Pathways of Bone Marrow Mononuclear Cell Differentiation after Transplantation into Postinfarction Heart

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We studied homing and differentiation fate of transplanted bone marrow mononuclears after non-selective intracoronary injection on day 30 after acute myocardial infarction in rats. Mononuclear cells migrated to the cicatrix zone where they differentiated into fibroblasts and myofibroblasts. Mononuclear cells did not differentiate into cardiomyocytes, endothelial cells, or smooth muscle cells of vascular media. Stimulation of angiogenesis and reparation of the myocardium was observed under these conditions.

Key Words: *bone marrow mononuclear cells; homing; differentiation; angiogenesis; myocardial reparation*

Cell therapy is not the radical treatment of acute and chronic cardiac diseases, but it can considerably improve the efficiency of therapeutic and surgical treatment [4]. Mononuclear cells (MNC) are considered as the optimal variant of cell transplant, because the procedure of their isolation is well developed, simple, and does not require special equipment or long-term expansion. There are ample experimental data on therapeutic efficiency of MNC and their effects on postinfarction heart [10,13,14], but the mechanisms of these influences are poorly understood. Our previous studies showed migration of MNC injected into coronary vessels into the cicatrix zone where they were not eliminated by host immune system, but survived leading to thickening of the heart wall in the cicatrix area and improving the function of the left ventricle [3]. Here we studied the differentiation fate of transplanted MNC and their role in angiogenesis and reparation of the myocardium.

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MATERIALS AND METHODS

Experiments were carried out on 36 male Sprague–Dawley rats. In all animals, transmural acute myocardial infarction (AMI) followed by reperfusion was modeled. On day 30 after AMI modeling, MNC were transplanted to experimental rats (n=20). Controls (n=16) received physiological saline. The animals were sacrificed by ether overdose on days 14 and 30 after cell transplantation [2]. At each stage of the experiment, 8-10 animals in the control and experimental groups were used.

BM MNC were isolated routinely in a density gradient [7]. The isolated nucleated cells were resuspended in 1 ml to a concentration of 5×10^6 cells per 1 ml physiological saline and labeled with a fluorescent membrane dye PkH26 (Red Fluorescent Cell Linker Mini Kit, Sigma) according to manufacturer's recommendations. The qualitative and quantitative evaluation of the distribution of labeled cells in organs and tissues was performed using a fluorescent microscope.

For histomorphometric study of rat heart, serial transverse sections of the left and right ventricles were prepared and stained with picrosirius red. For evalua-

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tion of angiogenesis after cell transplantation, the sections were stained after Mallory and the total number and volume density of blood vessels in the cicatricial area were determined.

For immunohistochemical study, monoclonal antibodies (Abcam) to Fapα (marker of reactive fibroblasts), CD68 (marker of macrophages), and α-SMA (marker of smooth muscle cell actin) were used according to manufacturer's protocol.

The mean and standard error of the mean were calculated. The Student *t* test (in case of normal data distribution), Mann–Whitney test (for data distribution not conforming the normal law), and Kruskal–Wallis one-way ANOVA on ranks were used. Statistical processing of the results was performed using SigmaPlot 11.0 software. The differences were significant at 5% significance level.

RESULTS

In our previous studies, homing of MNC into the damaged area, but not their equal distribution in organs was observed after non-selective administration of cells into the left and right coronary arteries 4 weeks after AMI [8]. Directed migration of MNC was also confirmed by localization of transplanted cells only in the cicatrix zone. Migration of BM cells to the infarction zone is thought to be induced by enhanced migration of stromal-derived factor-1 (SDF-1) by inflammatory cells [9]. The concentration of chemoattractants remains probably high at the state of cicatrisation, because the transplanted cells migrated to the damaged zone even 6 weeks after AMI where they participate in the formation and maturation of the cicatrix tissue [1]. Two weeks after intracoronary transplantation of MNC, they were detected primarily in the heart and spleen (55.5±3.2 and 52.3±1.7 cells per field of view, respectively), whereas in the liver and lungs only solitary cells were detected (2.9±0.5 и 1.4±0.4, respectively). After 4 weeks, 34.5±2.4, 43.8±2.4, 7.6±0.6, and 3.1±1.3 MNC were found in the heart, spleen, liver, and lungs, respectively [3].

In our study, the transplanted cells were not eliminated by the immune system and remained viable

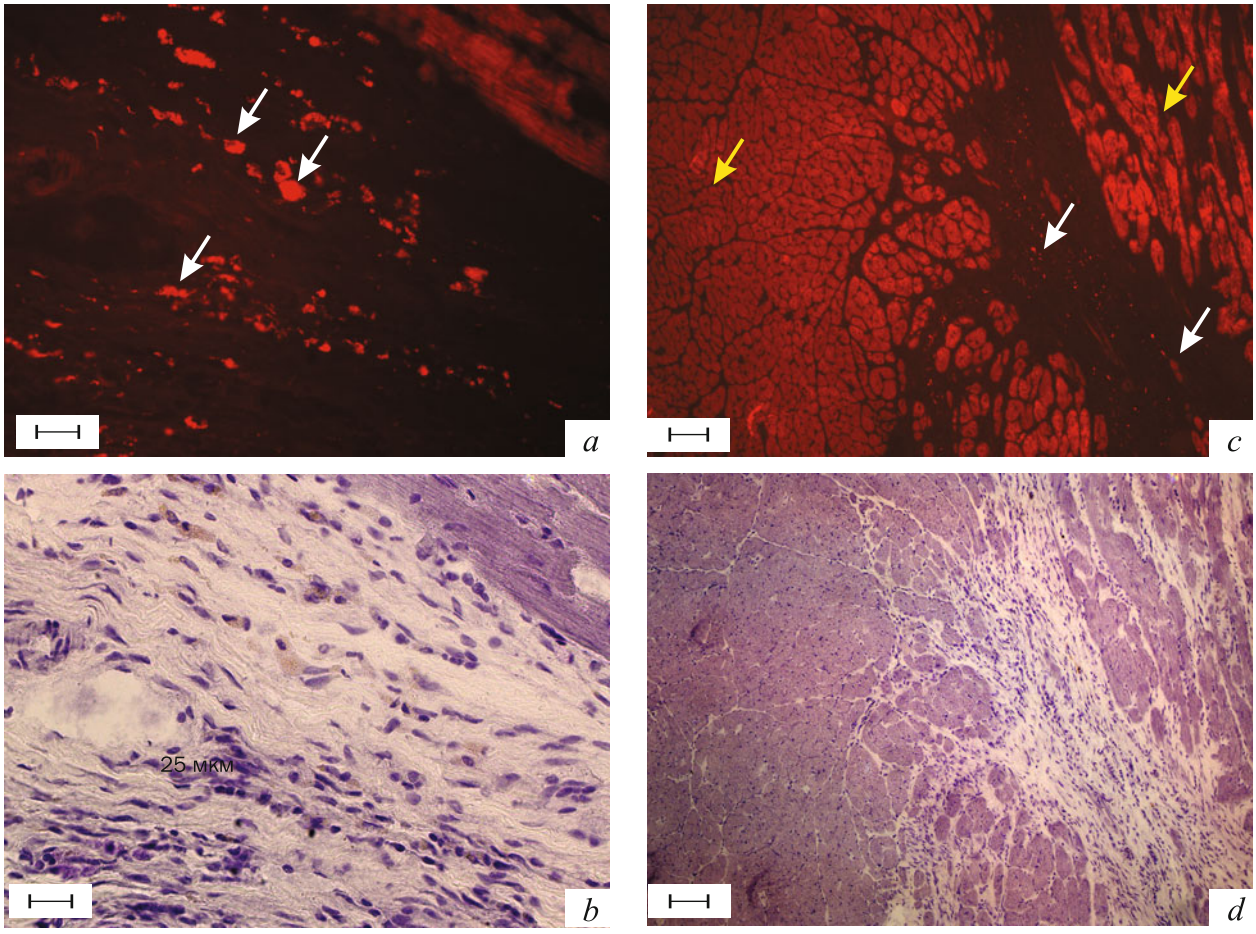

Fig. 1. Localization of labeled cells in the cicatrix and perimysium of the perifocal myocardium (*a*, *b*), fluorescent and light microscopy and the absence of labeled cells in undamaged myocardium (*c*, *d*). 30 days after transplantation. Yellow and white arrows show undamaged myocardium and labeled PkH26 cells in the cicatrix, respectively. Fluorescent and light microscopy. Scale: *a*) 10 μ; *b*) 10 μ; *c*) 100 μ; *d*) 100 μ.

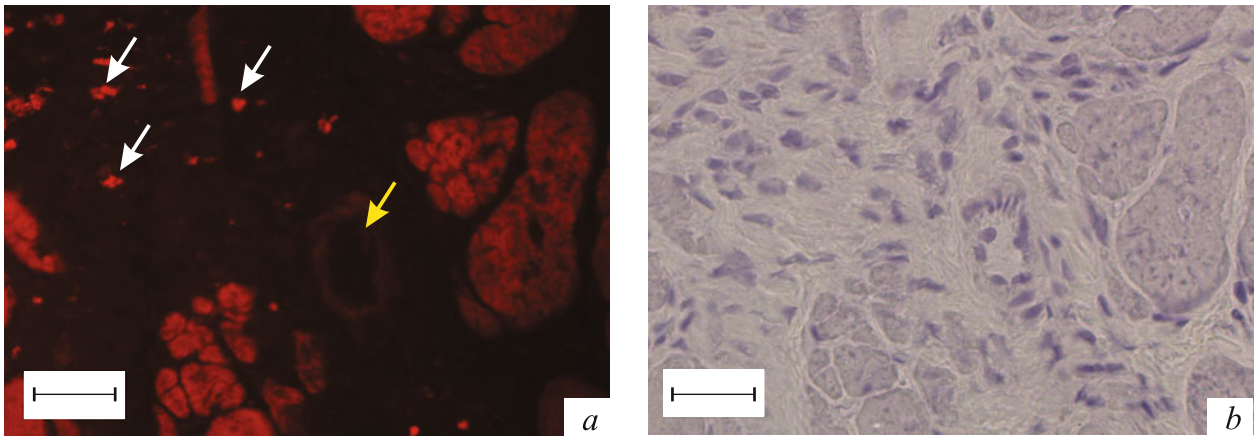

Fig. 2. Localization of labeled cells in the cicatrix and their absence in the vascular wall: 30 days after transplantation. White arrows show labeled cells and yellow arrow shows the vessel. Fluorescent (*a*) and light (*b*) microscopy. Scale: 25 μ.

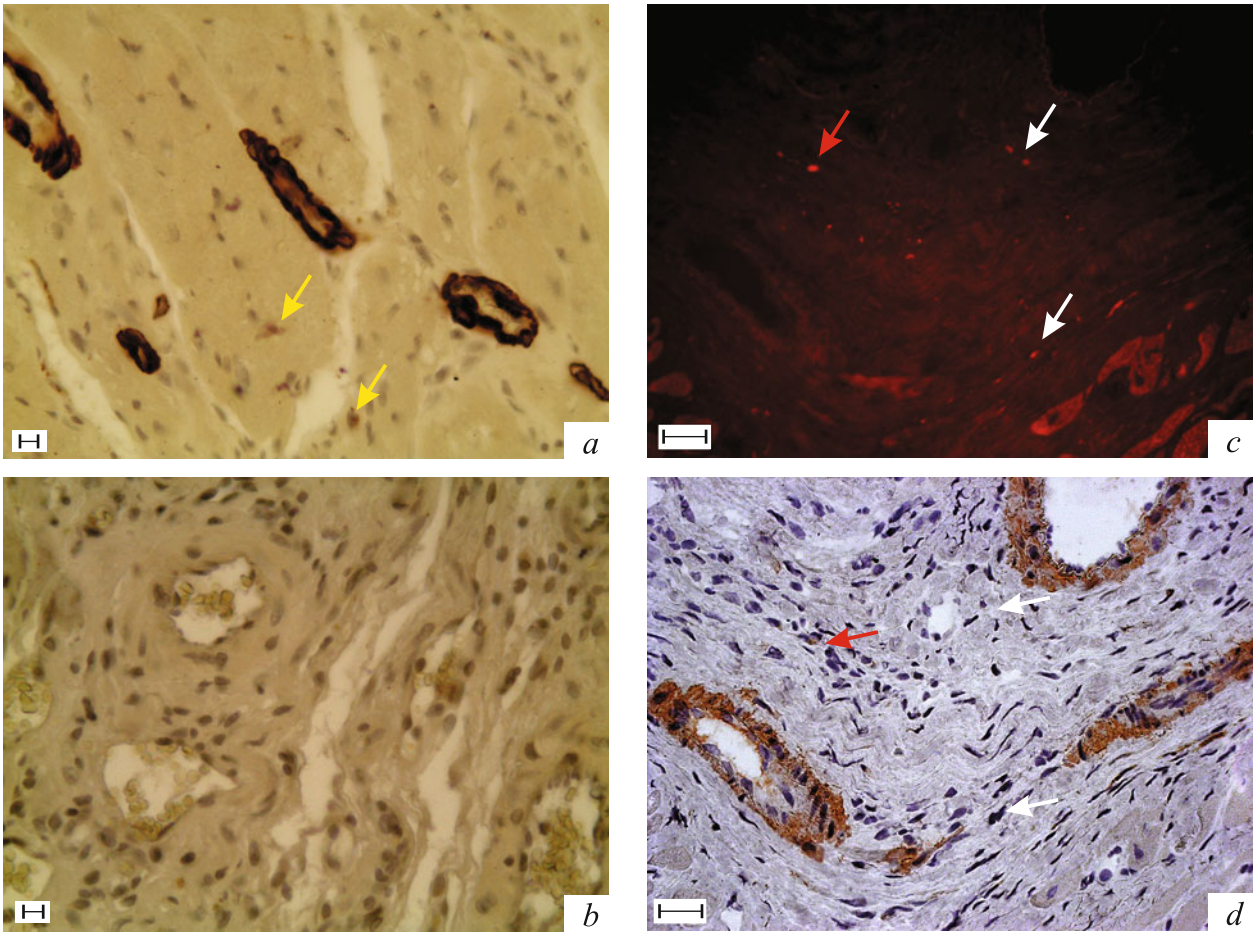

Fig. 3. Immunohistochemical staining with MAB to α-SMA. a) cicatrix tissue on day 30 after MNC transplantation. Yellow arrows: myofibroblasts; *b*) control without primary MAB; *c, d*) superposition of MAB-labeled MNC (*c*) and cells stained with MAB to α-SMA. Red and white arrows show labeled cells stained and not stained with MAB, respectively. Fluorescent and light microscopy. Scale: *a*) 10 μ; *b*) 10 μ; *c*) 25 μ; *d*) 25 μ.

4 weeks after transplantation. The cells were not damaged, no pronounced lymphocyte-macrophage infiltration was observed around them. The data of immunohistochemical analysis of monoclonal antibodies (MAB) to macrophage marker (CD68) indicate the presence of macrophages in both the control and experimental groups 2 weeks (13.17±0.54 and 13.16±0.56 cells, respectively; p=0.895) and 4 weeks (11.39±0.53 and 11.50±0.76, respectively; p=0.823) after transplantation. Solitary MAB-fluorescent-labeled

cells were probably eliminated by macrophages, but no morphological signs of transplant rejection were observed. Moreover, directed migration to the damaged zone confirmed cell viability.

We detected no working cardiomyocytes (CMC) carrying the fluorescent label. Not a single labeled cell was detected in the perimysium of undamaged myocardium (Fig. 1, *c, d*), while in the perifocal zone labeled MNC were seen in the connective tissue (Fig. 1, *a*, *b*)

No labeled MNC were found in the intima and media of newly formed blood vessels of the cicatrix (Fig. 2). Solitary labeled cells were detected in the adventitia, but we cannot say with confidence that they belonged to adventitia and not to the connective tissue surrounding the vessel. This suggests that transplanted MNC did not differentiate into endothelial and vascular smooth muscle cells and did not participate in the formation of new blood vessels.

This was also confirmed by immunohistochemical staining with MAB to α-SMA (Fig. 3). MAB selectively labeled vascular smooth muscle cells, but no labeled MNC were found among MAB-labeled vascular cells. Moreover, fibroblast-like cells labeled with MAB to α-SMA were found in the bulk of the cicatrix; they can be identified as myoblasts. These cells of the fibroblast differon not only synthesized the components of the connective tissue matrix, but also demonstrated contraction capacity. These cells play a key role in the formation and remodeling of the cicatrix tissue. Some MAB-labeled cells also carried the fluorescent label. It can be concluded that some transplanted cells differentiated into myoblasts.

Taking into account MNC localization and morphology, we hypothesized that transplanted cells differentiate into cicatrix fibroblasts. Because of the absence of fibroblasts-specific markers, the immunohistochemical assay was carried out using MAB to marker of reactive fibroblasts Fapα [6]. Fapα is α-subunit of membrane gelatinase, a member of serine proteinase family. This marker is specific for reactive fibroblasts appearing during wound healing, forma-

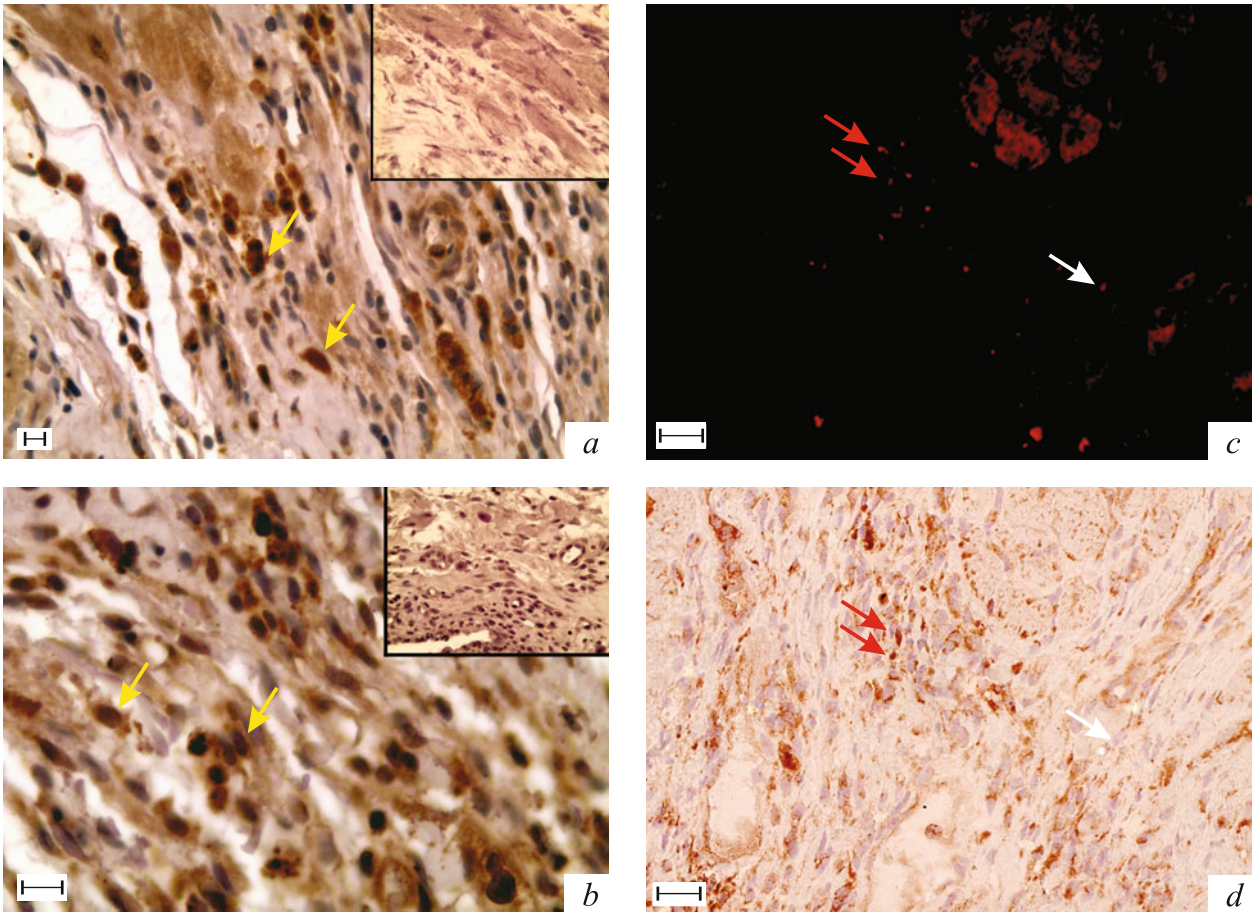

Fig. 4. Immunohistochemical staining with MAB to Fapα. *a*) cicatrix tissue on day 14 after MNC transplantation. Yellow arrows show reactive fibroblasts, insert: control without primary MAB; *b*) cicatrix tissue in the control group after 14 days. Yellow arrows: reactive fibroblasts; insert: control without primary MAB; *c, d*) superposition of labeled MNC (*c*) and cells stained with MAB to Fapα. Red and white arrows show labeled cells stained and not stained with MAB, respectively. Fluorescent and light microscopy. Scale: *a*) 10 μ; *b*) 10 μ; *c*) 25 μ; *d*) 25 μ.

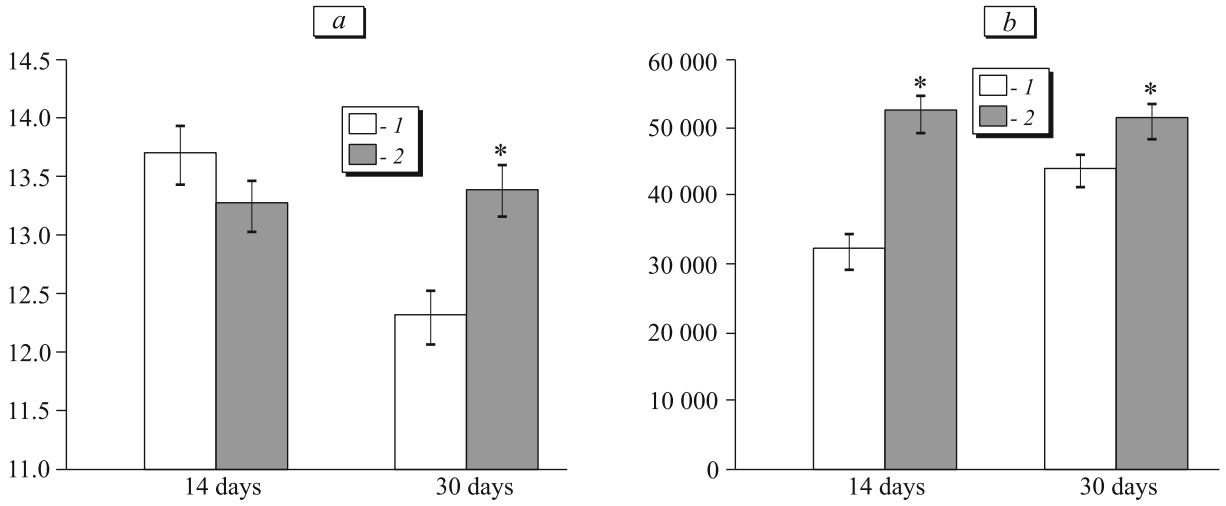

Fig. 5. Number (*a*) and volume density (*b*) of newly formed vessels (per field of view 786,432 μ) on days 14 and 30 after MNC transplantation. *1*) control group; *2*) experimental group. *$p<0.05$ in comparison with the control group.

tion of the granulation tissue, and some sarcomas and actively expressing Fapα homodimers.

Numerous Fapα-positive cells were detected in the cicatricial tissue in both the experimental and control groups. They apparently represent active fibroblasts participating in the synthesis of collagen. Some labeled MNC were stained with MAB to Fapα, which attests to differentiation of some transplanted cells into reactive fibroblasts (Fig. 4).

Despite the fact that transplanted cells did not differentiate into vascular wall cells, considerable stimulation of neoangiogenesis was observed. After 2 weeks, the number of blood vessels was similar in the control and experimental groups (p=0.244), but volume density of blood vessels was significantly higher ($p\leq0.001$) in the experimental group, which attested to greater caliber of newly formed vessels after MNC transplantation. On day 30, both the number ($p\leq0.001$) and volume density (p=0.004) of vessels in the experimental group were higher than in the control (Fig. 5).

In our study we did not observe differentiation of transplanted BM cells into CMC or vascular cells. We can also exclude fusion of transplanted cells with host cells, because no fluorescent label was detected in CMC, endothelial, and smooth muscle cells. Many published reports suggest otherwise [11,12]. However, the proofs of cardiomyogenesis and angiogenesis from transplanted cells are based only on the presence of differentiation markers in labeled cells, while the localization and morphology of cells are not taken into account [5,11]. In our study, BM MNC transplanted via intracoronary transventricular route migrated to the cicatrix zone where they differentiated into fibroblasts and myofibroblasts. This led to strengthening and thickening of the cicatrix wall and improvement of cardiac function [1]. We believe that angiogenesis was stimulated by paracrine factors released by transplanted cells.

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