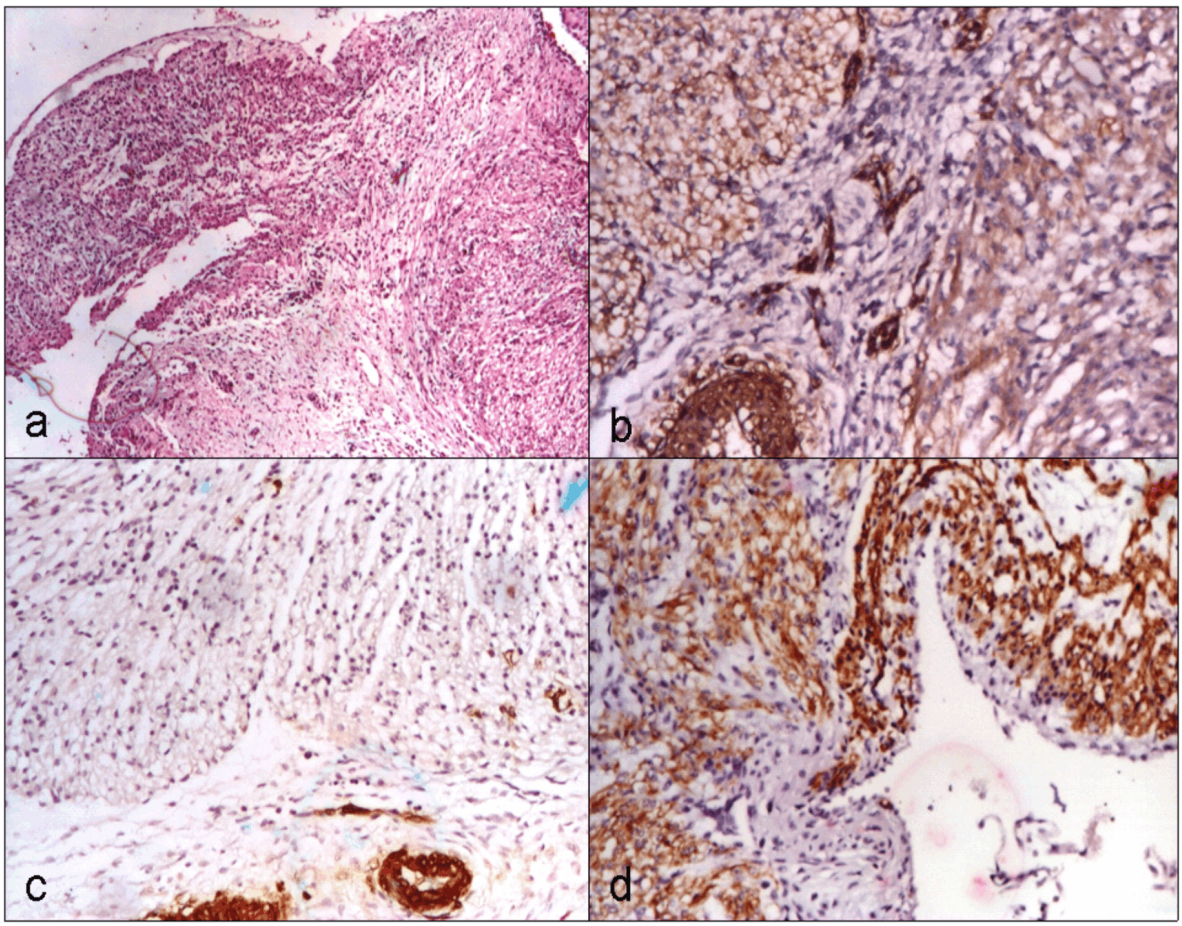

Figure 4 – ***Fetal myocardium, 17 weeks: (a) atrio-ventricular separation zone (HE stain, ob. 4×); (b) IHC staining for actin – positive in the atrial wall, and with weaker positivity in the ventricular wall (ob. 10×); (c) IHC staining for actin – negative reaction in ventricular wall (ob. 10×); (d) atrio-ventricular separation zone – variable IHC staining for desmin: intense reaction in the atrium, discrete reaction in the ventricle, and negative reaction in valve (ob. 10×).***

Vimentin was diffusely positive forming an ordered membrane or cytoplasmic network in interstitial cells and in myocardiocytes (Figure 5a). A decrease in vimentin expression was observed in the 25-week-old myocardium as compared to the 16 weeks samples (Figure 5b).

DDR2 presented a predominantly membrane expression in all fetal hearts in sparse fibroblasts (Figure 5c) and cardiomyocytes (Figure 5d), while valves and vascular walls were negative.

Connexin 43 had a relatively weak expression in cardiomyocytes, as fine granular deposits, with a regular disposition.

CD34 presented a constant and intense expression in all fragments of the fetal heart, regardless of gestational age, marking a much-branched capillary and stromal fibroblastic cells network. Connective bands entered the thickness of the myocardium, splitting it in lobular-like structures (Figure 6a). The vascular network expressing CD34 (Figure 6b) overlapped in large part the EGFR network (Figure 6c), but the latter could not be displayed in all cases – a positive reaction appeared in just 12 of the 18 cases investigated. The number of vessels highlighted with CD31 was much smaller as compared to the CD34 positive vessels (Figure 6d); the CD31 positive vessels were predominantly of adult type, while the CD34 positive vascular network was more of neoformation type.

A significant statistical correlation was obtained between the expression of CD34 in vessels and in stromal cells (fibroblasts) (r=0.787, p=0.0003). The expression of CD34 and vimentin in vessels showed that they were independent parameters (p=0.00075), similar to the vascular expression of CD34 compared to CD31 (p=0.00174). A poor but statistically significant correlation was observed between vimentin expression in vessels and in fibroblasts (r=0.517, p=0.015). Vimentin and desmin expressions in cardiomyocytes were independent parameters (p=0.065).

The **adult** heart fragments from patients **without clinical cardiac symptoms** presented in HE staining discrete or moderate centers of interstitial and peri-vascular fibrosis (Figure 7a), discrete lipomatosis, focal stasis and early arteriosclerotic lesions of intramyocardial coronary branches.

In adult hearts, smooth muscle alpha-actin was strongly expressed in all myocardial fragments in coronary vascular walls, but also in fine bundles of smooth muscle arranged especially subendocardially, or in excito-conductive structures without nodal disposition. The muscle actin (clone HHF35) appeared in the adult heart in both the myocardium and the vascular wall. Desmin was present in all the examined heart fragments, but cardiomyocytes staining was more extensive in adults (Figure 7b) as compared to fetuses. It was also expressed in the muscle tunica of the vessels.

The evaluation of DDR2 expression gave an unexpected result, most fragments presenting a positive reaction in the muscle fibers and less in the interstitial connective tissue and fibroblasts (Figure 7c). Vimentin was expressed similarly to the fetal hearts, in interstitial cells surrounding 1–2 layers of cardiomyocytes (Figure 7d).

Connexin 43 was expressed variably in adult normal myocardial fibers, much more intense than in fetal hearts and in a more ordered disposition (Figure 7e).

CD34 appeared positive in the vascular endothelium in all tested cases, revealing a dense, capillary network (Figure 7f). On the other hand, EGFR was positive in endothelial cells in only one of the four cases tested.

CD117 was expressed in mast cells and in rare interstitial cells with fibroblastic morphological aspect, with bipolar processes or very ramified and sinuous extensions.

The fragments with **chronic ischemic lesions** presented in **HE** (Figure 8a), van Gieson Elastic or Trichrome Masson stainings large zones of cardiosclerosis and fibrous microlesions. Perilesional cardiomyocytes had frequently hypertrophic nuclei of regenerative type and lipofuscin deposits in the cytoplasm.

Immunohistochemically, in the adult myocardium with ischemic lesions, the biomarkers expression was different from that of normal adult heart. Act was expressed in the vessels and in myofibroblastic/fibroblastic type cells of all the cicatricial lesions (Figure 8b). Muscle actin [HHF35] was diffusely present in cardiomyocytes and vessels. At the same time, vimentin was completely or almost completely negative in the fibrous areas or with only a discreet densification around the scar lesions in the ECM (Figure 8c) and in myofibroblastic/fibroblastic cells. Desmin was obviously less expressed in peri-cicatricial cardiomyocytes than in distant ones. DDR2 maintained an intense reaction in cardiomyocytes, but was expressed only scarcely in the interstitial fibroblasts and was always negative in the cicatricial zones (Figure 8d). Connexin 43 was variably expressed in cardiomyocytes forming a weak or strong peri-cicatricial network. CD34-positive capillary structures (Figure 9a) were well represented in all cases with chronic ischemic lesions forming rich networks of peri-scar vascular neoformation (Figure 9b), almost in parallel with the EGFR positive vascular network that appeared well expressed in nine of 14 cases tested (Figure 9c), with net peri-scar densification.

CD117 was positive only in mast cells and in rare spindle-shaped cells, in the wall of large coronary vessels.

CD31 was expressed only in the endothelium of large and medium vessels and isolated in the capillary network with neo-formation aspect (Figure 9d).

In the adult hearts with ischemic lesions, a statistically significant correlation was obtained between CD34 expression in vessels and fibroblasts (r=0.63, p=0.027). In addition, a poor but statistically significant correlation was obtained between CD34 and VIM expression in vessels (r=0.517, p=0.044). Another statistically significant correlation was established between the expression of VIM in vessels and in fibroblasts (r=0.83, p=0.03).

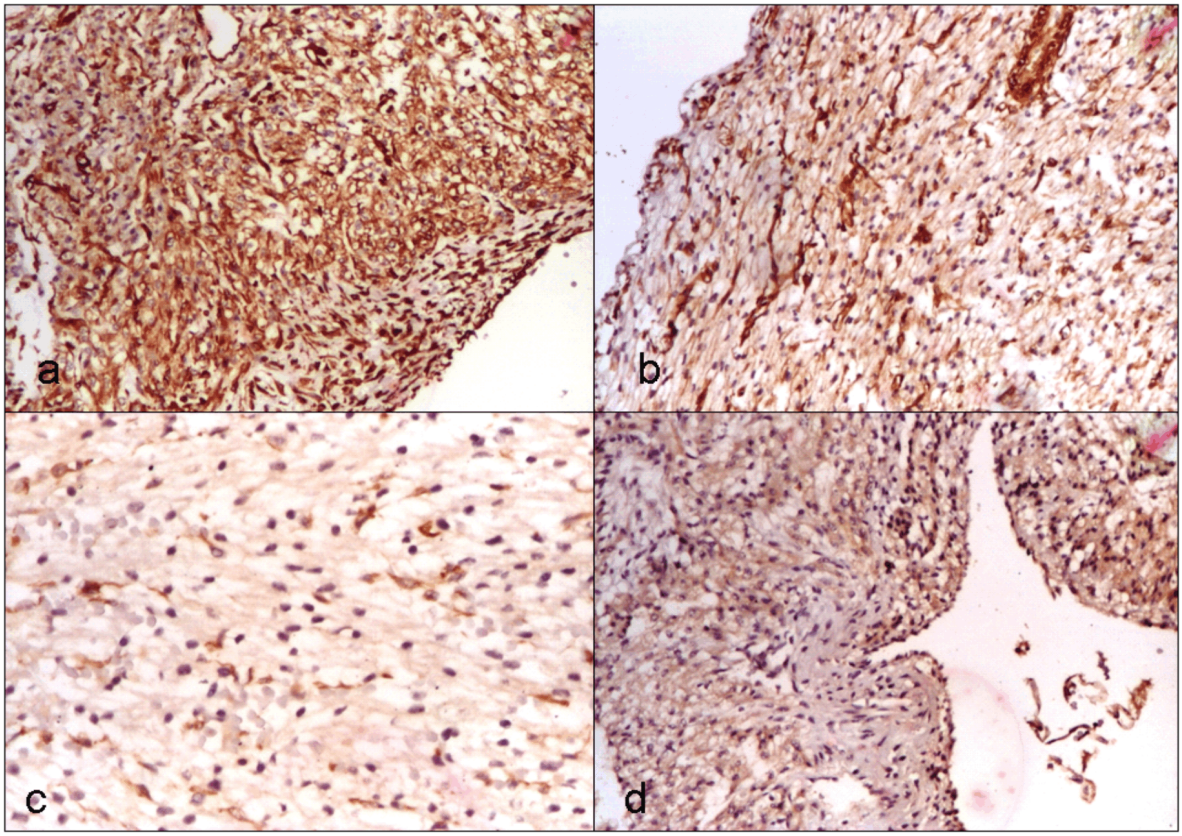

Figure 5 – ***Fetal myocardium: (a) IHC staining for vimentin in a 17 weeks fetus – intensely positive reaction in the ventricular myocardium (ob. 10×); (b) IHC staining for vimentin in a 23 weeks fetus – weak reaction in atrial wall (ob. 10×); (c) IHC staining for DDR2 in a 19 weeks fetus – positive reaction in interstitial cells (ob. 20×); (d) IHC staining for DDR2 in a 17 weeks fetus – positive reaction in myocardium, negative in valve (ob. 10×).***

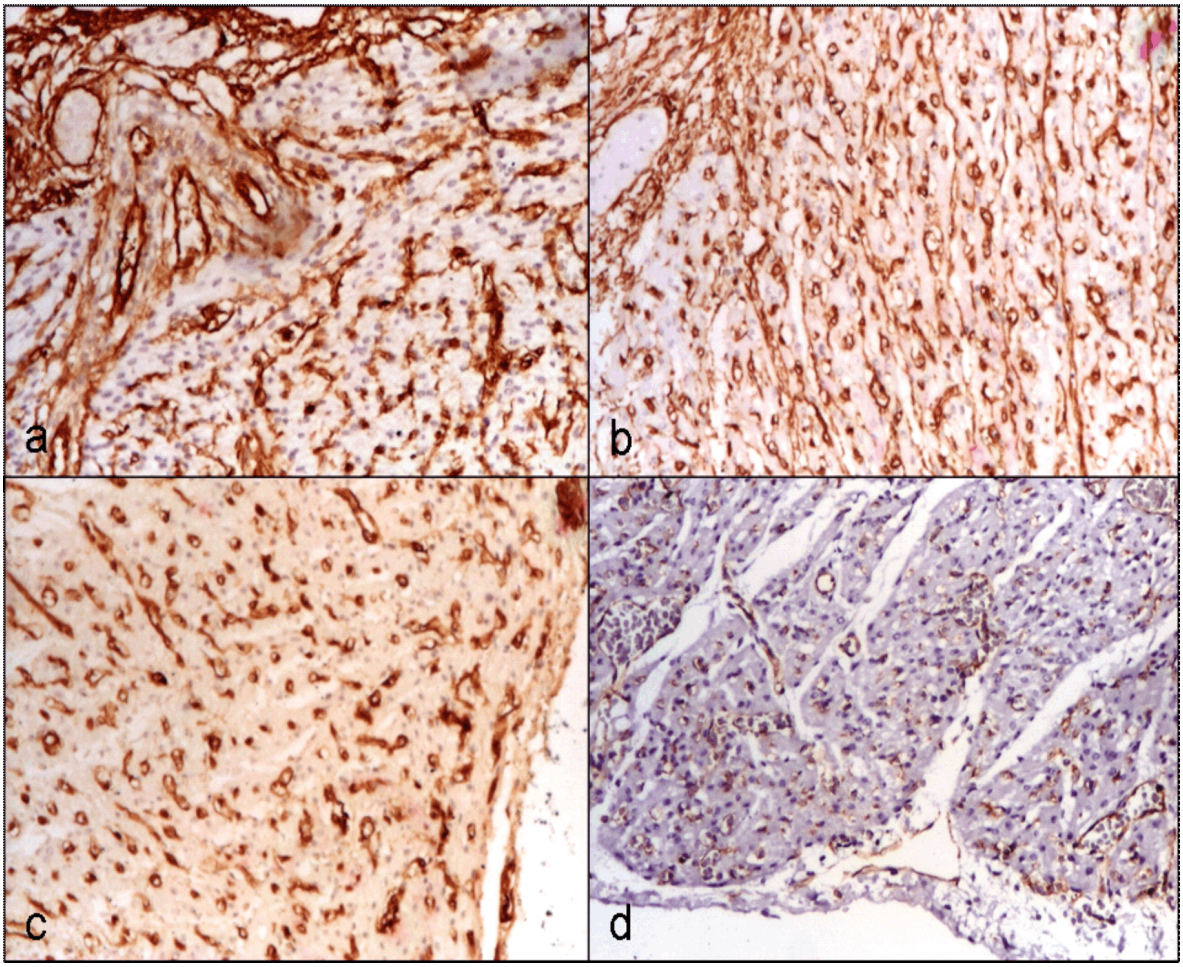

Figure 6 – ***Fetal myocardium: (a) IHC staining for CD34 in a 17 weeks fetus – intensely positive reaction in vessels and connective septa (ob. 10×); (b) IHC staining for CD34 in a 19 weeks fetus – marked expression in septa and vessels (ob. 10×); (c) IHC staining for EGFR in a 19 weeks fetus – rich expression in the capillary network (ob. 10×); (d) IHC staining for CD31 in a 23 weeks fetus – reduced reaction as compared to CD34 (ob. 10×).***

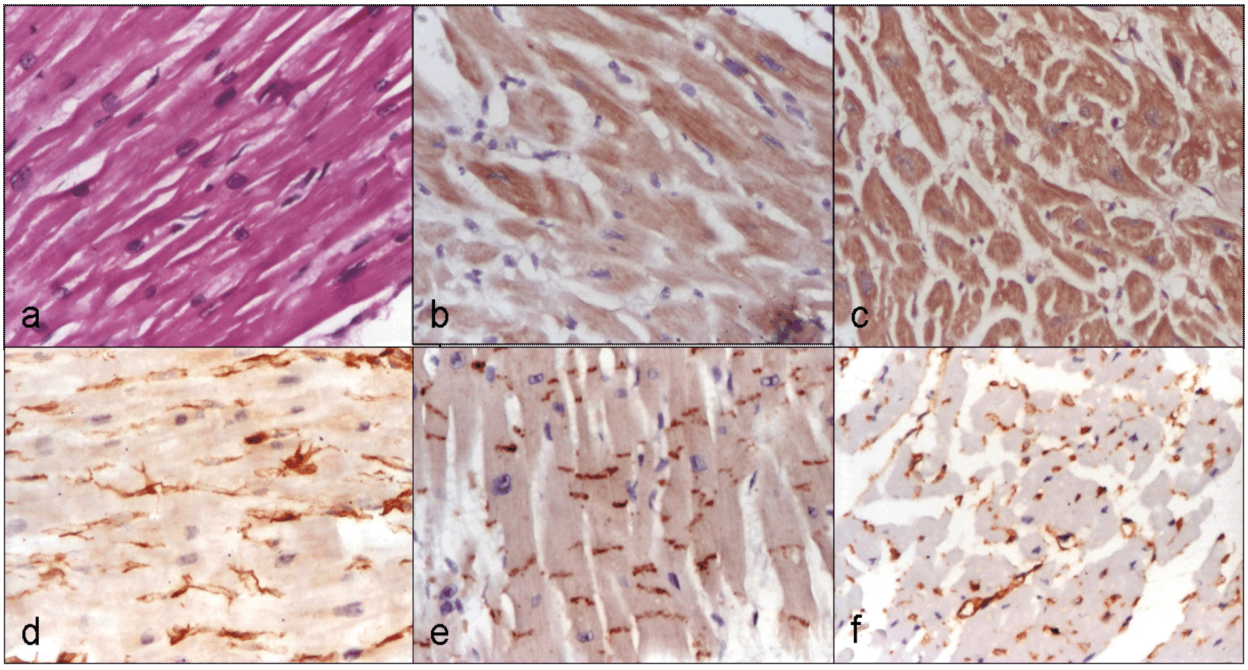

Figure 7 – ***Normal adult myocardium: (a) histological aspect of myocardial tissue without macroscopical changes (HE stain, ob. 10×); (b) positive IHC staining for desmin (ob. 20×); (c) diffuse positive IHC reaction for DDR2 in macroscopically normal adult myocardium (ob. 20×); (d) regular network of vimentin positive cells and fibers (ob. 20×); (e) positive IHC reaction for Cx43 in intercalary discs (ob. 20×); (f) positive IHC reaction for CD34 (ob. 20×).***

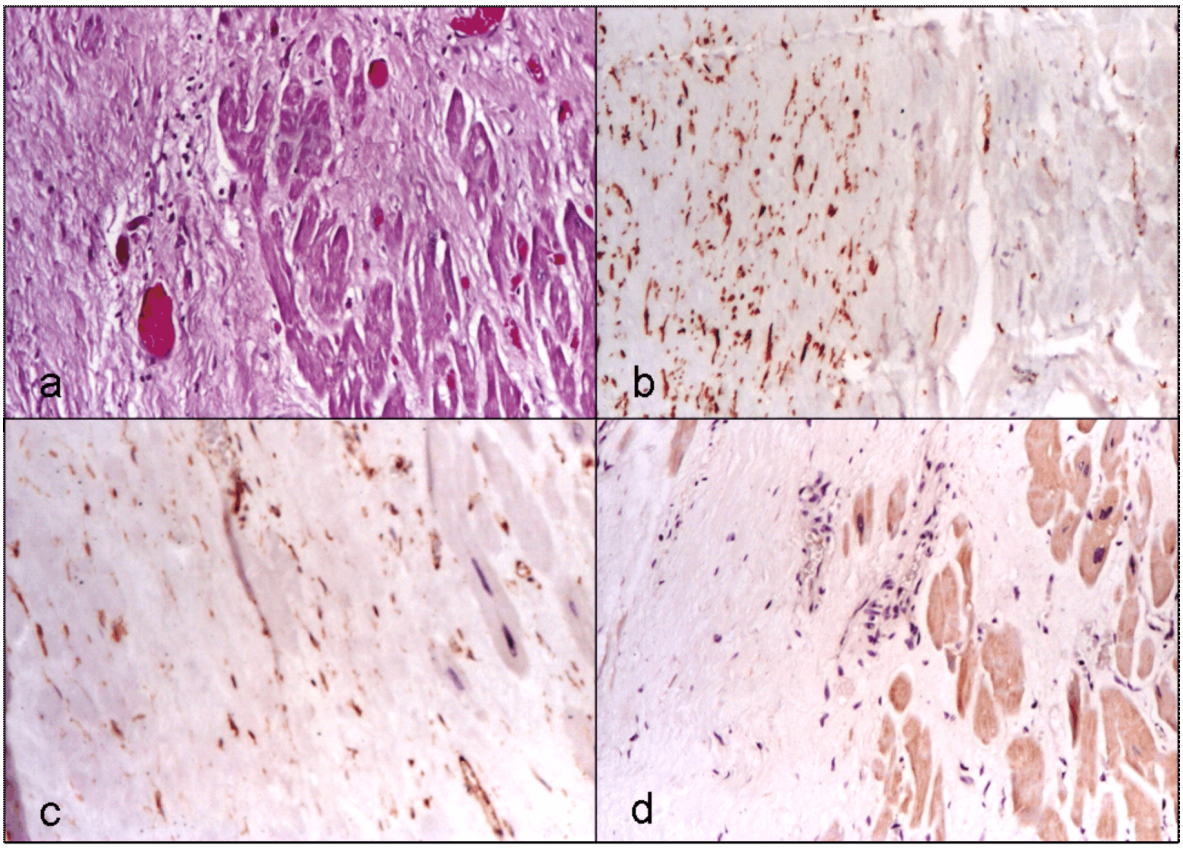

Figure 8 – ***Ischemic adult myocardium: (a) cicatricial zone in ventricular myocardium (HE stain, ob. 10×); (b) actin positivity in the cicatricial zone (ob. 10×); (c) IHC staining for vimentin – minimal reaction in the cicatricial zone (ob. 10×); (d) IHC staining for DDR2 – negative in cicatricial zone, positive in cardiomyocytes (ob. 10×).***

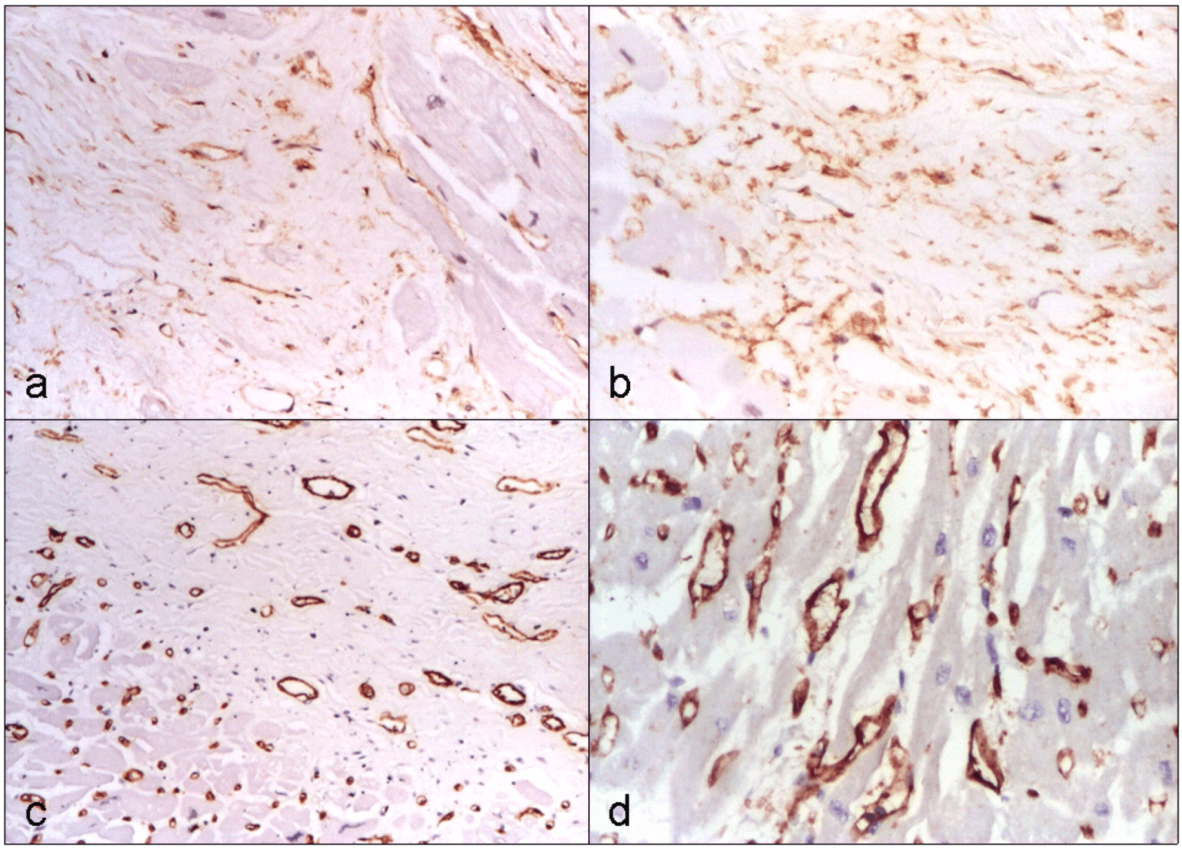

Figure 9 – ***Ischemic adult myocardium: (a) cicatricial zone with CD34 positive in vessels and interstitial cells (ob. 10×); (b) cicatricial zone with CD34 positive in vessels and interstitial cells – detail (ob. 20×); (c) IHC staining for EGFR – cicatricial zone with adult type large caliber vessels (ob. 10×); (d) positive IHC reaction for CD31 in cicatricial fibrosis (ob. 20×).***

Discussion

In the examined **embryos**, cardiomyocytes with frequent mitoses were clearly individualized, illustrating an intense proliferative activity. The mitotic activity was much lower in the primitive structures of large vessels than in the muscular ones. The expression of smooth muscle alpha-actin (Act) in scarce myocardiocytic groups in the atrial wall suggested the presence of some conductive structures dispersed at this level, a fact recently reported in literature [21]. These excito-conducting structures would possibly induce certain types of retrograde activation, a fact signaled by some authors that consider them as the cause of certain recurrent tachycardia forms in adults.

We also observed a more extensive Act expression in atrial interstitial cells as compared with ventricles, in accord with the results of an experimental study performed on atrial and ventricular fibroblast cultures that detected a higher atrial capacity to develop fibrous tissue as compared to the ventricle, difference detected even in the adult heart with cardiac failure [22, 24]. The absence of desmin expression in large vessels, while it was overexpressed in the atria in our cases, would suggest an initial dissociated maturation of the two structures, as reported by other authors which observed a simultaneous one after nine gestation weeks [29].

Towards the atrioventricular separation areas, we identified the endocardial cushions, formed by immature mesenchymal tissue, which gradually replace the remaining structures of the cardiac gelatin, representing the origin of atrioventricular valves. They presented a myxoid aspect and did not contain smooth muscle alpha-actin, unlike the rest of the myocardium. This result is similar with other studies that sustain the endocardial origin of the valves, through epithelial-mesenchymal transformation [18], while other opinions affirm the endothelial origin of the valves through a vasculogenesis process [19].

Throughout the heart development and maturation, although numerically they decrease at ~ 20–30% of the cardiac cell population due to the multiplication of non-cardiomyocytic cells, cardiomyocytes continue to represent more than 75% of the cardiac tissue mass [9]. The lack of fetal cardiomyocytes proliferation in the last stages of evolution is followed by their hypertrophy. They become much larger than embryonic cardiomyocytes. In our cases, we noticed morphological differences between the fetuses of 16–17 weeks and 23–28 weeks: in parallel with the gestational age, myocardial fibers showed a decrease in nuclear volume with shape modifications from round-oval to rod-like, an increased cytoplasmic volume and tinctoriality.

The fibroblastic population from the embryonic heart was very well represented, with an almost diffuse expression of vimentin in atria and large vessels, but much lower in ventricles. In this location, small vimentin positive fibroblasts with bipolar or multipolar processes formed a network around one or two rows of oval or polygonal cardiomyocytes, suggesting a lamellar disposition of cardiomyocytes similar to that reported in literature data [46].

CD34 expression limited to endocardium and EGFR absence in the embryonic heart suggested that the development mechanisms in which they are involved appear only in advanced stages. This finding is sustained by the very well developed vascular network in fetal hearts, especially the CD34 expressing ones, with capillary predominance.

The DDR2 distribution observed in our cases was somehow different from that reported in literature [45] because the endocardial cushions were completely negative; the atrial wall and the subepicardial ventricular zone positivity were in accord with other reports [59].

The CD117 positivity of the epicardial embryonic cells observed in our cases is in agreement with literature data, which found the protein encoded by c-Kit gene to be important in the epithelial-mesenchymal transformation process in embryos [49, 57]. Its expression was not found in fetal and adult heart, except for only sporadic cases, in fibroblast-like cells and often in mast cells.

The low expression of connexin 43 (Cx43) as disordered small granular deposits in muscle cells and the sporadic positivity in the embryonic fibroblastic cells could be explained by the young age of the embryo. The literature data showed the need of Cx43 presence for the subsequent development of the coronary vessels [39].

In the **fetal hearts**, we found a lower expression of the smooth muscle alpha-actin (Act) as compared to the embryo. Act was expressed in the wall of aorta, pulmonary and coronary arteries. It was also expressed in fine subendocardial smooth muscle bundles and in isolated myocardiocytes of the right atrium as a delicate fibrillary network, possibly representing a conductive structure, and was completely absent in ventricles. Unlike in the embryo, it was also expressed in valves and neighboring atrial cardiomyocytic layers even in 16 weeks fetuses, representing a first resemblance with the adult valves immunohistochemical profile and sustaining the possible development of the valves from the endocardium, according to some literature reports [19]. In over 20-week-old fetal hearts, smooth muscle alpha-actin expression was similar to that of an adult. HHF35b muscle actin was diffusely expressed in the fetal myocardium, similar to the adult one.

Vimentin expression was also close to that of the adult, decreasing with fetal age and realizing a regular membranous or cytoplasmic network in the fetal myocardium. Vimentin was clearly expressed in a rich network of spindle-shaped, fibroblast-like cells, with bipolar or multipolar processes. Their intimate relationship with cardiomyocytes was more apparent than in the adult heart. Desmin was expressed in all cases, regardless of fetal age, the reaction being more intense in atria than in ventricles and absent in the valvular structures. In large arterial vessels, desmin expression was very weak. DDR2, tested both with a monoclonal antibody and a polyclonal serum, presented an intense, largely membranous expression in cardiomyocytes of all fetal hearts, more intense than in embryonic myocardium, while connective structures of the valves, interstitial fibroblasts and vascular walls were negative. This DDR2 cardiomyocytic expression, that was common

in our cases, was reported recently in cardiomyocytes in the fetal and the adult period [45], contrasting with the early embryonic period, of the primitive cardiac tube formation, when DDR2 is very active and expressed in the fibroblasts that will induce the cardiomyocytes development [59]. Connexin 43 was weakly expressed in cardiomyocytes, but its disposition, in the form of regular bands, which seemed to draw the intercalated discs blueprints, sustains its localization in their gap junctions as reported in literature [40].

CD34 identified in all fetal hearts a very rich capillary network in parallel with an equally well-represented network of connective structures organized in fine septa with a tendency to separate the myocardium in lobules. This network was more apparent in the ventricular wall, similarly to other literature observations [46]. The EGFR positive vascular network was quantitatively reduced when compared to the CD34+ network, representing predominantly adult vessels as those expressing CD31.

In the **adult hearts**, the smooth muscle alpha-actin expression was expressed only in vessels and rarely in fine bundles of subendocardial smooth muscle and in some fine atrial muscle fibers without nodal organization, suggesting isolated conducting structures as also observed by some authors [21]. Desmin myocardial distribution was diffuse in the adult hearts from patients without cardiac symptoms and only focally in the hearts with ischemic lesions. The reaction was absent in areas with regenerative nuclei, suggesting that desmin is disappearing in the ischemic but not necessarily necrotic areas, a fact which contradicts some observations about the lack of desmin expression as a definite sign of necrosis [32].

DDR2 was positive diffusely in cardiomyocytes and in only isolated fibroblastic cells, in both **normal and ischemic** adult myocardium, a result different from other observations claiming that (a) DDR2 expression in fibroblasts is characteristic [9, 12] and (b) DDR2 is expressed only in the embryonic stages of heart development [59]. All scarred areas were always negative for DDR2.

In the myocardium with ischemic lesions, DDR2 expression was similar to that of vimentin. Vimentin was almost completely negative in the scarred areas and in regenerative interstitium, this observation being different from literature, which asserts that vimentin is expressed in regeneration and fibrotic processes [33]. Our finding could suggest that local stromal fibroblasts are not involved in the ischemic fibrotic scar.

On the contrary, smooth muscle alpha-actin was intensely and diffusely expressed in these cicatricial areas as well as CD34, suggesting the myofibroblasts intervention in postischemic fibrosis as also noted by other researchers [23] which, on the other hand, do not associate fibrosis with vimentin expression. We noticed, in all cases with ischemic lesions, a proliferation of the CD34 positive vascular network, in parallel, in most cases, with the EGFR positive network, different from those of the adult heart in asymptomatic patients, where the EGFR positive network was much weaker and inconstantly expressed. This aspect has not been reported in literature yet, to the best of our knowledge. However, the intense vascular EGFR expression was recently found to be associated with ischemia and ischemic post-conditioning [50], but might indicate an action of inducing cardiomyocyte hypertrophy as noted in literature [54], hypertrophic fibers with large regenerative type nuclei being present around the ischemic zones.

Conclusions

Our findings allow us to consider that the various components of myocardial cells, such as alpha smooth muscle actin, desmin, vimentin, DDR2 and CD34 are expressed differently in distinctive periods of cardiac development, having an undulating temporal-spatial evolution. Their expression is variable in intensity and location in ischemic myocardium as compared to the normal adult heart. DDR2 and vimentin expressions are lacking in ischemic fibrosis, which expresses smooth muscle alpha actin and CD34. This fact suggests a probable myofibroblastic, and not fibroblastic origin of ischemic scars. The EGFR positive vascular network is better represented in the ischemic heart as compared to the normal adult heart, a fact possibly related to EGFR implication in cardiac ischemic pre- and post-conditioning.

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