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Histochemical and immunohistochemical analyses of the myocardial scar fallowing acute myocardial infarction

Histohemijska i imunohistohemijska analiza ožiljka u miokardu posle akutnog infarkta miokarda

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Abstract

Background/Aim. The heart has traditionally been considered as a static organ without capacity of regeneration after trauma. Currently, the more and more often asked question is whether the heart has any intrinsic capacities to regenerate myocytes after myocardial infarction. The aim of this study was to present the existence of the preserved muscle fibers in the myocardial scar following myocardial infarction as well as the presence of numerous cells of various size and form that differently reacted to the used immunohistochemical antibodies. **Methods.** Histological, histochemical and immunohistochemical analyses of myocardial sections taken from 177 patients who had died of acute myocardial infarction and had the myocardial scar following myocardial infarction, were carried out. More sections taken both from the site of acute infarction and scar were examined by the following methods: hematoxylin-eosin (HE), periodic acid schiff (PAS), PAS-diastasis, Masson trichrom, Malory, van Gieson, vimentin, desmin, myosin, myoglobin, alpha actin, smoth muscle actin (SMA), p53, leukocyte common antigen (LCA), proliferating cell nuclear antigen (PCNA), Ki-67, actin HHF35, CD34, CD31, CD45, CD45Ro, CD8, CD20. **Results.** In all sections taken from the scar region, larger or smaller islets of the preserved muscle fibers with the signs of hypertrophy were found. In the scar, a large number of cells of various size and form: spindle, oval, elongated with abundant cytoplasm, small with one nucleus and cells with scanty cytoplasm, were found. The present cells differently reacted to histochemical and immunohistochemical methods. Large oval cells showed negative reaction to lymphocytic and leukocytic markers, and positive to alpha actin, actin HHF35, Ki-67, myosin, myoglobin and desmin. Elongated cells were also positive to those markers. Small mononuclear cells showed positive reaction to lymphocytic markers. Endothelial and smooth muscle cells in the blood vessel walls were positive to CD34 and CD31, and smooth muscle cells to SMA. Oval and elongated cells were positive to PCNA and Ki-67. The preserved muscle fibers in the scar were positive to myosin, myoglobin and desmin as well as elongated and oval cells. Other cells were negative to these markers. **Conclusion.** Our findings speak that myocardial regeneration is maybe possible and develops in human ischemic heart damages and that the myocardium is not a static organ without capacity of cell regeneration.

Key words:
myocardial infarction; cicatrix; myocardium; regeneration; myocytes, cardiac; immunohistochemistry; histological techniques.

Apstrakt

Uvod/Cilj. Tradicionalno, smatrano je da je srce statički organ i da je nesposobno da se regeneriše posle povrede. Danas, sve češće se postavlja pitanje da li srce ima unutrašnju sposobnost da regeneriše miocite posle infarkta miokarda. Cilj ove studije bio je da se prikaže postojanje očuvanih mišićnih vlakana u ožiljku srčanog mišića posle preležanog akutnog infarkta miokarda, kao i prisustvo mnogobrojnih ćelija različite veličine i oblika, koje su različito reagovale na primenu imunohistohemijskih antitela. **Metode.** Histološki, histohemijski i imunohistohemijski analizirani su isečci miokarda uzeti od 177 bolesnika umrlih od akutnog infarkta miokarda, koji su ranije već jednom preležali infarkt miokarda, i imali, kao posledicu, ožiljak u srčanom mišiću. Iz mesta akutnog infarkta, kao i iz ožiljka, uzimano je više isečaka miokarda, koji su tretirani sledećim metodama: HE, PAS, PAS-dijastaza, Masson trichrom, Malory, van Gieson,

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vimentin, dezmin, miozin, mioglobin, α-aktin, SMA, p53, LCA, PCNA, Ki-67, aktin HHF35, CD34, CD31, CD45, CD45Ro, CD8, CD20. **Rezultati.** U svim isečcima uzetim iz predela ožiljka nađena su veća ili manja ostrvca očuvanih mišićnih vlakana srca sa znacima hipertrofije. U ožiljku je nađen veliki broj ćelija različite veličine i oblika: vretenaste, ovalnog oblika, izdužene sa dosta citoplazme, sitne sa jednim jedrom i ćelije sa oskudnom citoplazmom. Prisutne ćelije su različito reagovale na primenu histohemijskih i imunohistohemijskih metoda. Velike, ovalne ćelije davale su negativnu reakciju na limfocitne i leukocitne markere, a pozitivne na alfa aktin, aktin HHF35, Ki-67, miozin, mioglobin i dezmin. Na ove markere bile su pozitivne i izdužene ćelije. Sitne monojedarne ćelije davale su pozitivnu reakciju na limfocitne markere. Endotelne ćelije i glatke mišićne ćelije u zidu krvnih sudova bile su pozitivne na CD34 i CD31, a glatke mišićne ćelije i na SMA. Ovalne i izdužene ćelije bile su pozitivne i na PCNA i Ki-67. Očuvana mišićna vlaka u ožiljku bila su pozitivna na miozin, mioglobin i dezmin, kao i izdužene i ovalne ćelije. Ostale ćelije bile su negativne na ove markere. **Zaključak**. Naš nalaz ide u prilog mišljenju da se miokardna regeneracija dešava u humanim ishemijskim povredama srca i da miokard nije statički organ bez ćelijske obnove.

Ključne reči:
infarkt miokarda; ožiljak; miokard; regeneracija; miocit srca; imunohistohemija; histološke tehnike.

Introduction

The heart has traditionally been considered as a static organ without capacity of regeneration after trauma [1, 2]. Currently, the more and more often asked question is whether the heart has any endogenous capacities to regenerate myocytes after myocardial infarction because the need for regeneration of damaged myocardium has been imposed [1, 3, 4]. There are numerous opinions that postnatal and adult hearts cannot regenerate and that the myocyte number present at birth dictates the lifetime heart function [1, 4–6]. These opinions confirm that the postnatal hearts are composed of a fixed myocyte number and if they die, they are permanently lost, so that the myocardium has to perform its vital function with a reduced number of cells, which will result in their hypertrophy and finally to their death. Still, some authors [1, 3, 4, 7–10] believe that there is some possibility of myocyte division in the pathologic heart. In the course of the previous years many authors [5, 8, 11–14] have documented the presence of mytotic forms in the human cardiomyocytes in acute and chronic ischemic cardiomyopathy, idiopathic dilatational cardiomyopathy, chronic aortic stenosis and ventricular dysfunction.

The aim of this study was to show the presence of the preserved muscle fibers in the myocardial scar after experienced acute myocardial infarction as well as the presence of numerous cells of various size and form that differently reacted to the used immunohistochemical antibodies.

Methods

Retrospective histological, histochemical and immunohistochemical analyses of sections from the myocardium of 177 deceased patients with acute myocardial infarction were carried out. Before death, all of them already had myocardial infarction and the cardiac muscle scar as its consequence.

From 1975 to 2000, at the Institute of Pathology and Forensic Medicine, Military Medical Academy, Belgrade, autopsies of 308 deceased patients due to acute myocardial infarction were performed. It was shown that 177 of them had already acute myocardial infarction. Among them there were 123 males and 54 females, aged 45–79 years, and most of them were aged 55–68 years.

The time period from the first infarction to death was 6 months to 2 years, and a period from death to autopsy was 7–12 hours. On the basis of data from the disease history, 163 of the autopsies showed systemic hypertension higher than 160 mmHg and 14 of them diabetes mellitus. Neither presence of some neoplasm nor chronic infection were found at any of 177 autopsies. Immediate cause of death in all patients was acute myocardial reinfarction.

At all autopsies, the mass of the heart was measured, localization, size of acute infarction and also the size of the scar as a consequence of previous infarction were precisely determined and measured. Several sections from the acute infarction site as well as from the scar and a borderline between the scar and the normal myocardial tissue were taken for analysis. Sections were fixed in 10% buffered neutral formalin, embedded into paraffin beds and cut by a microtome to 5–7 micrometers. The obtained sections were analysed by the following histologic, histochemical and immunohistochemical methods: hematoxylin-eosin (HE), periodic acid schiff (PAS), PAS diastasis, Masson trichrom, Malory, van Gieson, vimentin, desmin, myosin, myoglobin, alpha actin, smooth muscle actin (SMA), p53, leukocyte common antigen (LCA), proliferating cell nuclear antigen (PCNA), Ki-67, actin HHF35, CD34, CD31, CD45, CD8, CD20 and CD45-Ro.

Results

The mass of the deceased patients' hearts at autopsies was 350–1050 g, in most of them (n = 120) it was 350–650 g. Localization of the scar after the first cured infarction at 39 autopsies was in the region of the anterior left ventricular wall, at 50 of them in the region of the posterior left ventricular wall, at 55 in the region of the anterior left ventricular wall and interventricular septum, and at 33 autopsies in the region of the anterior and posterior left ventricular wall and interventricular septum. Serious atherosclerotic changes in the coronary blood vessels walls as well as obturation of their lumen were found at all autopsies. Obturations of the anterior descendent branch of the left coronary artery, then of the right coronary artery and of both the first and second marginal branches of the left coronary artery were the most frequent finding.

The reaction of different cells found in myocardial scar tissue to used markers is summarized in Table 1. Histochemical staining of various tissue is shown in Table 2.

The preserved muscle fibers in the scar were hypertrophic with large hypertrophic and hyperchromatic nucleus and with the preserved transversal stria (Figure 2).

Table 1

Reaction of cells present in the myocardial scar to used markers

Markers	Type of cells in the myocardial scar					
	Lympho-cytes	Leuko-cytes	Large oval cells abundant with cytoplasm	Elongated cells	Endothelial cells	Smooth muscle cells in the blood vessels wall
CD_8	+	–	–	–	–	–
CD_{20}	+	–	–	–	–	–
CD_{31}	–	–	+	+	+	–
CD_{34}	+	–	–	–	+	–
CD_{45}	+	+	–	–	–	–
CD_{45}-Ro	+	–	–	–	–	–
Alpha ACTIN	–	–	+	+	–	+
Ki-67	–	–	+	+	–	–
PCNA	–	–	+	+	–	–
ACTIN HHF_{35}	–	–	+	+	–	+
DESMIN	–	–	+	+	–	+
MYOGLOBIN	–	–	+	+	–	–
MYOSIN	–	–	+	+	–	–
LCA	+	+	–	–	–	–
SMA	–	–	–	–	–	+

PCNA – proliferating cell nuclear antigen; LCA – leukocyte common antigen; SMA – smooth muscle actin

Table 2

Tissue reactions to histochemical dyeing

Dyeing	Type of tissues		
	Heart muscle fibers	Collagen fibers	Elastic fibers
PAS	+	–	–
Masson trichrom	+ (red)	+ (green)	+ (green)
van Gieson	+ (yellow)	+ (red)	+ (red)
Malory	+ (red)	+ (green)	+ (green)

PAS – periodic acid schiff

Microscopic analysis was carried out for all sections from the myocardial scar of the deceased patients who already had bad myocardial infarction before the fatal one, revealed the preserved myocardial muscle fibers in the form of larger or smaller islets completely isolated from the surrounding preserved cardiac muscle, but surrounded by the connective tissue and without any contact with the normal muscle fibers (Figure 1).

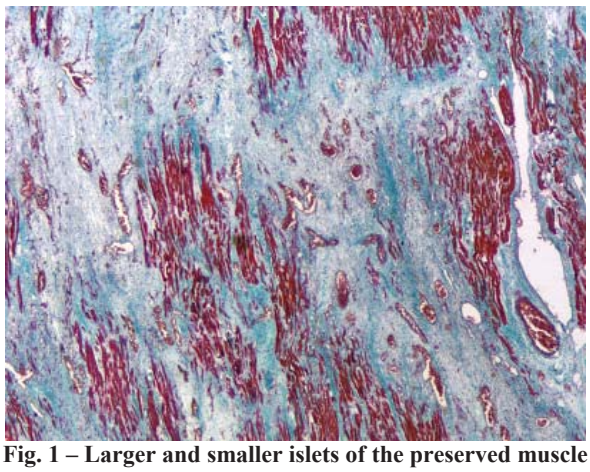

Fig. 1 – Larger and smaller islets of the preserved muscle fibers in the myocardial scar (Malory, x200)

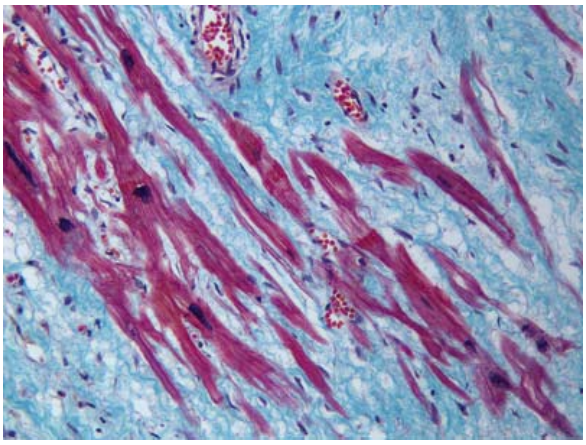

Fig. 2 – The preserved muscle fibers are hypertrophic with large hypertrophic and hyperchromatic nucleus (Masson trichrom, x400)

Around the preserved muscle fibers there was the enlarged newly formed connective tissue, in some sites very dense and in the others loose. The newly formed connective tissue was well vascularized by the numerous newly formed blood vessels with thin walls, coated with the monolayer endothelium. The lumens of newly formed blood vessels were

of various size and form and overfilled with blood. In the newly formed connective tissue (scar) there was a large number of both single cells and groups of cells of various sizes and forms (Figure 3).

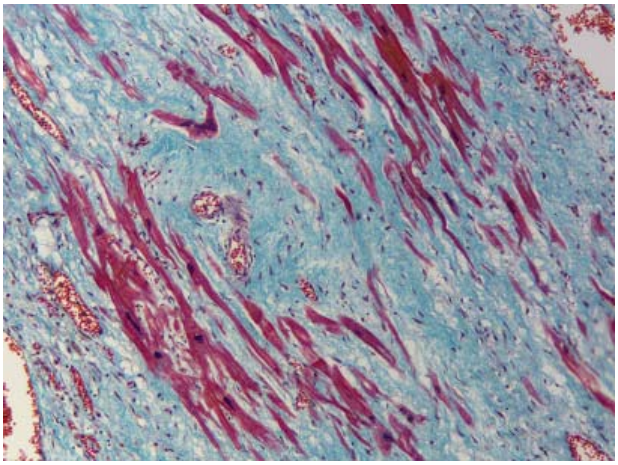

Fig. 3 – A large number of cells of various sizes and forms can be seen in the newly formed connective tissue (Masson trichrom, x200)

Some of the present cells were of the spindle form with a large hyperchromatic nucleus, the others were small with a little cytoplasm and one central nucleus. Some of these cells were oval with abundant cytoplasm and large circular nucleus. There were also elongated cells with abundant fine-grained cytoplasm and large circular nucleus. There were also cells with scanty cytoplasm so that only the nucleus was dominant. The present cells were mostly mononuclear and rarely with two or more nuclei. Only scattered blood vessels with a thick damaged wall could be seen in the scar. In more expressed vascularisation there was a larger number of cells. Myogenic, large oval cells with a large nucleus and abundant cytoplasm showed negative reaction to the lymphocytic and leukocytic markers CD34, CD45, CD20, CD8, CD45-Ro and LCA, but they were positive to alpha actin (Figure 4), actin HHF35 (Figure 5), Ki-67 (Figure 6), myosin, myoglobin (Figure 7) and desmin. Elongated myogenic cells were also positive to these markers (which would speak for cardiomyocytes). Other cells found in the scar were negative to these markers. Small mononuclear cells that would correspond to the lymphocytes as well as leukocytes presented in the scar, showed reaction both to the lymphocytic and leukocytic markers (Figure 8).

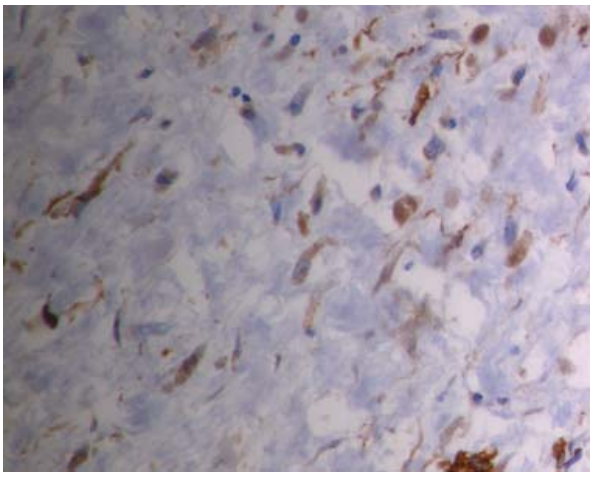

Fig. 4 – Large oval cells and elongated cells positive to alpha actin (x400)

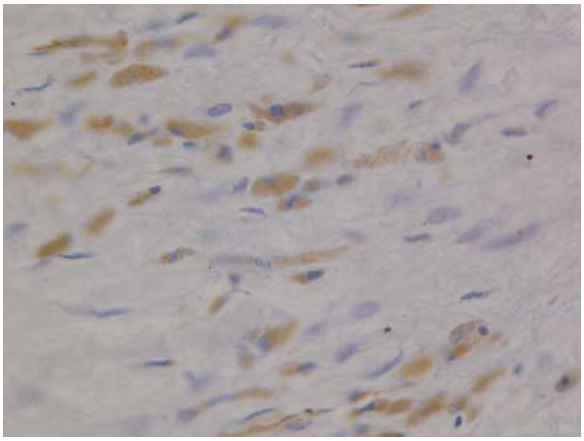

Fig. 5 – Oval and elongated cells in the scar positive to actin HHF35 (x400)

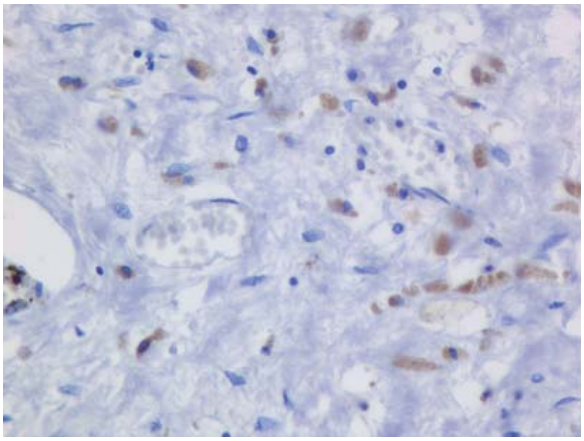

Fig. 6 – Large oval cells in the scar positive to Ki-67; other cells are negative (x400)

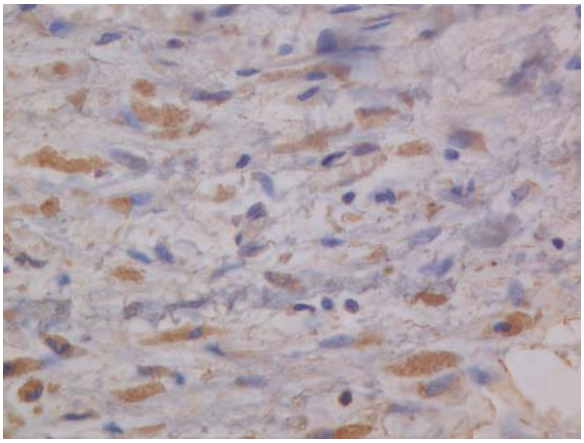

Fig. 7 – Large oval and elongated cells in the scar positive to myoglobin (x400)

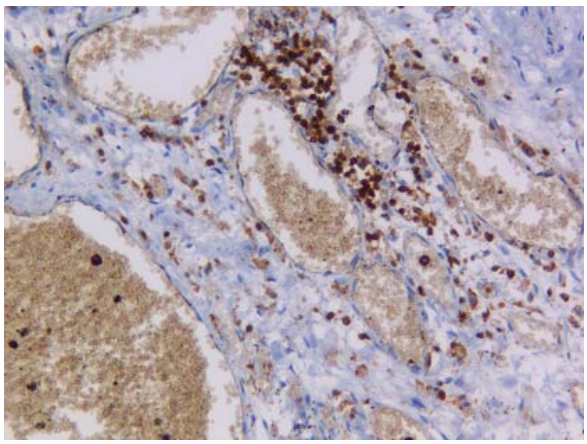

Fig. 8 – Lympocytes and leukocytes in the scar positive to LCA (x400)

cells showed positive reaction to PCNA markers (Figure 9), while spindle cells as well as other present cells were negative to PCNA marker. Expression to Ki-67 nuclear antigen was positive in all nuclei of large, oval and elongated myogenic cells (Figure 10).

Mitotic figures were noted in some nuclei of these cells suggesting cells division. These cells were also positive to PCNA marker for myocytic protein confirmation (Figures 9). The usage of myoglobulin, myosin and desmin markers showed positive reaction to the preserved muscle fibers in the scar (Figure 11), as well as to the elongated mononuclear cells and large, oval cells abundant with cytoplasm and with the large nucleus suggesting the presence of cardiac muscle fibers and most probably cardiomyocytes. Other cells were negative to these markers (Figures 1 and 2).

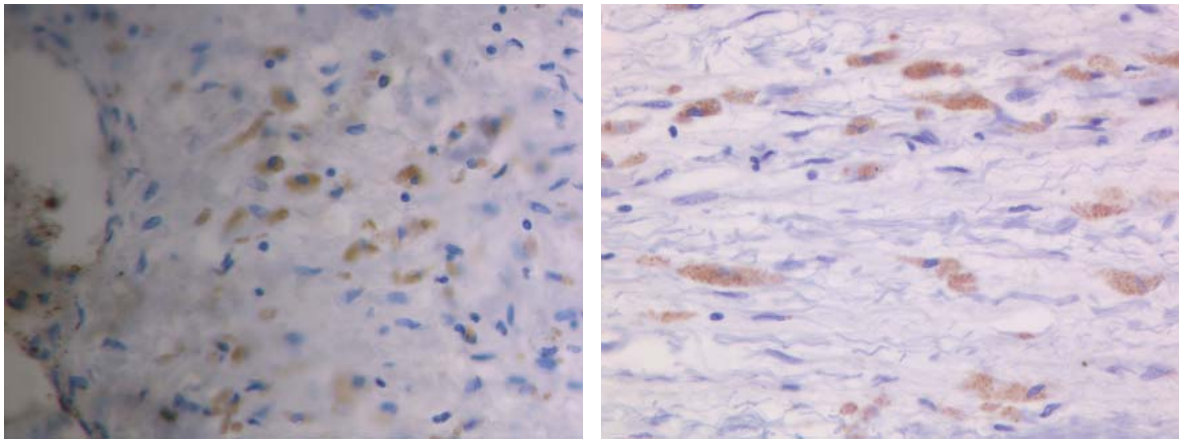

Fig. 9 – Oval and elongated cells in the scar positive to PCNA; other cells were negative (x400)

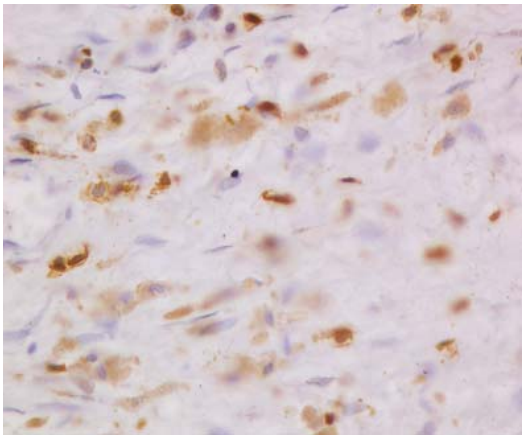

Fig. 10 – Oval and elongated cells in the scar positive to Ki-67 (x400)

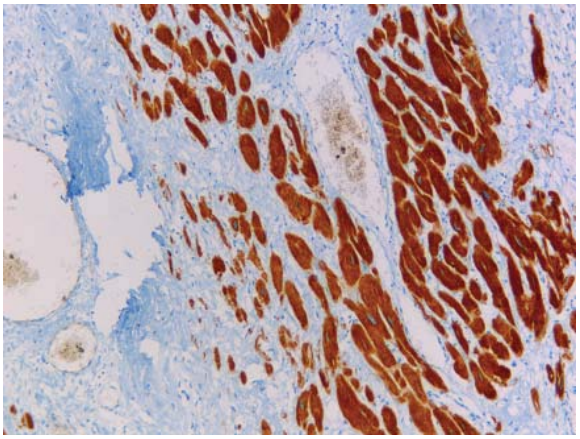

Fig. 11 – Preserved muscle fibers in the scar positive to desmin (x400)

CD34 and CD31 markers showed positive reactions to endothelial cells and smooth muscle cells in walls of the newly formed blood vessels in the scar. The usage of SMA markers resulted in positive reaction for the presence of the smooth muscle cells in walls of the newly formed blood vessels. The present oval cells abundant with cytoplasm and with the large nucleus as well as elongated mononuclear

Discussion

Cardiac failure is the leading cause of both morbidity and mortality in developed countries [10]. Hearts of adult persons are well supplied with blood and are capable to maintain tissue integrity during physiologic cell function [1, 3, 4]. Soon after birth the cardiac function is maintained by in-

creasing both the number and volume of myocytes which together contribute to development of the adult heart [2]. The question is whether the heart possesses endogenous capacity to regenerate myocytes after myocardial infarction [1, 3, 4]. Most of human bodily organs contain divisible and indivisible cells. However, indivisible cells can be in the Go phase and reenter the cellular cycle or become terminally differentiated without possible further division [3]. But, these mechanisms of cell growth have not been accepted so far when the myocardium is concerned and it is believed that cardiomyocytes cannot be regenerated in cells by the division because in mammals their division was discontinued immediately after birth [1, 4–6, 15]. On this basis human myocytes can live for the lifetime even if it were 100 years. It seems exceptionally extravagant that cardiomyocytes contracting 70 times a minute do that continually for 100 years. During that period they would contract 3.7 billion times [1, 3]. If it is true, then cardiomyocytes are immortal cells [1, 3]. It has been accepted that myocytes can enlarge their volume but not the number (the cardiac mass of a 42-weeks-old infant is 2.9 ± 6.2 g).

Nevertheless, some authors [3, 16] accept the possibility of myocyte division. In their studies they have measured the volume and number of human cardiac myocytes of patients died of decompensated cardiac hypertension and congestive cardiac failure. Mass of all the examined hearts was 500 g and more and it was characterized by noticeably increased myocyte number, more than cellular hypertrophy [3, 16]. Some other authors [17] claim that methods used by these authors can only suggest, but not confirm the real increase in the cell number. It was believed that cardiac hypertrophy is a consequence of the present myocytes hypertrophy [4, 8, 18] and that the heart is a terminally differentiated postmitotic organ. After these studies had been published, generations of a great number of pathologists and cardiovascular experts accepted the fact that myocyte mitosis was not seen in the adult myocardium. However, in the course of the last 10–12 years a greater number of authors [1, 3–5, 7–11] have documented the presence of mitotic figures in human cardiomyocytes in cases of acute and chronic ischemic cardiomyopathy, idiopathic dilatational cardiomyopathy, chronic aortic stenosis and ventricular dysfunction. Other authors [11, 19–21] emphasize that the formed parenchymatous cells are permanent in the normal myocardium, but that myocyte regeneration is also present in pathologic hearts, which they have microscopically confirmed.

Recognition that stem cells are present in the myocardium raised a question of their role in the treatment of the affected human heart [9, 10, 17, 18, 22]. Question related to the presence and role of adult cardiac stem cells is based upon the fact that these stem cells regenerate *in vivo* in cases of the myocardial infarction [9]. Urbanek et al. [22] emphasizes that the number of cardiac stem cells was increased in chronic myocardial infarction, and Quaini et al. [4] prove that the ventricular myocytes are not terminally differentiated cells. Beltrami et al. [8] have analyzed sections taken from three patients died 4–12 days after myocardial infarction and compared findings of sections taken from the normal hearts of 9 control groups of patients. By using methods of immunohistochemistry in sections taken from the deceased after myocardial infarction, the following was found: cardiomyocytes with elements characteristic for the cell division such as mitotic spindle and contractile ring formations, karyokinesis and cytokinesis. Similar findings were also found out in our own research. These findings confirm that there is a mitotic proliferation after myocardial infarction, and myocyte regeneration can contribute to increase in myocardial mass. This suggests that a prolonged cardiac failure can progressively influence upon mitotic activity. Regeneration initiation time depends on the span survival period after infarction and it starts already on the 10th day after experienced infarction. It reduces infarction by 40%–50% after 20 days. Ten days after infarction develops myocyte and blood vessels regeneration being improved in time [2, 8, 9.]

The human heart, just like the brain, is mostly composed of terminally differentiated cells, but it is not a terminally differentiated organ because it contains stem cells that can develop its regeneration [2, 3, 13, 23–27]. By the usage of Ki-67 nuclear antigen, Beltrami et al. [8] has found out positive reaction associated with cell division in all nuclei of the large oval and elongated cells. Similar results were found in our research. These cells were negative to lymphocyte, leukocyte and endothelial markers. Ki-67 is a nuclear antigen and in our research it was positive in myocytes of an infarcted heart suggesting that cardimyocytes are divisional. By using alpha actin Beltrami et al. [8] have found in these cells its accumulation in the contractile ring what was also confirmed in our study. These results show that in the adult heart after experienced infarction, there is a myocyte subpopulation not terminally differentiated and capable to divide soon after infarcton. In our research cells that were positive to Ki-67 nuclear antigen were also positive to the usage of: PCNA, alpha actin, actin HHF35, myoglobin, myosin and desmin, but showed negative reaction to lymphocyte, leukocyte and endothelial markers. Other authors' [1, 5, 8] finding are the same suggesting possible myocyte regeneration after myocardial infarction [1, 2, 5, 8]. It has been proved that heart stem cells are capable to differentiate into three types of heart cells: cardiomyocytes, smooth muscle cells in the blood vessel wall and endothelial cells [12]. We have showed the presence of both smooth muscle and endothelial cells in the walls of the newly formed blood vessels in the scar after infarction. Stem cell factor can affect and activate all the three mentioned cells during myocardial ischemia and result in a significant increase in new myocyte formations [2, 12]. Myocardium regeneration requires myocyte and blood vessels formation because myocytes cannot live and grow without blood vessels. However, blood vessels formation alone will not regenerate the dead myocardium and its contractile activity after infarction [7, 28, 29]. The preserved muscle fibers that we have found isolated in the myocardial scar after infarction were hypertrophic with the large nucleus suggesting their participation in synchronous contractions of heart muscle and possibly in prevention of the heart aneurysm development.

A finding that the mammalian heart contains stem cells that regenerate the myocytes and blood vessels represents a unique potential of the dead myocardium reconstruction after

infarction [6, 18, 30]. Myocardium regeneration occurs in human ischemic and nonischemic heart damages. Heart stem cells localized within infarction or close to it can be divided and differentiated reconstructing consequently the myocardium. This response can increase the number of myocyte and blood vessels reducing in that way infarction size, improving its function and reducing mortality rate [6, 18, 30]. The fact that myocardium is not a static organ and that cell reconstruction is not limited as well as both endothelial and vascular smooth muscle cells, requires reinterpretation of the heart biology and mechanism of its life.

Conclusion

The islets of the preserved cardiomyocytes (together with some other cells) surrounded by the connective tissue in the myocardial scar following myocardial infarction were found in our investigations. That it is about cardiomyocytes (and also about other cells) we have confirmed not only by clasical histopathologic dyeing but also by immunohistochemical methods and by broad battery of tests to corresponding markers. On the other hand, the mentioned islets of the preserved cardiomyocytes were not found in sections taken from necrotic tissue in the repeated acute myocardial infarction resulting in the fatal outcome. The question is what, in fact, the found islets of the preserved cardiomyocytes in the scar of the preceded infarction represent? Are they the remains of the preserved myocardium in the infarcted region supplied by some arterial collateral? Is it correlated with the regenerated myocardium due to progenitory cells delivered by blood and differentiated into cardiomyocytetes, or with proliferated cardiac stem sells themselves? Also, what is the significance of these preserved islets of cardiomyocytes? Do they take part in myocardial contractions? Are they the cause of cardiac arrhythmias in pathologic cases? The findings obtained and confirmed by this study are clear and therefore important and interesting, but further studies are necessary to answer the above raised questions.

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