Stimulation of Angiogenesis in Rat Ischemic Limb by Intramuscular Implantation of Mononuclear Fraction Cells from Autologous Bone Marrow

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We studied angiogenic effects of two variants of indirect revascularization (transplantation of mononuclear fraction cells from autologous bone marrow alone or in combination with laser tunneling) after modeled ischemia of the lower limbs in rats. Doppler sonography and immunofluorescence studies showed that intramuscular implantation of isolated fraction of autologous bone marrow mononuclear cells in combination with laser tunneling of the muscles is most effective and can be recommended as a method of angiogenesis stimulation in non-reconstructable distal vascular pathology of the extremities.

Key Words: *ischemia of extremities; revascularization; bone marrow mononuclear fraction; laser tunneling of muscles; immunofluorescence*

Chronic ischemia of the lower extremities (CILE) against the background of atherosclerosis is annually diagnosed in 1.8 men and 0.6 women per 1000 examined individuals at the age of 45-54 years, in 5.1 men and 1.9 women at the age of 55-64 years, and in 6.3 men and 3.8 women at the age of 65-74 years [16]. According to WHO prognosis, the number of patients with this pathology is expected to considerably increase (by 5-7%), while the need in reconstructive surgery will attain 200-300 per one million population [10]. The need in reconstructive surgeries on the arterial system of the lower extremities in Russia in 1998 was 930 per 1 million population; however, no more that 22% surgeries from the required number is annually performed [1].

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Since 2000, about 10,000 surgical interventions for correction of peripheral arterial diseases of the lower extremities are annually performed. The main peculiarity of this pathology is steadily progressing course characterized by aggravation of intermittent claudication to rest pain and/or gangrene, which develops in 15-20% patients [14]. The results of treatment remain unsatisfactory: up to 30% patients die, in 30% above-knee amputations are performed, and in only 40% patients positive results are achieved [13]. These data suggest that despite the wide choice of revascularization methods, treatment of CILE remains an unsolved problem. Compensation of CILE in distal occlusive and stenotic lesions and unsatisfactory results of direct vascularization are still pressing problems. New alternative approaches to blood flow stimulation based on the use of cell and laser technologies and gene therapy are now intensively developed [4,7,12,18,19]. Cell and laser technologies are most widely applied for revascularization of the myocardium and stimulation of car-

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diomyogenesis in ischemic and cytopathic lesions [5-8]. Induction of angiogenesis and myogenesis in skeletal muscles is less studied [15].

We performed a comparative morphological study of the angiogenic effects of different variants of indirect revascularization in experimental lower extremity ischemia.

MATERIALS AND METHODS

The experiments were carried out on Wistar rats. At stage 1, ligation of the femoral artery with collaterals was performed in all animals under ether narcosis [4]. Ischemia was documented by laser Doppler flowmetry using capillary blood flow analyzer (LAKK-01, Lazma).

In series I, cell homing was evaluated in 15 animals receiving an injection of bone marrow mononuclear cells (BM MNC) fractionated by adhesion to plastic into the ischemic extremity on day 25 after femoral artery ligation. BM MNC were isolated from BM aspirate by density gradient centrifugation [3] in a volume of 0.1 ml per injection. Four injections per extremity were performed, each contained from 1.2×10^6 to 1.5×10^6 MNC. Before implantation, BM MNC were labeled with 5-bromodeoxyuridine (BRDU). The relative content of BRDU-positive cells was 9.8% of their total *in vitro* content before injection. The animals were sacrificed after 6, 12, and 24 h (5 animals per term).

In series II (comparison of revascularization variants), the animals were divided into 3 groups. Group 1 animals (n=7) received BM MNC (4 injections into the right ischemic extremity, each injection contained from 1.2×10^6 to 1.5×10^6 MNC) 25 days after ligation of the femoral artery. In group 2 animals (n=8), the cells were implanted after laser tunneling of the muscles on the ischemic extremity as described elsewhere [4]. The laser tunnels were created using an IRE-Polus-1.56 μ semiconductor laser operated in continuous mode using a quartz light guide with a diameter of 600 μ. In group 3 animals (control, n=6), no manipulations were performed after ischemia modeling. The left (contralateral) non-ischemic extremity served as internal control in each animal. The animals were sacrificed by ether overdosage on day 35 after indirect vascularization.

For morphological analysis, the tissue samples from the thigh and shin were fixed in 10% neutral formalin. For light and immunofluorescent microscopy, serial cryostat sections were stained with hematoxylin and eosin and used for immunohistochemical analysis. The initial level of label incorporation was evaluated using anti-BRDU monoclonal antibodies (Sigma) in a working dilution of 1:200, secondary species-specific FITC-labeled antibodies were used in a dilution of 1:50 (Sigma). Fluorescent microscopy was performed on an Axioskop FL 40 using AxioVision 4.7 and Filter Set 09 software. We also used primary monoclonal anti-CD31 antibodies (working dilution 1:100) followed by staining with secondary goat anti-mouse antibodies conjugated with Cy-5 (1:100 working dilution). The total number of capillaries per unit area 1 mm^2 was calculated (Axioskop FL-40 with AxioVision 4.7 and Filter Set 32 software).

Doppler measurements were performed at emission wavelength of 0.63 μ, the time of laser Doppler flowgram recording was 3 min. The amplitude-frequency spectrum of perfusion fluctuations was analyzed using software supplied with LAKK-01 analyzer. During the analysis of Doppler sonogram, the dynamics of microcirculation by the mean blood flow over the recording period (M, perfusion units), mean square deviation (δ), and coefficient of variation (C_v) were evaluated. Coefficient of variation was calculated by the formula: $C_v=\delta/M\times100\%$.

Three frequency ranges of vascular wall modulation were analyzed [17]. Slow oscillations (LF range) are determined by activity of proper microcirculatory bed components. Fast oscillations (HF range) coincide with respiratory rhythm and depend on venous blood flow oscillations caused by changes in thoracic pressure during expiration and inspiration. Cardiorhythms (CF range) correspond to pulse blood flow oscillations. Since the absolute values of oscillation amplitudes in a particular frequency range are not always convenient for practical purposes, in our study we analyzed A_{max}HF and A_{max}CF/A_{max}LF. Index of microcirculation efficiency (IME), an integral parameter characterizing the ratio of mechanisms of active and passive modulation [2], was also evaluated. IME was calculated by the formula: IME=A(LF)+A(VLF)/A(CF)+A(HF), where A are amplitudes of the corresponding rhythms.

Statistical processing of the results was performed using Excel 2007 and Origin 7.5 Pro software.

RESULTS

Light microscopy and immunofluorescent analysis revealed massive cell accumulations in interstitial spaces adjacent to the sites of BM MNC implantation 6, 12, and 24 h postinjection. Thus, the relative content of BRDU-positive cells in cell infiltrates after 6, 12, and 24 h was 9.4, 8.8, and 7.1%, respectively. After 6 h, BRDU-positive BM MNC were present not only in cell conglomerates, but also at a certain distance from the site of injection (Fig. 1).

In experimental series II, the indexes of microcirculation in the shin of right (ischemic) leg in control animals (group 3) approached those in the left (non-ischemic) leg by day 35 after surgery. In the right shin,

parameter δ characterizing blood flow modulation in the microcirculatory bed increased by 56% and IME increased by 32% (Table 1). The increase in C_v reflected improvement of circulation. This improvement can be determined by an increase in vasomotor activity of microvessels, which is seen from elevation of A_{max}LF/M and A_{max}LF by 86 and 89%, respectively, and greater contribution of passive (respiratory) mechanism of capillary blood flow regulation depending on variations of venous blood flow caused by changes in thoracic pressure during expiration and inspiration (increase in A_{max}HF by 85%). All these changes in microcirculation occurred against the background of reduced contribution of the cardiac mechanism of capillary blood flow regulation: A_{max}CF/A_{max}LF and A_{max}CF/δ decreased by 42 and 39%, respectively (Table 1).

In group 1 rats, the index of microcirculation in the right shin on day 25 after BM MNC implantation approached that in the left (non-ischemic) shin, which was similar to the picture observed in the group without correction. The main contribution into the regulation of capillary blood flow in the ischemic extremity was made by the passive (cardiac) mechanism: A_{max}CF/A_{max}LF increased by 2.7 times and A_{max}CF by 88% compared to the left extremity. Compared to the group without correction, myogenic activity of vasomotors decreased (A_{max}LF/M decreased by 46%) and the contribution of cardiac mechanism of capillary blood flow regulation increased (A_{max}CF/A_{max}LF increased by 2.1 times).

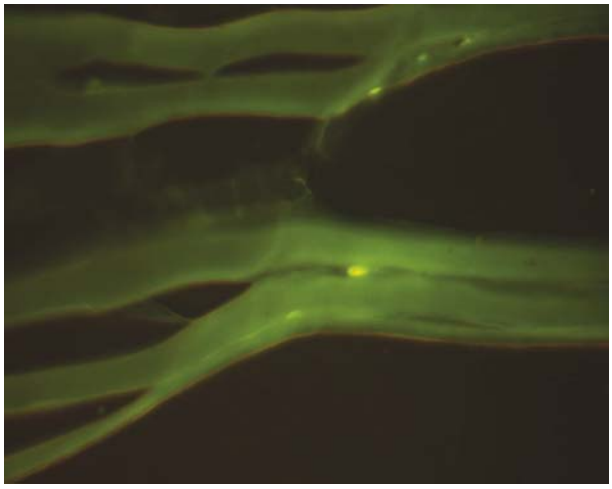

Fig. 1. BRDU-positive cells in interstitial intermuscular space after 24 h. BRDU staining, FITC labeled secondary antibodies, Filter-Set 09, ×260.

In group 2 rats (MNC implantation on day 25 of the experiment and laser tunneling), the index of microcirculation in the right shin on day 35 after BM MNC implantation approached that in the left (non-ischemic) shin, which was similar to the picture observed in the group without correction. The contribution of cardiac and respiratory mechanisms of capillary blood flow regulation decreased compared to the contralateral leg: A_{max}CF/A_{max}LF, A_{max}CF/δ, and A_{max}HF/A_{max}LF decreased by 61, 43, and 29%, respectively. The microcirculation index in the group with MNC

TABLE 1. Parameters of Microcirculation in Low Extremities on Day 35 after Modeling of Unilateral Ischemia and Indirect Revascularization ($M \pm \Delta tm$)

Parameter	Control (group 3)		Group 1		Group 2	
	LE	RE	LE	RE	LE	RE
Index of microcirculation (perfusion units)	14.75±0.88	14.92±1.86	14.72±2.01	17.1±3.23	22.92±2.46	20.48±1.35$^+$
Square deviation (δ)	1.14±0.08	1.78±0.45	0.86±0.23	1.24±0.41	1.0±0.21	1.16±0.09$^+$
C_v	7.64±1.73	11.87±2.87	5.38±0.97	6.41±0.76$^+$	4.53±1.03	5.79±0.61$^+$
IME	1.08±0.06	1.42±0.26	1.49±0.14	1.22±0.21	0.95±0.1	1.58±0.15*
A_{max}CF/A_{max}LF	0.45±0.05	0.26±0.06*	0.20±0.04	0.54±0.15*$^+$	0.51±0.13	0.20±0.03*
A_{max}HF/A_{max}LF	0.60±0.12	0.56±0.09	0.52±0.06	0.53±0.05	0.62±0.10	0.44±0.04*
A_{max}LF/M	0.07±0.01	0.13±0.03*	0.07±0.01	0.07±0.02$^+$	0.04±0.01	0.07±0.01$^+$
δ/A_{max}LF	1.07±0.11	0.89±0.24	0.83±0.13	0.99±0.33	1.18±0.23	0.8±0.1
A_{max}CF/δ	0.33±0.04	0.20±0.05*	0.20±0.03	0.26±0.06	0.42±0.08	0.24±0.02*
A_{max}HF/δ	0.47±0.02	0.55±0.12	0.56±0.09	0.44±0.13	0.48±0.07	0.54±0.09

Note. A_{max}: maximum amplitude; CF: cardiorhythms; LF: slow oscillations; HF: fast oscillations; LE: left extremity; RE: right extremity. *p<0.05 compared to left extremity in this group; ^+p<0.05 compared to right extremity in the control group.

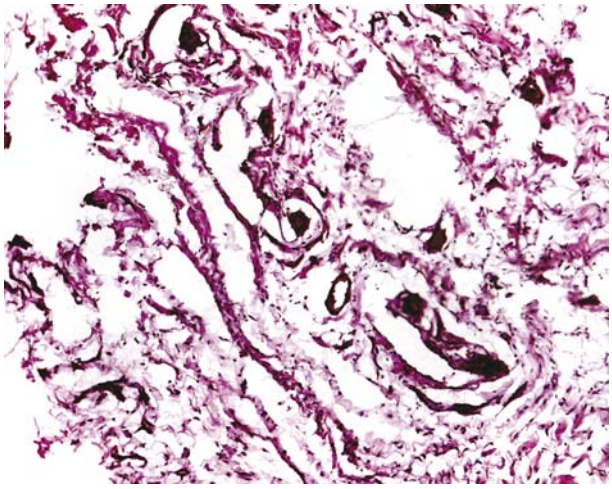

Fig. 2. Microvessels with loosened edematous walls in the zone of cell implantation into laser channels. Hematoxylin and eosin staining, ×260.

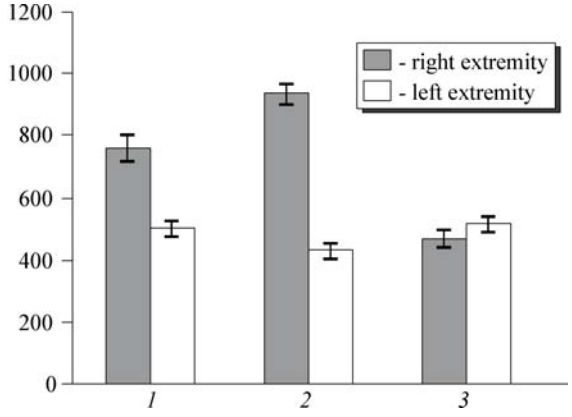

Fig. 3. Specific density of capillaries on day 35 after indirect revascularization. 1) experimental ischemia of the right extremity followed by BM MNC transplantation; 2) experimental ischemia of the right extremity followed by laser tunneling and BM MNC transplantation; 3) experimental ischemia of the right extremity without subsequent manipulations.

implantation and laser tunneling increased compared to that in the control group against the background of reduced vasomotor activity (A_{max}LF/M decreased by 46%).

Light microscopy of the laser vascularization and BM MBC implantation zone revealed foci of sclerosis and fibrosis (cicatrices) with different degree of neovascularization at the boundary with cicatrical zones and directly in the cicatrices. In the implantation zones we observed numerous microvessels (arterioles) with wall loosening and tunica media edema (Fig. 2).

Morphometric parameters of the capillary blood flow at these terms corresponded to the laser Doppler flowmetry data (Fig. 3). The density of capillaries by day 35 after indirect revascularization in group 1 (BM MNC implantation) was 758.33±40.84 per 1 mm^2, in group 2 (laser tunneling with cell implantation) 935.79±32.19 per 1 mm^2 (p<0.05), and in the control group (without manipulations) 465.74±28.15 per 1 mm^2. These findings attest to a pronounced angiogenic effect of both variants of indirect revascularization. The method combining laser tunneling and BM MNC implantation was most effective: the density of microvessels per 1 mm^2 increased 2-fold compared to the control.

Thus, the relative content of BRDU-positive MNC in sites of injections did not significantly changed over 1 day, which attested to predominant retention of implanted cells in the ischemic zone. According to Doppler sonography data, implantation of autologous MNC into the ischemic extremity (with or without laser tunneling) stimulates angiogenesis and promotes recovery of microcirculation in sites of implantation. The combination of laser tunneling with MNC implantation into channels was most effective for intensification of microcirculation. These findings agree with out previous data on more effective revascularization of the ischemic myocardium after combined application of laser and cell technologies [4,8,11].

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