Morphological and Molecular Analysis of Angiogenesis after Intramyocardial Transplantation of Autologous Bone Marrow Mononuclear Cells

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We studied the peculiarities of angiogenesis in the postinfarction period after transmyocardial laser revascularization and intramyocardial implantation of mononuclear bone marrow cells into the pericicatrical zone of the left ventricular myocardium in dogs. Morphological manifestation of angiogenesis in the myocardium after application of laser and cell technologies are angiomatosis, formation of large thin-wall vessels and sinusoids. The angiogenic effect of implanted mononuclear bone marrow cells is determined by high content (43-47%) of $CD31^+$ cells in both adherent and nonadherent fractions. More pronounced angiogenic potential of nonadherent cells is determined by intensive expression of cytokine VEGF-B and D mRNA essential for arterial vessels growth. Immunohistochemical studies showed that about 90% cells of the nonadherent fraction are endothelial precursors expressing endothelial cell markers isolectin B_4 and VEGF-R2. It was found that the use of adherent mononuclear bone marrow cells during the postinfarction period induces ossification of the epicardium and subepicardial myocardium layer, formation of cartilage plates, and focal calcification. Implantation of nonadherent mononuclear bone marrow cells into transmyocardial laser channels did not induce ectopic ossification of the myocardium.

Key Words: *ischemia and revascularization of the myocardium; laser and cell technologies; vasoendothelial growth factor*

The use of cell technologies in experiments and clinical practice for stimulation of neoangio- and vasculogenesis in ischemic zones of the myocardium demonstrated high efficiency of this approach. At the same time, different efficiency of angio- and cardiomyogenesis after application of different cell population (bone marrow stem cells, endothelial precursor cells, *etc.*) and different methods of their delivery to the site of damage substantiate the necessity of further evaluation of molecular characteristics of cells used for implantation. For instance, many investigators in their attempts to regulate parameters of angiovasculogenesis pay mach attention to evaluation of molecular characteristics of various cell populations of the bone marrow (BM), *e.g.* obtained by their fractionation. There are numerous reports on different angiogenic efficiency of various fractions of mononuclear cells (MNC) of BM, their combinations, genetic modifications, and methods of their delivery to the ischemic area. Implantation of cells into blind laser transmyo-

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cardial channels is one of the most promising methods of their delivery to the focus of damage [3,9,12].

An important step in substantiation of the possibility of therapeutic application of MNC for correction of ischemia aftereffects is the study of the molecular mechanisms of possible atypical vasculogenesis and the search for optimal methods of isolation of endothelial precursor cells with required characteristics. Of particular importance is the development of approaches for reducing side effects of cell therapy and for combined application of different methods of revascularization. This dictates the need of studying the peculiarities of reparative regeneration of the myocardium under conditions of chronic ischemia after intramyocardial implantation of various BM MNC populations.

Here we studied the peculiarities of reparative regeneration of the myocardium and molecular mechanisms of angiogenesis in modeled ischemic heart disease and transmyocardial laser revascularization (TMLR) with intramyocardial implantation of BM MNC.

MATERIALS AND METHODS

Ischemic damage to the myocardium was modeled in 12 mongrel male and female dogs (age 3-5 years, body weight 15-20 kg) by ligation of the anterior interventricular artery and collateral branches of the first diagonal artery under conditions of intubation narcosis. During the postoperation period, all animals received analgesics and antibacterial drugs; the formation of myocardium infarction was monitored by ECC. After 3 months, two series of experiments for revascularization of the myocardium were performed: intramyocardial implantation of adherent fraction of BM MNC into the pericicatricial zone (4 animals) and TMLR with implantation of nonadherent fraction of MNC (8 animals).

Laser tunneling was performed using a semiconductor laser IRE-Polus (λ=1.56 μ). Laser radiation with a power 6-8 W was delivered through a quartz lightguide in a continuous mode [1,4]. Eight to twelve blind (not penetrating into the cavity of the left ventricle) oblique laser channels were made in the perifocal zone of postinfarction cicatrix on the anteriolateral wall of the left ventricle.

BM was obtained via puncture the posterior superior iliac spine before repeated surgery. BM MNC were centrifuged in Ficoll-urografin density gradient (1.077 g/ml at 400*g* for 30 min). For isolation of adherent and nonadherent fractions of BM cells, the suspension was washed with buffered physiological saline and incubated for 30 min in culture flasks with RPMI-1640 medium. Nonadherent cells were collected and centrifuged in buffered physiological saline. The adherent cells were processed similarly except short-term preincubation with Versen solution with trypsin. Before implantation, nonseparated BM MNC and nonadherent cells were diluted with 2 ml physiological saline to a concentration of 5×10^6 cell/ml. The suspension was implanted into preliminary created laser channels; the external opening was sutured with a previously applied purse-string suture. The remaining cell material was analyzed by immunocytochemical methods and by RT-PCR.

Immunophenotypical characteristics of adherent and nonadherent BM MNC were analyzed on a FACSCalibur flow cytofluorometer Becton Dickinson using a panel of antibodies to CD31/CD34, CD34/CD45, CD73, CD90, and CD105 (Becton Dickinson) according to manufactirer's instructions.

The animals were euthanized 4 weeks after repeated surgery. For microscopic examination, the myocardium of the anteriolateral wall and apex of the left ventricle was taken. Histological sections were prepared on a Microm HM-550 cryostat (Carl Zeiss) and BioVitrum materials. Serial cryostat sections (7 μ) perpendicular to the axis of the transmyocardial channel were stained with hematoxylin and eosin and after van Gieson. Morphological analysis of the sections was performed using hardware-software complexes on the basis of Axioskop FL-40 (AxioCam MRc camera and AxioVision 3.1 software with MultiChannel module) and Axiovert M200 microscopes (AxioCam HRc camera and AxioVision 4.7 software, Carl Zeiss).

For the analysis of the expression of VEGF and SDF-1 gene mRNA in BM MNC, a method of quantitative real-time RT-PCR was developed. To this end, databases of canine RNA and genome sequences were analyzed. On the basis of homology analysis of nucleotide sequences for the chosen genes and sequenced genome fragments, the corresponding canine genes and mRNA were identified. Pairs of primers for these sequences were designed allowing specific amplification of cDNA fragments of VEGF and SDF-1 genes and β-actin gene (standard). Axygen reagents and Bio-Rad equipment (iQ5 Real-Time PCR Detection System) were used for RT-PCR.

For evaluation of "endothelial potential" of nonadherent BM MNC, immunocytochemical analysis of these cells after incubation in differentiation media EndoCult and MesenCult (StemCell Inc.) was performed. Antibodies to markers of immature endothelium (isolectin B_4) and *de-novo* formed arterial vessels (type 2 vasoendothelial growth factor, VEGF-R2) were purchased from Invitrogen.

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

RESULTS

Immunophenotyping of BM MNC fractions by flow cytofluorometry. Analysis of immunophenotypic characteristics of BM MNC revealed predominance of cells with phenotypes $CD34^{-}/45^{+}$ and $CD34^{-}/45^{-}$, the latter are regarded by many authorities as true pluripotent cells. The percent of $CD31^{+}$ cells was also high (43-47%); under conditions of developing ischemia, this phenomenon can attest to compensatory enhancement of MB cell proliferation and their differentiation into endothelial cells. Analysis of cell fractions showed that the relative content of $CD34^{-}/45^{-}$ and $CD34^{-}/45^{+}$ cells was higher in the fraction of nonadherent cells (20.6% and 87.4%, respectively, *vs.* 11.6% and 78.6% in the fraction of adherent cells, $p<0.05$). Both BM MNC fractions contained mesenchymal stem cells (CD73, CD90, CD105), which are precursors of various connective tissue cells and can be responsible for ectopic regeneration after their intramyocardial implantation.

Morphological changes in ischemic myocardium after revascularization with the use of laser and cell technologies. Intramyocardial implantation of adherent fraction of BM MNC induced diffuse ossification of the epicardium and subepicardial layer of the myocardium; diffuse growth of osteal trabeculae oriented primarily towards the epicardium and subepicardial layer [2]. In some cases, cartilage plates were formed and focal calcification not accompanied by the formation of osteal and cartilage structures was observed (Fig. 1, *a*, *b*) In sites of ossification, osteoclast reaction of different intensity was observed (Fig. 1, *c*). The induced reparative regeneration of the myocardium under these conditions was also characterized by focal proliferation of smooth muscle cells (one more morphological symptom). They separate muscle fibers of the myocardium without their invasion.

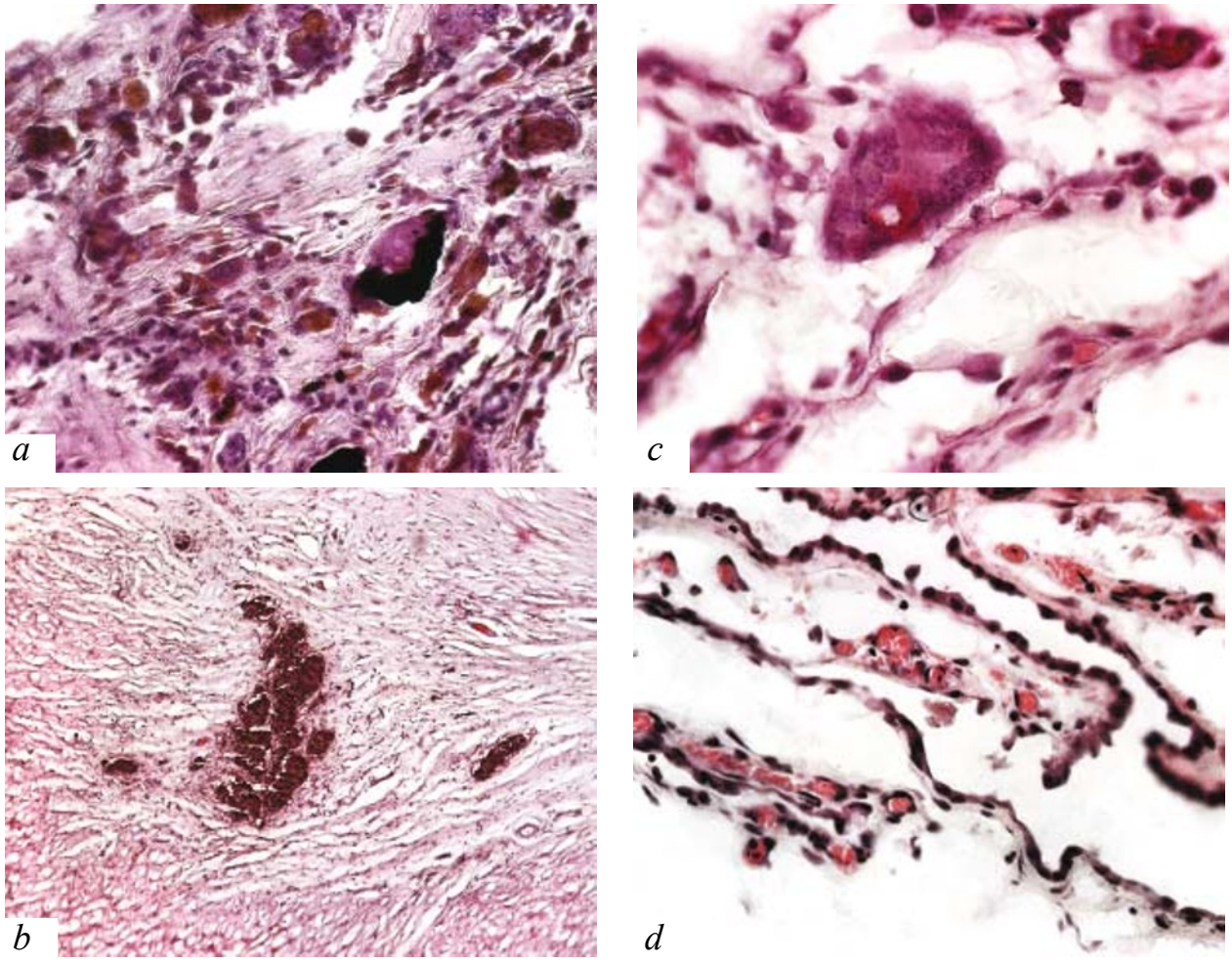

Fig. 1. Angiomatosis and peculiarities of reparative regeneration of the myocardium in intramyocardial implantation of BM MNC. Hematoxylin and eosin staining. *a*) cartilaginous lamellae with partial mineralization (×400); *b*) focal calcification in the subepicardial layer (×100); *c*) multinuclear osteoclast cell surrounding a small bone trabecule (×1000); *d*) angiomatosis, vessels of the sinusoid type, "vessels-in-vessels" formation (×160).

Induced neovasculogenesis in zones of BM MNC implantation manifested in angiomatosis (Fig. 1, *d*). In zones of implantation, diffuse growth of small monomorphic vessels with hyperchromatic endothelium was observed, "vascular buds" were found in these zones, vessel recalibration and "vessel-in-vessel" formation were often seen. The cells forming angiomatosis structures often retain their proliferative activity. Focal blast cell infiltrates were often seen in the zones of BM MNC implantation.

Some peculiarities of neoangiogenesis were noted after combined laser treatment and implantation of nonadherent BM MNC: appearance of zones with high density of neovascularization, formation of large thin-walled vessels and sinuses, and angiomatosis. Intramyocardial angiomatosis was most significant and common morphogenetic event in the postinfarction remodeling of the myocardium under conditions of TMLR with implantation of BM MNC. In these cases, vessels-in-vessels, diffusely located monomorphic vessels, and vascular buds typical of angiomatosis were also observed. Focal accumulations of blast (sometimes multinuclear) cells, often with proliferative activity, were seen in the zones of correction treatment. No complications of intramyocardial implantation of BM NMC (ossification and calcinosis) were seen.

The detection of diffuse ossification of the myocardium after transplantation of adherent BM MNC and atypical angiomatosis after implantation of nonadherent cells dictated the need of special molecular, genetic, and cytological analysis of these fractions. We previously showed that ectopic ossification of the subepicardium after transplantation of adherent BM MNC fraction is determined by intensive expression of genes involved into osteo- and chondrogenesis (aggrecan, lumican, and osteopontin) in these cells [2].

Evaluation of the expression of VEGF family gene mRNA in separated cells of the mononuclear fraction of BM. Quantitative PCR analysis of VEGF family A, B, C, and D gene expression in cells of the supernatants (nonadherent fraction) revealed enhanced expression of mRNA for genes VEGF-D, VEGF-B, and to a lesser extent VEGF-A essential primarily for vasculogenesis of arterial vessels (Fig. 2, *a*). Adherent cells were characterized by enhanced expression of VEGF-C and VEGF-B; products of these genes induce lymphoangiogenesis [10,13]. These findings confirm the necessity of fractionation of BM MNC for directed vasculogenesis. Intramyocardial implantation of nonadherent BM MNC fraction not only reduces the risk of the formation of bone elements in the myocardium, but also increases their angiogenic potential.

Evaluation of the expression of VEGF-A and SDF gene mRNA in ischemic myocardium and in zones of revascularization. Analysis of the effect of implantation of nonadherent BM cells into laser channels on the expression of VEGF-A and SDF-1 gene mRNA in the zones of complex revascularization of the myocardium demonstrated reliably enhanced production of these cytokines. Four weeks after implantation of BM MNC into laser channels, the expression of VEGF-A and SDF-1 gene mRNA in the myocardium increased by 4 and 10 times, respectively (Fig. 2, *b*).

These findings suggest that TMLR with implantation of nonadherent fraction of BM MNC drastically activates the synthesis of VEGF-A and SDF-1, the cytokines responsible for paracrine regulation of mobilization of endothelial progenitor cells from BM into ischemic focus [15], increased permeability of the vascular wall in the ischemic zone, active proliferation

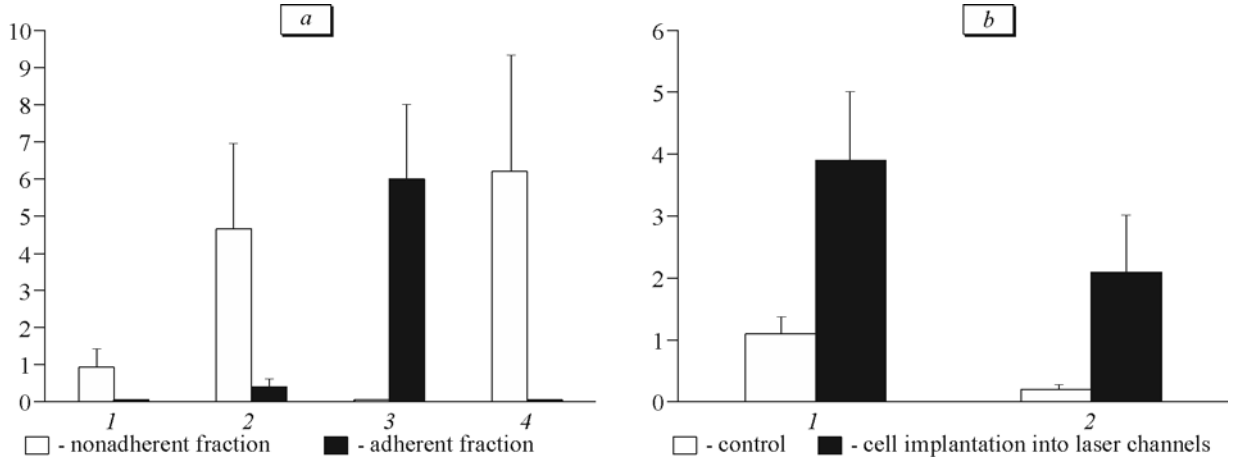

Fig. 2. Expression of vascular endothelial growth factor (VEGF) mRNA and stromal factor (SDF-1) mRNA. *a*) relative expression of mRNA for VEGF-A (*1*), VEGF-B (*2*), VEGF-C (*3*), and VEGF-D (*4*) in cells of nonadherent and adherent fractions of BM MNC; *b*) relative expression of mRNA for VEGF-A (*1*) and SDF-1 (*2*) genes in ischemic myocardium in the zone of implantation of nonadherent BM MNC fraction. The values are presented in arbitrary units ($p<0.05$).

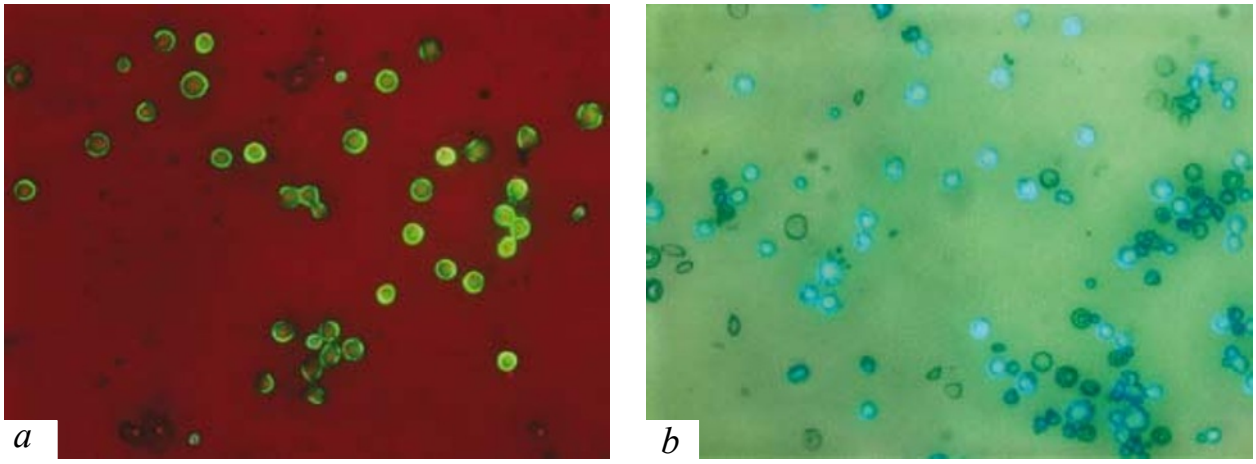

Fig. 3. Immunocytochemical staining of nonadherent fraction of BM MNC after culturing in differentiation media with isolectin-B_4 and FITC-labeled antibodies to VEGF-R2 (×630). *a*) fluorescence channel for FITC-labeled antibodies to VEGF-R2; *b*) colocalization of VEGF-R2 and isolectin B_4; three-channel image. Fluorescence with phase contrast.

and migration of endotheliocytes, formation of tubular structures by these cells [5], and adhesion of precursor cells to endotheliocytes and stromal elements [6].

Analysis of cell populations in differentiation media. Not only mobilization and chemotactic activity of BM MNC, but also their differentiation capacity in the focus of damage is important for induction of neovasculogenesis, a morphological phenomenon of the employed revascularization technologies. In light of this, we studied "endothelial potential" of nonadherent BM MNC, *i.e.* their capacity to transdifferentiate into endotheliocytes. To this end, immunohistochemical study of these cells was performed after incubation in differentiation media followed by staining against markers of immature endothelium (isolectin-B_4) and endothelium of *de novo* formed arterial vessels (VEGF-R2; Fig. 3). The percent of positively stained cells was the same in both groups, which attested to co-localization of these markers (Table 1). Our experiments showed that about 90% cells of the nonadherent fraction belong to endothelial precursors, which was seen from simultaneous detection of isolectin B_4 and VEGF-R2 in these cells.

When discussing the peculiarities of neoangiogenesis, atypical vessel formation, in particular, formation of angiomatous structures and large sinusoid-type vessels (from 300 to 1000 μ) should be noted. The appearance of similar dilated and often tortuous vessels of the sinusoid type was described after injections of recombinant VEGF-A [14]. These sinusoids can be later transformed into smaller vessels due to formation of endothelial bridges or development of vascular wall, which leads to the appearance of large arteries and veins [7].

The formation of similar vascular structures was observed in tumor-associated angiogenesis and (to a lesser extent) in reparative processes in the myocardium and during wound healing [8,11]. The formation of angiomatous structures and large vessels of the sinusoid type is most likely determined by the action of VEGF-A. This assumption agrees with RT-PCR results, which confirmed high level of VEGF-A expression in the myocardium 4 weeks after implantation of nonadherent MNC. This mechanism probably underlies excessive growth of dilated tortuous vessels probably belonging to the system of lymphatic vessels.

TABLE 1. Results of Immunocytochemical Staining of Induced BM MNC Fractions

Culture medium	Isolectin B_4			VEGF-R2		
	total	positive		total	positive	
		abs.	%		abs.	%
EndoCult	510	470	92.1	520	460	88.5
MesenCult	440	410	93.2	420	410	97.6
DMEM/F12	510	460	90.2	500	460	92.0

Thus, intramyocardial implantation of nonadherent BM MNC during the postinfarction period promotes induction of angiogenesis in the pericicatricial zone of the myocardium in dogs. The diffuse growth of vessels in subepicardial layers of the myocardium and angiomatosis observed by us provide the morphological basis for the recorded improvement of cardiac function. Short-term separation of BM MNC on plastic yields cell fractions with different angiogenic and osteogenic potential. Morphological and molecular analysis showed that nonadherent cells are characterized by higher angiogenic potential and lower osteo- and chondrogenic potential than adherent cells.

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