Cardiomyocyte Regeneration in Pericicatricial Zone of the Myocardium after Laser Tunneling and Implantation of Bone Marrow Mononuclear Cells

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Using canine model of chronic postinfarction myocardial ischemia we evaluated angiogenesis and cardiomyogenesis after combined revascularization by laser tunneling of the pericicatricial zone followed by implantation of bone marrow mononuclear cells into the channels. It was shown that the numerical density of arterioles and capillaries considerably increased 1 month after revascularization. We also observed a considerable increase in the density of cardiomyocyte nuclei in the test area, a 2-fold increase in the total area of nuclei, and an increase in the mean area of cardiomyocyte nuclei. The number of PNCA-positive cardiomyocytes significantly increased after combined revascularization. Our findings suggest that combined revascularization of the pericicatricial zone of the myocardium promoted angiogenesis and stimulates regeneration of cardiomyocytes.

Key Words: *revascularization of the myocardium; microcirculation; regeneration of cardiomyocytes*

The development of approaches to stimulation of regeneration in the myocardium, especially in large-focal myocardial damage, is an important problem of modern medicine. In light of this, evaluation of regeneration potential of cardiomyocytes (CMC) and other cell populations of the myocardium, and improvement of the methods of evaluation of their proliferative activity is of crucial importance. It has long been accepted that CMC of adult mammals lose proliferation capacity (hyperplastic regeneration) soon after birth and then are capable of intracellular regeneration only. Experimental and clinical morphological studies of the last decade demonstrated the possibility of cellular forms of CMC regeneration at different periods of ontogeny and after myocardial damage of different genesis [4,6]. After diffuse or focal apoptotic and necrotic death of CMC their number can be restored at the expense of either proliferation of resident cardiac stem cells and non-terminally differentiated CMC, or transdifferentiation of cells originating from the bone marrow into CMC [7,8,15].

It was shown that the use of cell technologies for regeneration of the myocardium in the postinfarction period led to the appearance of newly-formed CMC in the cicatrix and pericicatricial zone. However, many investigators consider that implantation of mononuclear bone marrow cells to patients with chronic coronary heart disease can improve perfusion of the myocardium, but does not practically affect the size of the cicatrix, *i.e.* does not considerably stimulate CMC regeneration. This substantiates the need in more

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effective approaches to stimulation of cardiomyogenesis and more informative criteria for evaluation of regeneration reactions of CMC.

Here we analyzed reparative processes in CMC of the pericicatricial zone of the myocardium in dogs under conditions of changed microcirculation after laser revascularization with implantation of bone marrow mononuclear cells (BMMC) into laser channels.

MATERIALS AND METHODS

The study was performed on 14 mongrel dogs weighing 11-14 kg. Myocardial infarction was modeled by ligation of the anterior interventriocular coronary artery and collateral branches of the first diagonal artery under conditions of intubation narcosis. After 3 months, combined revascularization of the pericicatricial zone of the myocardium was performed in 9 animals. To this end, 5×10^6 autologous BMMC were implanted into 8-12 laser channels [1,3,5]. For prevention of ectopic ossification, BMMC were separated on plastic for 30 min [2,3]. The animals were sacrificed 1 month after combined revascularization. Five animals with postinfarction cicatrix served as the control. Ischemia exposure time was 3 months.

Myocardial samples from the left ventricle were obtained for morphological examination. The density of arterioles was evaluated by the positive reaction to alkaline phosphatase. For detection of alkaline phosphatase the following medium was used: 25 mg naphthol SA-BI phosphate in 0.5 ml N,N-dimethylformamide, 50 ml 0.1 M tris-HCl buffer, pH 9.2. The reaction was evaluated visually only of cross-sections of vessels.

For evaluation of microcirculatory bed, the sections were stained with isolectin GS-IB_4 labeled with AlexaFluor 594 fluorochrome (Invitrogen Lot. 404250, Fig. 1) diluted 1:100 in 0.1 M phosphate buffer using a Multi-Shaker PSU-20 for 11 h at room temperature. When counting capillaries in the myocardium we counted the fibers with cross profile, which were included into the analyzed area during their selection. Images were analyzed using a hardware-software complex on the basis of an Axioskop FL-40 microscope with an AxioCam MRc camera (Carl Zeiss) and AxioVision 3.1 software with manual probing modules. A MultiChannel module, Plan-Neofluar objective (×40), final magnification 460, filter set 00 BR 530-585, FT 600, LP615, and filter set 09 BR 450-490, FT 510, LP515 (Carl Zeiss) were used for image acquisition.

Fluorescent karyometry of the myocardium was performed (Fig. 2). The sections were stained with ethidium bromide (10 μg/ml aqueous solution) for 9 min at room temperature. The following parameters were analyzed: numerical density of CMC nuclei in the test area, integral area of nuclei in the test area (in μ^2), area of nuclei in the test area of myocardium (in %), area of one nucleus (in μ^2), "ideal" nucleus diameter based on the mean area of the myocardium (in μ^2). For the studied group, the densitometric maximum of CMC nuclei and densitometric minimum were similar. Morphometry was performed at ×630, the test area was 39437 μ^2.

The images were acquired and analyzed using an M200 microscope with AxioCam HRc camera (Carl Zeiss), AxioVision 4.7 software, and program module for automatic measurements; the module was programmed with activation of Sigma, Threshold, Size, Sharpening, and Segmentation mask filters (for inclusion or exclusion of objects during the analysis). We used single-phase regimen and a Neofluar objective (×63) with filter set 00 BR 530-585, FT600, LP615.

Immunohistochemical reaction for detection of proliferating cell nuclear antigen (PCNA) was performed using a double-staining technique. Primary antibodies were diluted 1:90 (PCNA, Rb. pAb, Lot.473985, ab. 2426-1); the sections were incubated overnight at 4°C. After washout, the sections were stained with second antibodies (FITC-labeled Goat pAb to Rb IgG, Lot.459401, ab. 6717-1) diluted 1:200 for 2 h at room temperature using an orbital shaker. After washout, the nuclei were stained with DAPI (Sigma, 50 μg/ml, aqueous solution) for 30 min. Two-channel images were obtained using Multichenal Zeiss program module with filter set 38 BR470-40, FT495, BR525-50 for FITC channel and filter set 49 G 365, FT395, BR 445-50 for DAPI channel, Neofluar objective (×63), and filter set 00 BR 530-585, FT600, LP615.

The data were processed statistically by ANOVA tests at $p<0.05$ using Microsoft Excel and OriginPro 7.0 software.

RESULTS

Three months after modeling myocardial infarction in dogs, a large-focal transmural cardiosclerosis developed distally to the site of coronary artery ligation (usually, extensive cicatricial fields). The extensive connective tissue cicatrices were characterized by devastation of the vascular bed (large arteries, veins, arterioles, venules were devastated). Remodeling of the vascular bed observed in the myocardium during the postinfarction period consisted in recalibration and/or formation of multibarrelled vessels. Changes in the vascular bed outside the cicatricial tissue were also characterized by the appearance of locking-type vessels. Replacement of muscle fibers with the adipose tissue growing primarily from the subepicardial to the medium layer of the myocardium was often seen in the postinfarction myocardium. Pronounced edema, the presence of focal lymphocyte infiltrates in the cicatri-

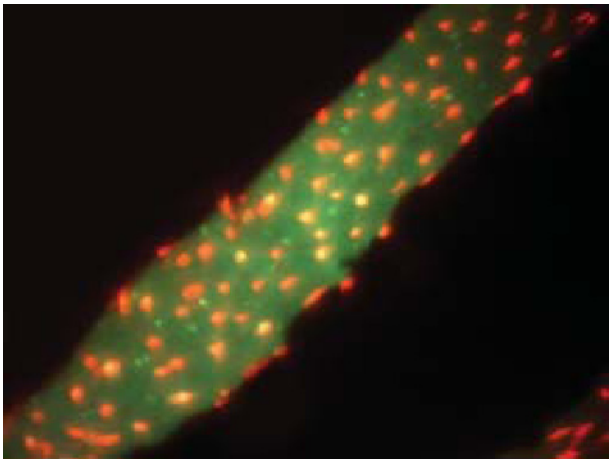


Fig. 1. Fluorescent label Alex-flu for isolectin B4; capillaries of the myocardium are stained red-orange. Contrast: autofluorescence (×460).

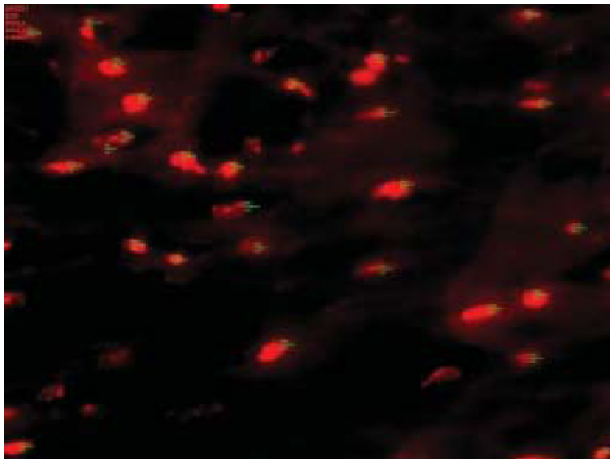
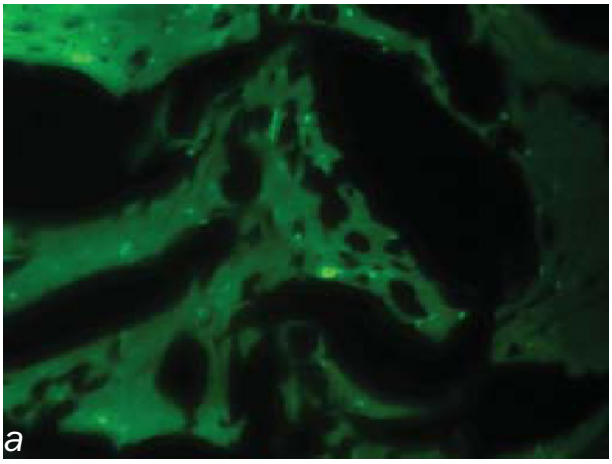
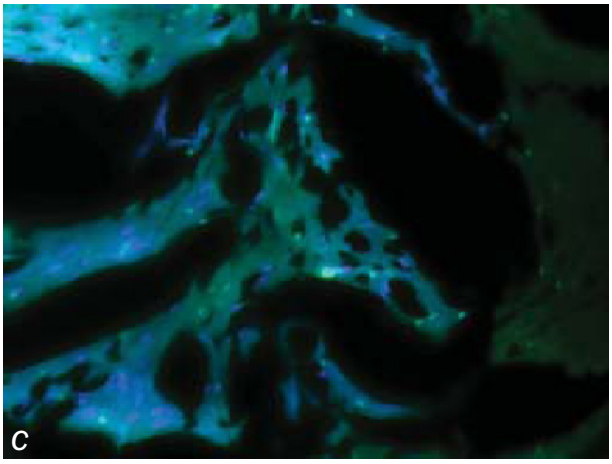


Fig. 2. Ethidium bromide staining of the myocardium. Densitometric parameters are shown on nuclei (×630).





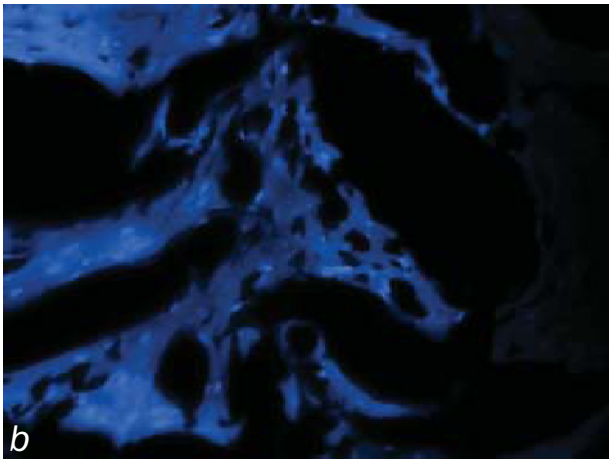


Fig. 3. Diploid PCNA-positive CMC nucleus (×630). *a*) FITC channel; *b*) DAPI channel; *c*) two-channel image.

cial zone and residual myocardium, and hypertrophy of some muscle fibers were important pathomorphological characteristics of postinfarction myocardium.

One month after combined revascularization of the pericicatricial (periinfarction) zone of the myocardium, the mean numerical density of arterioles in this area increased by 30% (124.0±4.7/mm^2 *vs*. 95.0±0.4/mm^2 in the control group, $p<0.05$; Table 1). Our data on increased numerical density of arterioles after combined revascularization agree with the results of other studies, where transmyocardial laser revascularization was performed in large animals [9,10]. The mean

TABLE 1. Morphometric Characteristics of Vessels and CMC Nuclei in Pericicatricial Zone of the Myocardium (*M*±*m*)

Parameter	Control (chronic coronary heart disease)	Combined revascularization (laser tunneling followed by implantation of BMMC)
Numerical density of arterioles, per 1 mm^2	95.0±0.4	124.0±4.7*
Numerical density of capillaries, per 1 mm^2	2144.0±93.6	2938.0±68.8*
Total area of CMC nuclei in the test area, μ^2	465.6±37.1	972.3±98.2*
Relative area of CMC nuclei, %	1.180±0.094	2.190±0.122*
Numerical density of CMC nuclei in the test area	22.00±1.52	29.70±1.32*
Numerical density of PCNA-positive CMC nuclei in the test area	3.66±0.23	5.83±0.36*
Area of CMC nucleus, μ^2	21.00±0.38	32.60±0.64*
"Ideal" diameter of CMC nucleus, μ	5.02±0.04	6.04±0.04*

Note. *p<0.05 compared to control group.

numerical density of capillaries in the pericicatricial zone after combined revascularization increased by 37% (from 2144.0±93.6/mm^2 in the control group to 2938.0±68.8/mm^2; p<0.05). Similar, but less pronounced increase in capillary density (by 25%) was observed in swine with experimental chronic myocardial ischemia 12 weeks after transplantation of BMMC into the myocardium (1132±69/mm^2 *vs.* 903±44/mm^2 in the control) [14].

Activation of neoangiogenesis after combined revascularization of the pericicatricial zone of the myocardium was accompanied by intensification of regenerative processes in CMC. In the myocardium of dogs subjected to transmyocardial laser revascularization and implantation of BMMC into laser channel, the total area of CMC nuclei increased practically 2-fold, the relative area of CMC nuclei and their numerical density increased by 86 and 35%, respectively (Table 1). Moreover, CMC nuclei increased in size, in particular, the mean "ideal" diameter and the mean area of CMC nuclei increased after combined revascularization by 20 and 25%, respectively.

The increase in the numerical density of CMC nuclei and their size in the group with combined revascularization probably attests to realization of cellular and intracellular forms of CMC regeneration under these conditions. The increase in numerical density of PCNA-positive CMC nuclei in the pericicatricial zone compared to the control (by 59%) confirms enhanced proliferative activity of CMC (Table 1). A considerable number of PCNA-positive CMC were diploid (Fig. 3). These findings suggest that regeneration of the myocardium in the pericicatricial zone is primarily realized via the formation of mono- and binucleated diploid CMC and to a lesser extent via the formation of polyploid cells. According to the reports of some investigators studying different variants of cardiac pathology in experiments, the expression of PCNA related to the level of DNA synthesis and enlargement of CMC nuclei positively correlate with systolic function of the heart and can negatively correlate with diastolic function [11,12].

The increase in numerical density of CMC nuclei can also be determined by reduced apoptotic death of CMC after combined revascularization of the pericicatricial zone. Activation of antiapoptotic mechanisms and stimulation CMC regeneration in the pericicatricial zone after cell therapy are most likely determined by paracrine effects of implanted cells [13].

Thus, our experiments demonstrated the possibility of stimulating cellular forms of CMC regeneration (including proliferative activity) in the pericicatricial zone after combined revascularization (laser tunneling of the left ventricular wall followed by implantation of BMMC into the channels). These findings suggest that restoration of the vascular bed is an obligatory condition of activation of CMC regeneration.

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