Reparation of the Myocardium after Transplantation of Mononuclear Bone Marrow Cells

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Safety and efficiency of intracoronary transventricular transplantation of autologous mononuclear bone marrow cells in rats with postinfarction cardiosclerosis were studied. The cells migrated to the damaged area and were detected only in the cicatricial tissue; they have fibroblast-like phenotype and some of them were stained with Fapα (marker of reactive fibroblasts). More active proliferation of non-muscular cells and formation and maturation of collagen fibers in the cicatricial tissue were observed after transplantation of mononuclear cells. This led to thickening of the cicatricial wall, but the size of the scar and index of dilatation of the left ventricle remained unchanged. The number and volume density of newly formed blood vessels in the damaged area increased after transplantation, but no labeled cells were seen in the vascular walls. It can be hypothesized that stimulation of neoangiogenesis is mediated by paracrine mechanisms, which also explains improvement of global contractility of the left ventricle (increased contractility index in functional tests). Thus, transplantation of mononuclear bone marrow cells leads to thickening and strengthening of the cicatricial wall, stimulates angiogenesis, and improves global myocardial contractility. However, no morphological signs of reverse remodeling of the left-ventricular myocardium were revealed.

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

Cell therapy of heart diseases is a promising trend in modern medicine. It is currently accepted that cell therapy is not a radical method leading to complete recovery, but been a component of complex treatment it can serve as a bridge to surgical treatment or even to heart transplantation [1].

The mechanism of the therapeutic effect of stem/progenitor cells is still a matter of discussion. Some investigators claim that transplanted cells can differentiate into cardiomyocytes or vascular cells and can provide a replacement effect [11], while others believe that stimulation of angiogenesis and reparative processes are determined by paracrine regulatory factors synthesized by transplanted cells without their differentiation into specialized myocardial cells [16].

Mononuclear cells (MNC) of the red bone marrow (BM) are now the most common transplant for cell therapy, because the technique of their isolation is routine and simple and does not require special conditions, equipment, and time for expansion. We previously showed that transplanted MNC after nonselective intracoronary injection migrated primarily into the heart and spleen [2]. In the myocardium, the labeled transplanted cells were detected only in the

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cicatricial tissue and were not incorporated into the vascular walls, but were surrounded by thick bundles of collagen fibers and probably underwent differentiation into fibroblasts [2].

It is clear that autologous BM MNC exhibit high angiogenic activity [18], but their effect on postinfarction remodeling of the myocardium of the left ventricle (LV) is little studied. Here we studied the differentiation pathways of transplanted cells and their role in stimulation of angiogenesis and reparative processes in the myocardium.

MATERIALS AND METHODS

Surgical manipulations for modeling myocardial infarction (MI) and chronic cardiosclerosis and transplantation of MNC were performed in a Laboratory of Biological Testing, Branch of Institute of Bioorganic Chemistry, Russian Academy of Sciences under GLP conditions. Animal experiments were carried out in accordance to principles of bioethics, rules for laboratory studies, and ethical norms. The experiments were approved by Bioethics Committee, Institute of Human Morphology, Russian Academy of Sciences and Branch of Institute of Bioorganic Chemistry, Russian Academy of Sciences.

Experiments were performed on male CD rats. BM was isolated by exfusion of both femoral and tibial bones and then the animals were randomly divided into 2 groups: experimental (n=20) and control (n=16). The rats were sacrificed 14 and 30 days after cell transplantation by narcotic analgesic overgosage.

Surgery for MI modeling. For modeling of postinfarction cardiosclerosis and chronic cardiac failure, transmural infarction of the anterior wall and apex of VL followed by reperfusion was reproduced 30 days before cell transplantation [2].

Preparation of cell transplant. BM MNC were isolated routinely in a density gradient [10]. The isolated nucleated cells were resuspended in 1 ml 9% physiological saline to a concentration of 5×10^6 cells/ml. Before transplantation, the cells from 4 experimental rats were labeled with fluorescent membrane dye PkH26 (Red Fluorescent Cell Linker Mini Kit, Sigma) according to manufacturer's recommendations.

Cell transplantation. Cell transplant (CT) was administered once 30 days after acute MI into LV cavity via a catheter introduced through the left carotid artery with simultaneous clamping of the aorta (for ≤10 sec). For each animal, the volume of CT was calculated according to animal body weight (25×10^6 cells/kg, concentration 5 mln/ml) [2].

Histological examination. For histomorphometry of heart tissues from rats (n=32), serial cross-sections at 10 levels of LV and right ventricle were prepared (the distance between the sections was 500 μ). Paraffin sections were stained with picrosirius red for quantitative analysis of collagen content and LV morphometry. For evaluation of neoangiogenesis after cell transplantation, the sections were stained after Mallori and the total number and volume density of blood vessels in the cicatricial area were determined. Histological preparations were examined under a light microscope and in polarized light for evaluation of the relative content of mature collagen.

For counting PkH26-labeled cells and immunohistochemical study, the organs from 4 rats were frozen in liquid nitrogen, cryosections were prepared, nuclei were post-stained with Karachi hematoxylin, and examined under a fluorescent microscope.

Immunohistochemical study. For immunohistochemical study, monoclonal antibodies (MAB, Abcam) to CD68 (membrane marker of macrophages), Ki67 (nuclear proliferation marker), and Fapα (membrane marker of reactive fibroblasts) were used. After deparaffinization, the sections were successively treated with citrate buffer (pH 6.0 for 30 min at T≈100°C), endogenous peroxidase blocker, and 10% BSA. Incubation with primary antibodies in dilution recommended by the manufacturer was carried out for 24 h at T≈4°C. Biotin–streptavidin–peroxidase complex was used as the visualization system (20-min incubation). The nuclei were poststained with Karachi hematoxylin.

Morphometric study. Morphometry was performed on histological preparations stained with picrosirius red. For evaluation of reverse remodeling of LV, the following parameters were measured: cicatricial tissue area, LV wall thickness in the scar zone, LV wall thickness in the perifocal area, area of LV cavity, total LV area, and area of LV wall. From these parameters, the following indexes were calculated: scar size (ratio of cicatricial tissue area to LV wall area×100), index of LV dilatation (ratio of LV cavity area to total LV area×100), and index of wall thickness in the scar area (ratio of LV wall thicknesses in the scar to that in the perifocal area×100). Angiogenesis was evaluated by the number of blood vessels in the field of view and their volume density (ratio of the area of all blood vessels to the area of field of view).

Statistical analysis. The means and standard errors of the mean for the absolute and relative (%) parameters were calculated. Absolute values were compared using Student's test (in case of their normal distribution) or using Mann–Whitney test (if the distribution did not conform the normal law). Parameters expressed in percents were compared using one-way dispersion Kruskal–Wallis test. Statistical processing of the results was performed using SigmaPlot 11.0 software. The differences were significant at 5% significance level.

RESULTS

We previously showed that MNC after their intracoronary administration migrate into the myocardium and spleen and were present there for at least 1 month [2]. Similar results were obtained during comparison of cell distribution after intracoronary and intravenous administration [13,14]. In the myocardium, the transplanted cells were detected only in the cicatricial tissue. These findings suggest that the method of transventricular intracoronary cell transplantation developed by us is effective and ensures cell delivery to the damaged zone. Localization of labeled cells in the cicatricial zone attests to their homing into the damaged zone despite equal distribution into the right and left coronary artery during nonselective administration.

The absence of inflammation and the presence of labeled cells in the cicatricial zone for 1 month without changes in their localization and morphology attest to the absence of transplant rejection reaction and cell elimination. Staining with MAB to CD68 revealed the presence of macrophages in the cicatricial area in both the experimental and control groups (Fig. 1). Solitary labeled cells were stained with MAB to CD68, which probably suggested that they either were phagocytozed, or belonged to myeloid cells. These findings suggest that transplanted cells did not induce more pronounced macrophage infiltration of the cicatricial tissue compared to the control group.

In contrast to some reports [4,12,13], no evidence of differentiation of the transplanted cells into cardiomyocytes or vascular wall cells was obtained in our study. The transplanted labeled cells were detected only in the cicatricial tissue: none cells were detected in normal and perifocal myocardium. The absence of fluorescent label in cardiomyocytes and vascular endothelial and smooth muscle cells suggest that transplanted cells do not fuse and do not differentiate into cardiomyocytes and vascular wall cells. The labeled cells had a fibroblast-like phenotype and were surrounded by boundless of collagen fibers and seem to differentiate into fibroblasts. To confirm this assumption, we carried out an immunohistochemical analysis

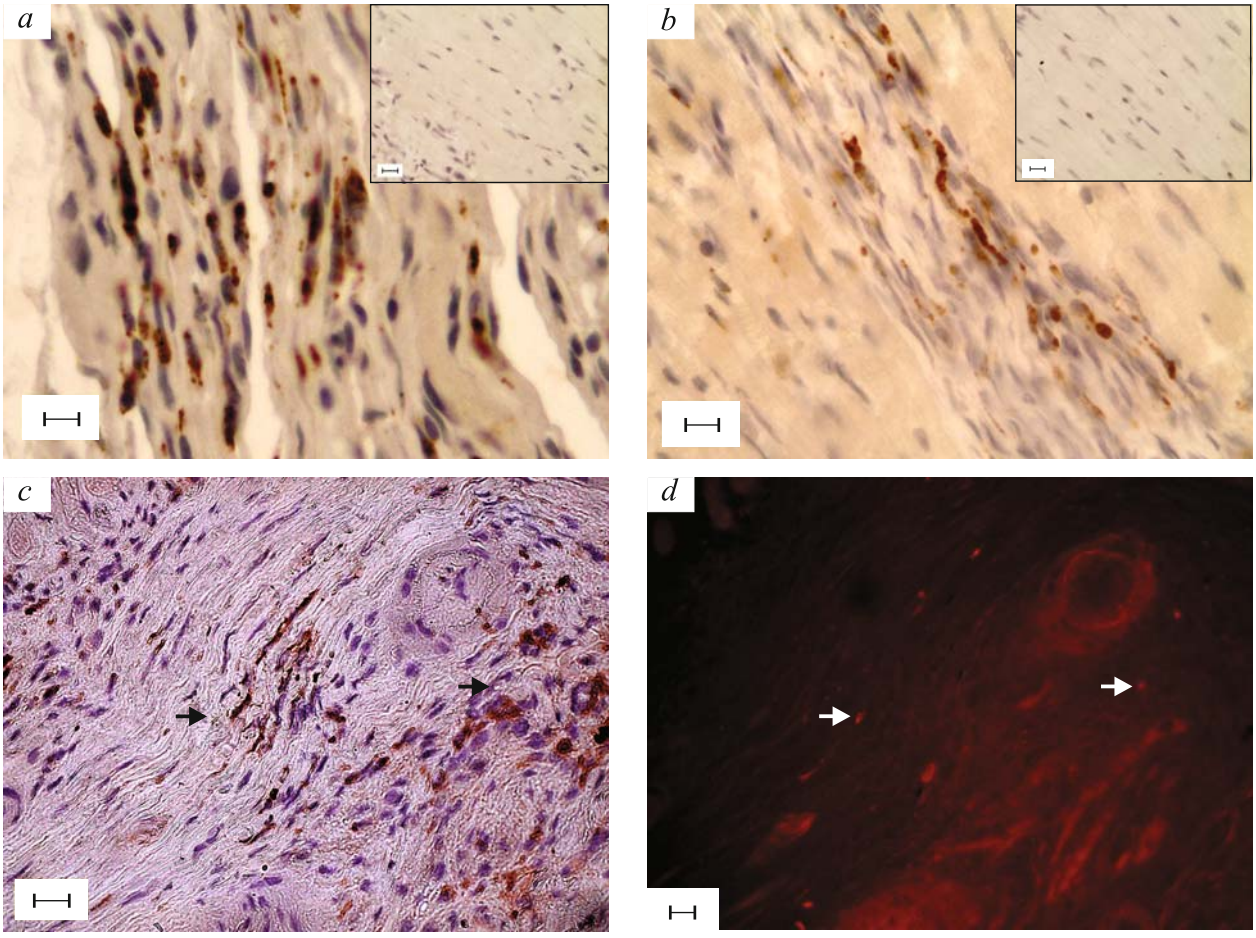

Fig. 1. Immunohistochemical staining with MAB to CD68, ×40. *a*) cicatricial tissue 2 weeks after injection of MNC, insert: control without primary MAB; *b*) cicatricial tissue after 2 weeks in the control group, insert: control without primary MAB; *c, d*) comparison of labeled MNC (*d*) and cells stained with MAB to CD68 (*c*). Arrows: coincidence of labeled cells and cells stained with MAB to CD68. Scale: 10 μ.

for the presence of reactive fibroblast marker Fapα [5]. Fapα is α-subunit of membrane gelatinase, a membrane of serine proteinase family. Reactive fibroblasts appear during wound healing, formation of the granulation tissue, and some sarcomas and actively express Fapα homodimers. In our experiment, numerous Fapα-positive cells were detected in the cicatricial tissue in both the experimental and control groups. These cells correspond to active fibroblasts synthesizing collagen and actively participating in scar formation. The great part of labeled cells was stained with MAB to Fapα, which attests to differentiation of some transplanted cells into reactive fibroblasts participating in scar formation (Fig. 2).

In the cicatricial tissue, about 85% fibrillar extracellular matter is formed by type I collagen forming thick collagen bundles and 11% collagen fibers are formed by type III collagen organized in thin bundles. During scar formation, the total amount of collagen fibers and the collagen I/collagen III ratio increased, which can be considered as a marker of maturity of the cicatricial tissue [3]. Examination of histological preparations stained with picrosirius red in polarized light showed red thick (type I) and green thin (type III) collagen bundles [8]. The proportion between red and green collagen fibers was considered as morphological marker of cicatricial tissue maturity. Comparison of the thickness of collagen fibers in scars in the experimental and control groups showed that the relative content of mature collagen on day 14 was higher in the experimental group (p=0.003; Fig. 3). On day 30 this parameter became similar in the experimental and control group, while the relative amount of thick bundles of collagen fibers in the control corresponded to that in the experimental group (p=0.767). Thus, transplanted cells accelerated scar maturation.

In the experimental group, cell density in the cicatricial tissue was significantly ($p \leq 0.001$) higher than in the control (78.8±2.8 *vs*. 54.7±1.8 cells per field of view 2 weeks after transplantation and 74.4±1.3 *vs*. 51.6±1.3 cells per field of view 4 weeks after transplantation). On day 14, transplanted cells predomi-

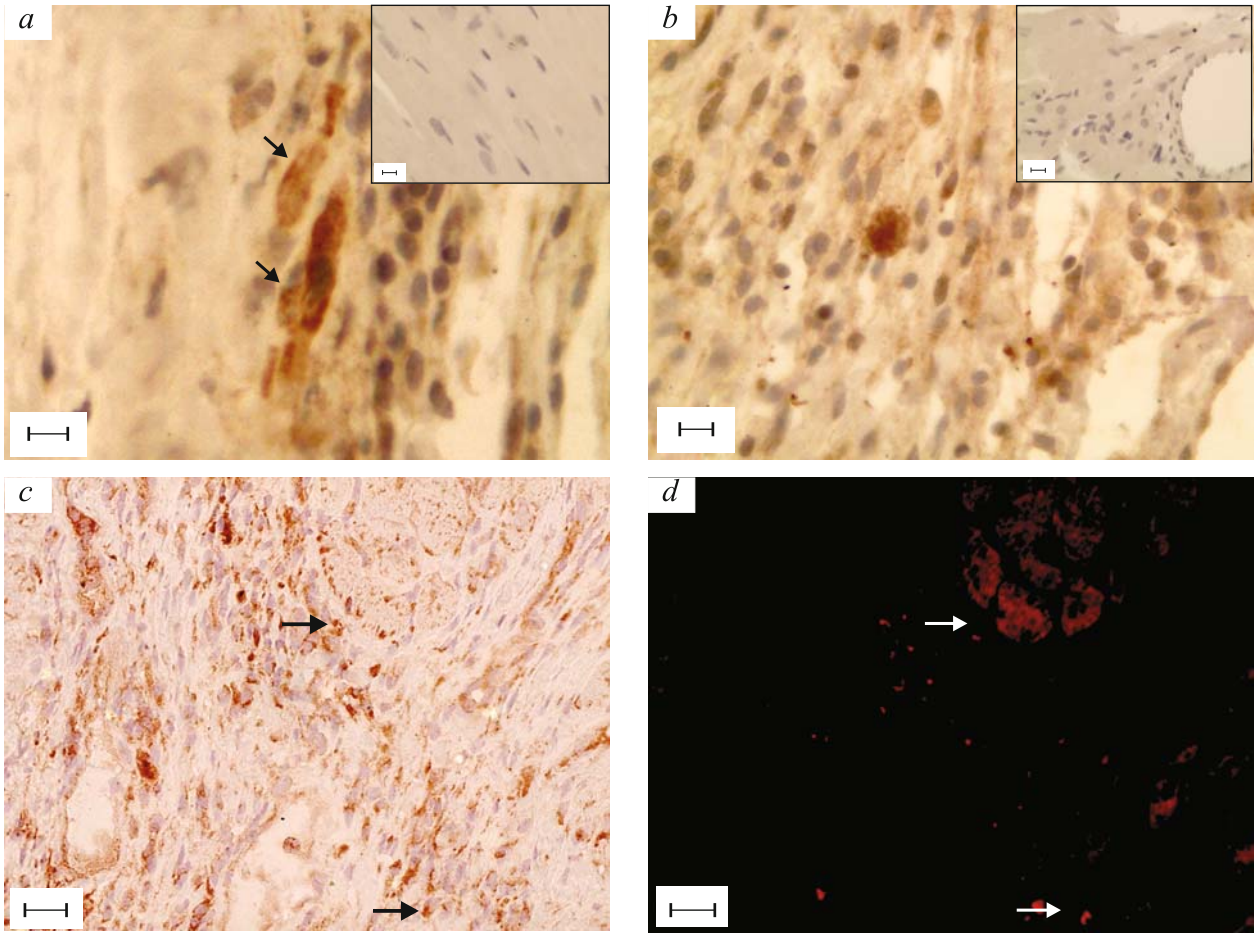

Fig. 2. Immunohistochemical staining with MAB to Fapα, ×40. *a*) cicatricial tissue 2 weeks after injection of MNC, insert: control without primary MAB. Arrows: cells stained with MAB to Fapα; *b*) cicatricial tissue after 2 weeks in the control group, insert: control without primary MAB; *c, d*) comparison of labeled MNC (*d*) and cells stained with MAB to Fapα (*c*). Arrows: coincidence of labeled cells and cells stained with MAB to Fapα. Scale: 10 μ.

nated (55.5±3.2 per field of view), but on day 30 the percent of labeled cells decreased (34.5±2.4 per field of view). Thus, high cellularity and maturity of the scar in the experimental group are largely determined by transplanted cells capable of synthesizing collagen or stimulating its synthesis by other cells, which leads to thickening and strengthening of LV wall in the scar zone.

Staining with MAB to proliferation marker Ki67 revealed numerous proliferating cells in the scar, epicardium, and vascular walls in both the control and experimental group (Fig. 4). In perifocal and normal myocardium, only non-muscular cells in the perimysium were Ki67-positive. In the scar, mainly transplanted cell proliferated, which was confirmed by higher cellularity of the cicatricial tissue after transplantation and high relative content of labeled cells among all cells in the scar in the experimental group.

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 did not differ in the control and experimental groups (p=0.244), but volume density of blood vessels was significantly higher (p≤0.001) in the experimental group, which attested to greater caliber of newly formed vessels after MNC transplantation. On day 30, both the number (p≤0.001) and volume density (p=0.004) of vessels in

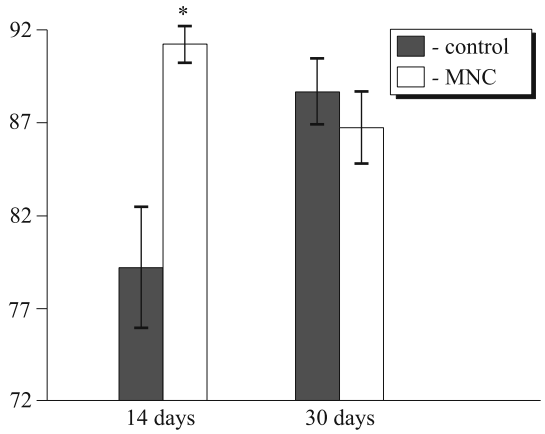

Fig. 3. Proportion between thick and thin collagen bundles in the cicatricial area 2 and 4 weeks after transplantation.

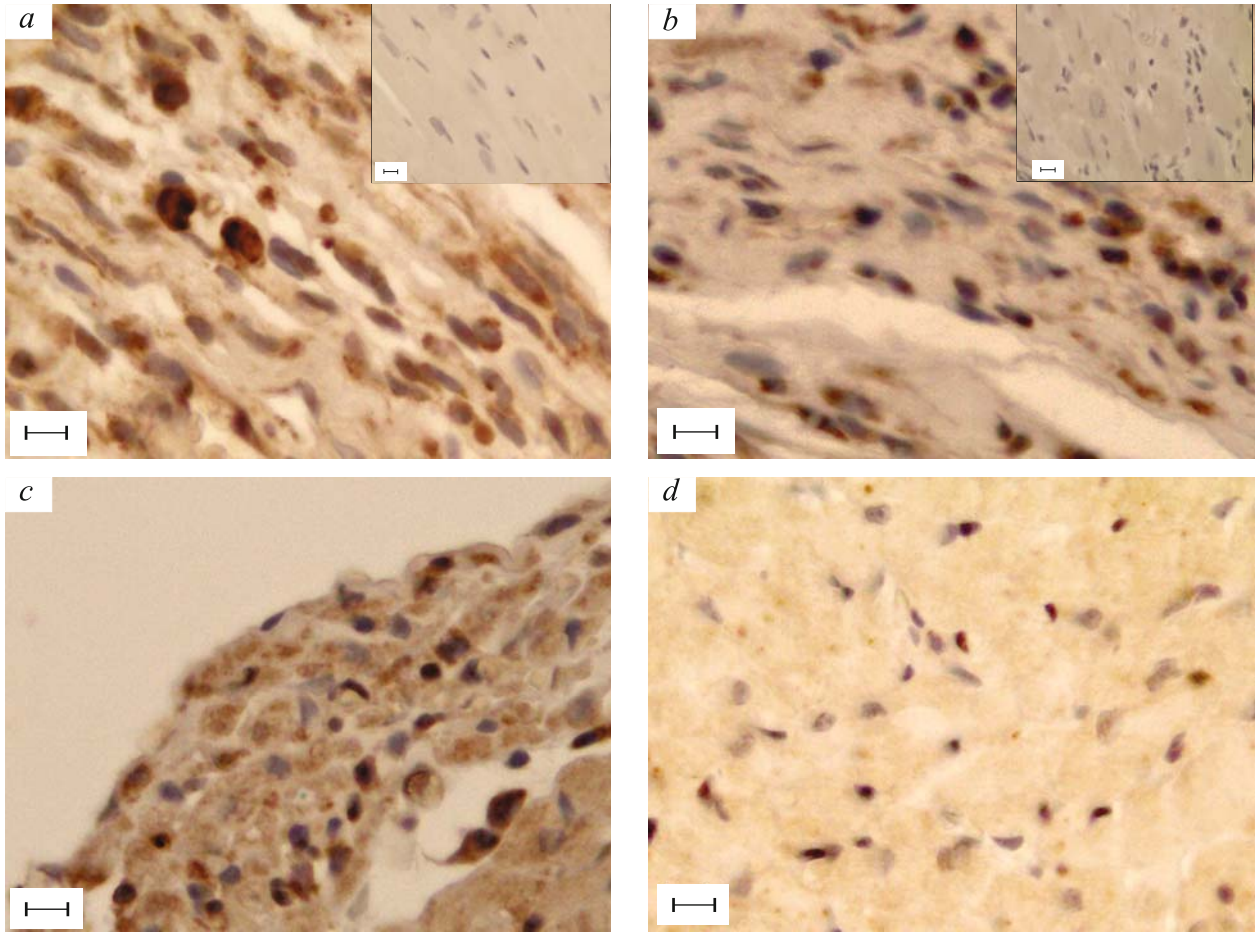

Fig. 4. Immunohistochemical staining with MAB to Ki67, ×40. *a*) cicatricial tissue 2 weeks after injection of MNC, insert: control without primary MAB; *b*) cicatricial tissue after 2 weeks in the control group, insert: control without primary MAB; *c*) epicardium in the experimental group; *d*) normal myocardium in the experimental group. Scale: 10 μ.

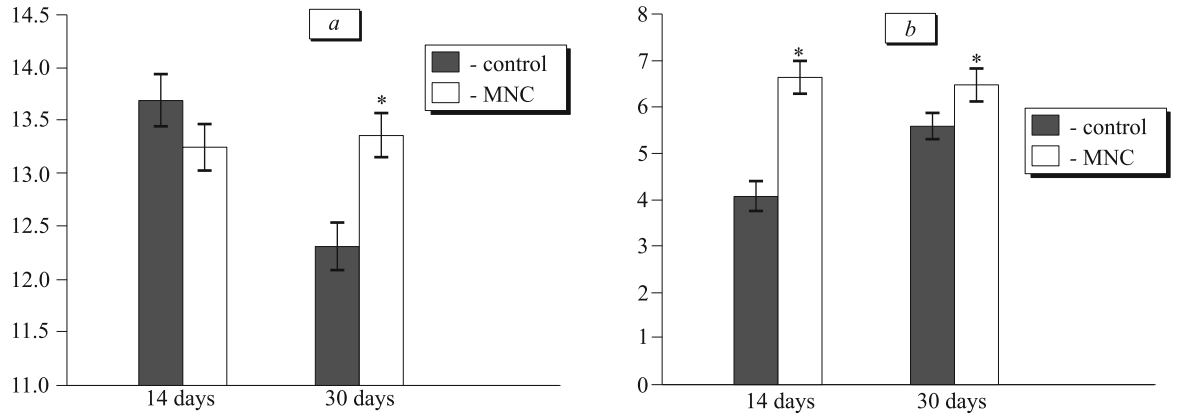

Fig. 5. Blood vessels 2 and 4 weeks after transplantation (total area of the field of view 786 432 pixels). *a*) number; *b*) volume density. Here and on Fig. 6: *$p \leq 0.05$ compared to the control.

the experimental group was higher than in the control (Fig. 5).

Morphological analysis revealed no signs of reverse remodeling of LV after cell transplantation (scar size and index of dilatation remained unchanged, Table 1). However, changes in the thickness of LV wall in the scar zone were found. After 2 and 4 weeks, the thickness of LV wall in the experimental group was higher than in the control ($p \leq 0.05$). This can be determined by higher cellularity and maturity of the cicatricial tissue in the experimental group compared to the control. Changes in the linear size (shortening) of the scar without changes in its area, but with wall thickening cannot also be excluded.

Despite the absence of reverse remodeling, such functional parameters of cardiac activity as mean systemic pressure, maximum LV pressure, and index of contractility increased more rapidly after cell transplantation (Fig. 6).

Two main mechanisms of participation of stem/progenitor cells in myocardial reparation are discussed in published reports. Some investigators reported a replacement effect of transplantation leading to replacement of the cicatricial tissue by newly formed contractile elements. The evidence presented by these authors is based on incorporation of the label present in transplanted cells into cardiomyocytes or blood vessel cells, which is not always convincingly demonstrated and can attest to cell fusion [18]. Now, more and more authors incline towards the induction mechanism of stimulation of angiogenesis and reparative processes [17].

In our study we did not observe differentiation of transplanted cells into cardiomyocytes or blood vessel cells. Moreover, the absence of fluorescent label in cardiomyocytes and vascular endothelial and smooth muscle cells suggests that transplanted cells do not fuse with cardiomyocytes and vascular wall cells. Some BM MNC (probably fibroblasts or their precursors) migrated into the scar and were detected only there; they differentiated into fibroblasts, proliferate, and actively synthesized extracellular matrix components of the cicatricial tissue. This led to thickening and consolidation of the cicatricial wall, but the area of the scar did not change, and this was most likely due to contraction of the scar caused by myofibroblast activity.

The cicatricial tissue is not an inert structure, as it was believed earlier, its active reorganization is going on even 6-8 weeks after acute MI. The key role in scar remodeling is played by fibroblasts and myofibroblasts. The latter apart from the synthesis of extracellular matrix is capable of contraction. Insufficient production and progressive degradation of the

TABLE 1. Size of Scar, Index of LV Dilatation, and Wall Thickness in the Scar Zone 2 and 4 Weeks after Transplantation

Parameter	Solvent		Cell transplant	
	14 days after injection	30 days after injection	14 days after injection	30 days after injection
Scar size, %	7.1±4.1	7.1±3.3	5.8±3.3	9.7±3.3
Index of dilatation, %	23.4±6.7	28.4±5.9	20.3±5.7	33.4±6.1
Wall thickness in the zone of scar, %	37.2±5.1	26.5±3.1	48.9±4.7	29.5±3.1

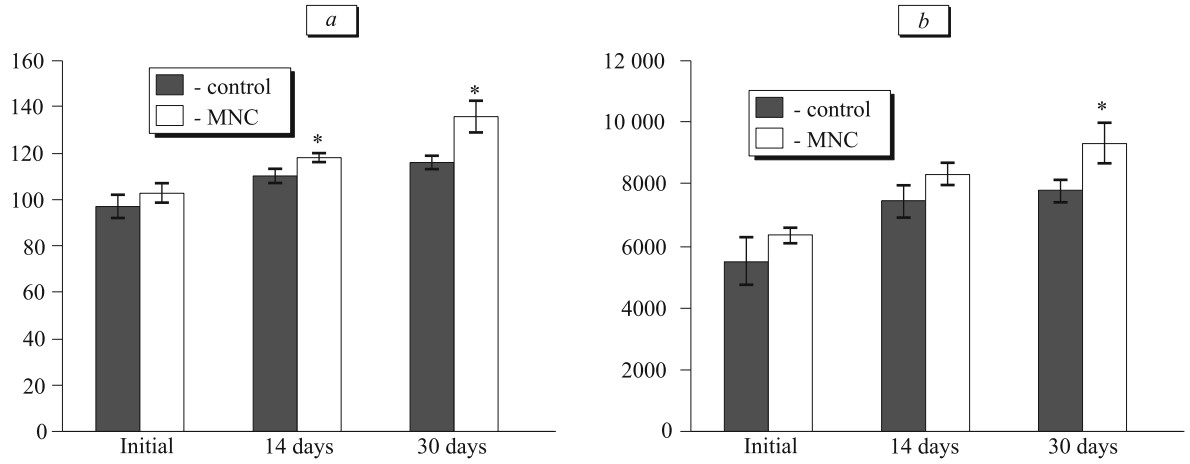

Fig. 6. Left ventricle. *a*) dynamics of maximum pressure; *b*) dynamics of contractility index.

extracellular collagen matrix in the infarction zone are now believed to be the causes of pathological remodeling of LV and heart failure after MI [14]. These changes can lead to the formation and rupture of an aneurism and patient's death. Therefore, stimulation of fibroplastic processes in the infarction zone aimed at thickening and strengthening of the scar and prevention or deceleration of pathological remodeling is a promising trend in anti-remodeling therapy. Thickening of LV wall is thought to reduce its strain according to Laplace law and decreases systolic dilatation of LV [15]. Our experiments showed that MNC transplantation can be one of these methods.

Moreover, transplanted cells potentiated *de novo* formation of blood vessels in the scar, but do not directly participate in their formation. None labeled transplanted cells were detected in the vascular walls. It is most likely that stimulation of angiogenesis is determined by induction (paracrine) mechanisms and release of angiogenic factors. Participation of red BM cells in angiogenesis is related to secretion of angiogenic factors VEGF, bFGF, HGF, TGF-β, angiopoietin-1, *etc*. by these cells [6].

Transplantation of MNC improved global contractility of LV, which manifested in elevation of systemic blood pressure, maximum LV pressure, and index of contractility (14 and 30 days after cell transplantation); these shifts were not observed in animals receiving physiological saline. However, no morphometric signs of reverse LV remodeling were revealed, therefore we can hypothesize that improvement of cardiac function is determined by induction mechanisms and strengthening of the scar wall.

The drawback of our study is that we obtained only indirect evidence of differentiation of transplanted MNC into fibroblasts. The problem consists in the absence of specific immunophenotypical markers of fibroblasts; therefore we consider cells as fibroblasts on the basis of their morphological characteristics, localization, and staining with MAB to Fapα. It remains unclear, which cell population among nonfractionated mononuclears migrated into the scar and differentiated into fibroblasts. In our experiments, nether quantitative nor qualitative characteristics of induction (paracrine) factors stimulating angiogenesis and reparation, which are known to play the key role in therapeutic activity of MNC, were obtained. These problems are the subjects of further researches.

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