The Use Angiogenesis Stimulators for the Treatment of Chronic Ischemia of Lower Extremities

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We present the results of treatment of chronic ischemia of the lower extremities (distal form) with angiogenesis stimulators, autologous endothelioblast precursors (CD133+) and gene preparation of vascular endothelial growth factor $VEGF_{165}$ (angiostimulin). Good clinical effect was attained in all patients, which was confirmed instrumentally 1, 3, and 6 months after administration of the stimulant: transcutaneous oxygen tension on the foot, index of malleolar pressure, and walking duration increased, parameters of microcirculation improved, the number of newly formed collateral arteries increased (angiography findings), quality of life improved (SF 36 questionnaire), and parameters of coagulogram also improved. The maximum positive dynamics was observed by month 3 of the study.

Key Words: *angiogenesis; CD133+ endothelioblast precursor cells; gene therapy; $VEGF_{165}$; chronic ischemia of lower extremities*

Despite successful application of methods of surgical revascularization and drug therapy for the treatment of cardiovascular diseases, chronic ischemia of lower extremities (CILE) is still a central problem in angiology and vascular surgery [4]. The necessity of improving macrohemodynamics in the involved limb by surgical methods is beyond doubts, but reconstructive operation can be impossible because of wide spreading of the pathological process and involvement of the distal vascular bed [5]. Therefore, methods of non-surgical revascularization of extremities are now intensively investigated. A concept of stimulation of angiogenesis (the process of capillary network development from pre-existing vessels) in the ischemic organ (so-called therapeutic angiogenesis) is now successfully developed [1,4,7,11,14]. To this end, angiogenic factors, genes encoding these factors, and precursor cells (from the bone marrow and peripheral blood) are used. Special interest is focused on vascular endothelial growth factor ($VEGF_{165}$), which interacts with receptors on endothelial cells and induces their migration, growth, and proliferation, thus stimulating the formation of new capillaries [6,14]. Recent experimental studies showed that autologous bone marrow precursor cells implanted into the ischemic zone (muscles of lower extremities) induce neoangiogenesis and that local concentration of growth factors and angiogenic cytokines considerably increases in these areas, *i.e.* endothelioblast precursors not only incorporate into the pre-existing capillary network, but also secrete numerous angiogenic cytokines and growth factors, thus stimulating angiogenesis [2,6,8-10,13,14]. The search for most effective stimulators of angiogenesis (cells and gene preparations), their combinations, and methods of their delivery into tissues, as well as determination of the terms of their

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repeated administration are the subjects of modern researches.

Here we compared the results of the use of various stimulators (autologous precursor cells and gene of vascular endothelial growth factor) and different methods of the delivery (intraarterial injection into the major artery proximal to the occlusion zone and intramuscular injections into the sural muscle).

MATERIALS AND METHODS

Studies of angiogenesis stimulators were carried out at the Department of Surgical Treatment of Arterial Pathology, A. N. Bakulev Research Center of Cardiovascular Surgery. We used autologous CD133+ endothelioblast precursor cells (group 1) and angiostimulin, a gene preparation of vascular endothelial growth factor $VEGF_{165}$, (group 2). Precursor cells were isolated from a standard volume of the bone marrow (40 ml) obtained from the iliac bone during aspiration biopsy 1-2 days before injection of the stimulants (disposable bone marrow needles were used). The mean number of injected cells was 850,000 (from 300,000 to 1.2 mln). Angiostimulin was injected in a dose of 1000 μg.

Angiogenesis stimulator was administered as single injection into the major artery of the involved extremity or multiple injections into sural muscle (every 4-5 injections). A total of 52 patients were treated by this method: 41 men (79%) and 11 women (21%) aging 60.1±11.4 years (from 30 to 80 years). The patients were divided into two groups including 15 (group 1) and 37 patients (group 2). The causes of CILE were atherosclerosis (87%) or thromboangiitis obliterans (13%). The duration of CILE in the majority of patients surpassed 3 years. At the moment of inclusion into the study, chronic critical ischemia of the lower extremities was diagnosed in 67% patients (stage 3 in 21 patients and stage 4 in 14 patients), stage 2B (subcompensation) was found in 33% (17 patients). Occlusion of the superficial femoral artery was found in 87% patients, in 46% patients it was associated with anatomic and functional insufficiency of the deep femoral artery and in 100% cases with occlusion-stenotic damage to the popliteal segment and crural arteries. Mean transcutaneous oxygen pressure was 27.3 mm Hg, index of malleolar pressure was below 0.59.

The data were processed statistically using Statistica 6.0 software.

RESULTS

No side effects after injection of angiogenesis stimulators were noted, biochemical and clinical tests confirmed safety of angiogenesis stimulators for the patients.

Examination 1 and 3 months after injection of angiogenesis stimulators showed that clinical improvement in the state of the lower extremity was attained in 80 and 100% cases, respectively ($p<0.05$). Healing of trophic ulcers or positive dynamics in nonhealing postoperative wounds was noted in all 14 patients with stage 4 CILE, pain at rest disappeared in 50% patients with critical ischemia.

One month after treatment, better effects were observed in group 1 patients (Fig. 1): clinical improvement was attained in 90% cases (*vs.* 73% in group 2). Three months after treatment, 100% clinical effect was observed in both groups. In groups 1 and 2, moderate improvement was noted in 78

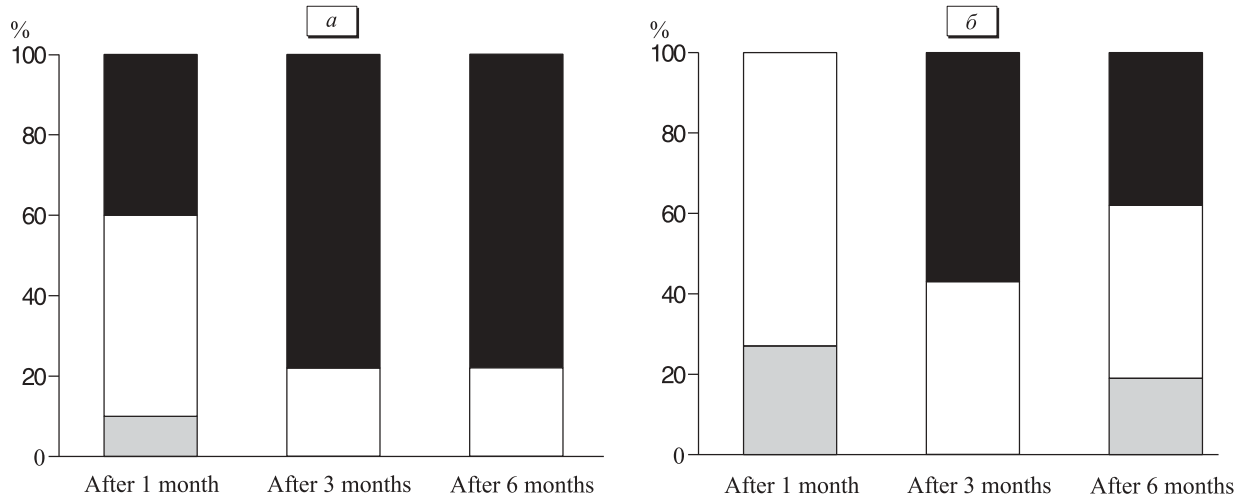

Fig. 1. Dynamics of clinical state in group 1 (*a*) and group 2 (*b*) patients. Moderate improvement (dark segment), minimum improvement (light segment), without improvement (grey segment).

TABLE 1. Transcutaneous Oxygen Pressure in Dynamics

Observation term	Group 1 (cell therapy)		Group 2 (gene therapy)	
	median	range	median	range
Initial	25.4	23-30	23.8	19-26
After 1 month	32.9*	25-44	30.3*	26-35
Increment over 1 month	7.5	4-12	6.5	4-8
After 3 months	41.1*+	42-47	34.7*+	28-41
Increment over 3 months	14.0	10-8	10.4	6-15
After 6 months	46.1*+x	44-52	37.8*+	34-44
Increment over 6 months	16.0	12-19	13	4-18

Note. Here and in Table 2: range of median (25;75). $p<0.05$ compared to: *initial level, +1 month; x3 months.

TABLE 2. Index of Malleolar Pressure in Dynamics

Observation term	Group 1 (cell therapy)		Group 2 (gene therapy)	
	median	range	median	range
Initial	0.50	0.36; 0.61	0.43	0.30; 0.62
After 1 month	0.53	0.41; 0.60	0.47	0.33; 0.57
After 3 months	0.60*	0.57; 0.67	0.43	0.34; 0.50
After 6 months	0.68*+	0.59; 0.75	0.53*	0.38; 0.71

and 57% patients, respectively, and minimum improvement was observed in 22 and 43% patients, respectively. After 6 months the effect was retained in all group 1 patients and in 81% group 2 patients. The positive dynamics in both groups did not exceed +2 according to Rutherford scale (moderate improvement), but no deterioration were noted either.

The dynamics of clinical state was confirmed by instrumental methods. Transcutaneous monitoring of oxygen tension in the first interdigital space on the dorsal surface of the food most significantly reflected changes in the clinical state of the extremity and viability of tissues (Table 1).

In groups 1 and 2, the mean increment of oxygen tension was 7.5 and 6.5 mm Hg after 1 month, 14.0 and 10.4 mm Hg after 3 months, and 16.0 and 13.0 mm Hg after 6 months, respectively. The mean increment of oxygen tension in group 1 was higher at all terms of observation, but the difference was significant only after 3 months.

The mean increment of Po_2 after intramuscular injection of precursor cells was higher at all terms of observation, but no significant differences were found between the groups with different administration routes. The mean increment of Po_2 1 month after intramuscular injection of angiostimulin was significantly higher than after its intraarterial injection, but at later terms no differences were revealed. Thus, intramuscular injection of angiostimulin produced more rapid effect.

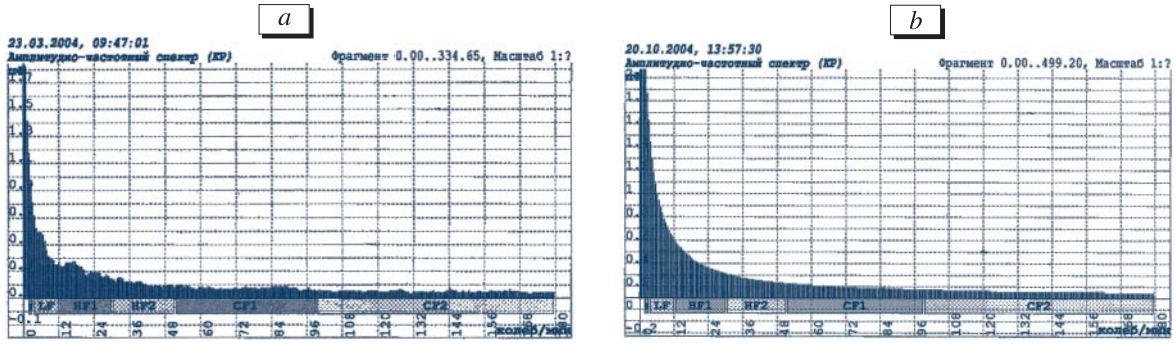

Fig. 2. Frequency-amplitude spectrum (Fourier analysis). *a*) initial; *b*) after 3 months.

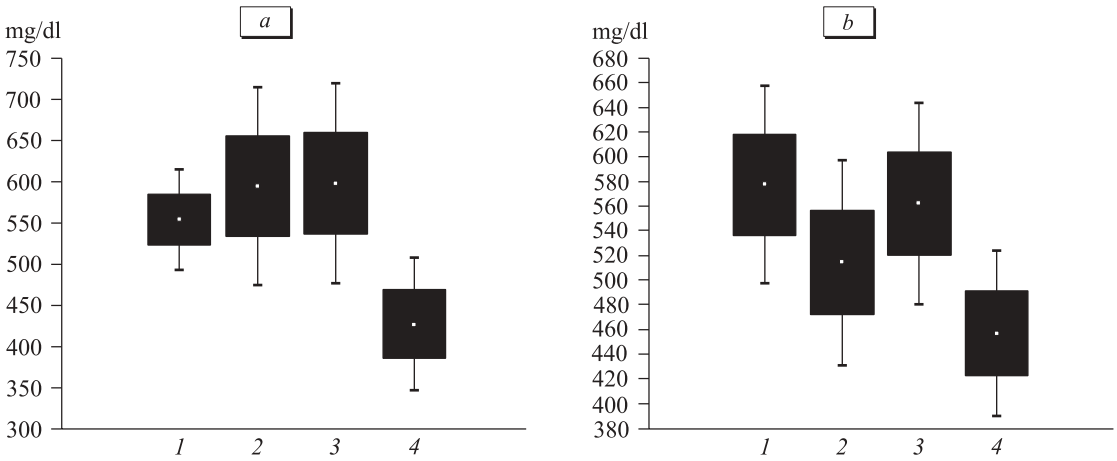

Fig. 3. Fibrinogen level in group 1 (*a*) and group 2 (*b*) patients. *1*) initial; *2*) after 1 month; *3*) after 3 months; *4*) after 6 months.

According to laser Doppler flowmetry (LDF), the mean index of microcirculation (M) reflecting the intensity of perfusion in the microcirculatory bed successively gradually in both groups. Reliable increase was attained after 3 and 6 months. Fourier transform analysis of LDF signal revealed a tendency to uniform distribution of the amplitude-frequency spectrum of blood flow oscillations in both groups after 3 months (Fig. 2), which was confirmed by normalization of the index of microcirculation efficiency, an integral parameter of microhemodynamics.

The increase in endothelium activity was observed 1 month after implantation of precursor cells,

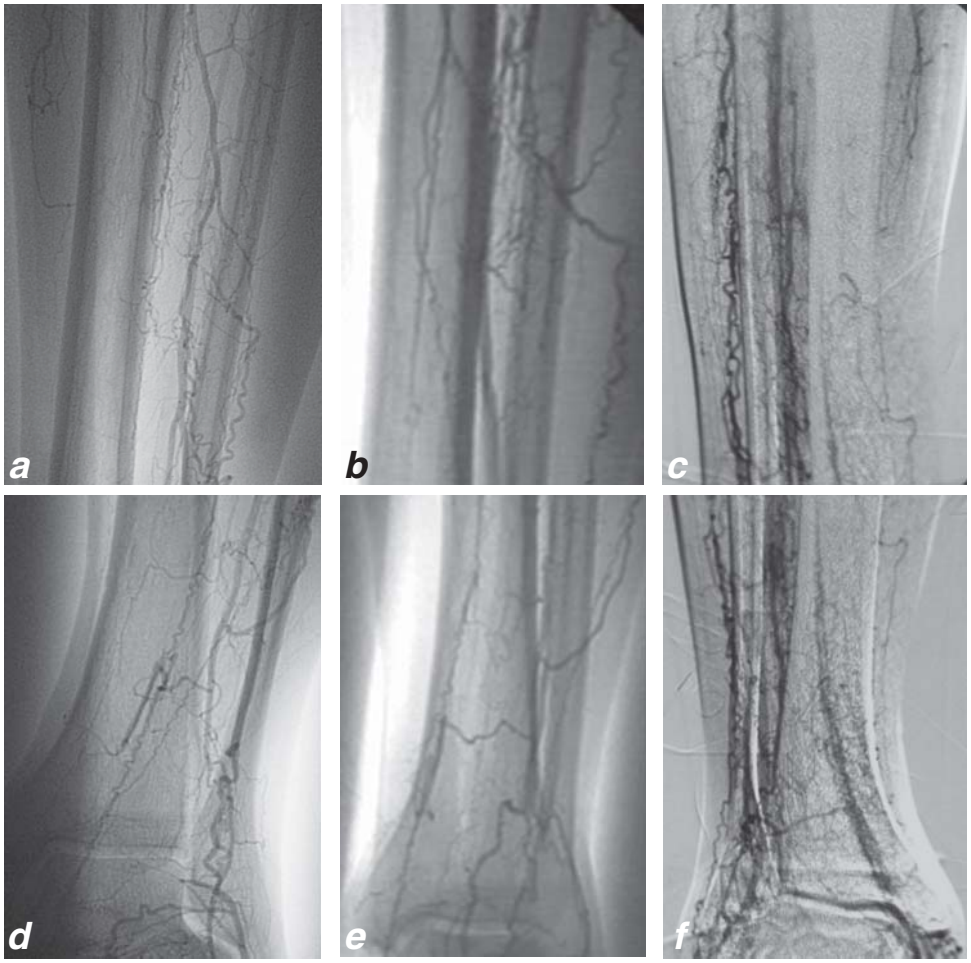

Fig. 4. Patient K, 30 years. Angiography of the lower extremity before (*a, d*) and 3 months (*b,e*) and 2 years (*c, f*) after implantation of autologous precursor cells.

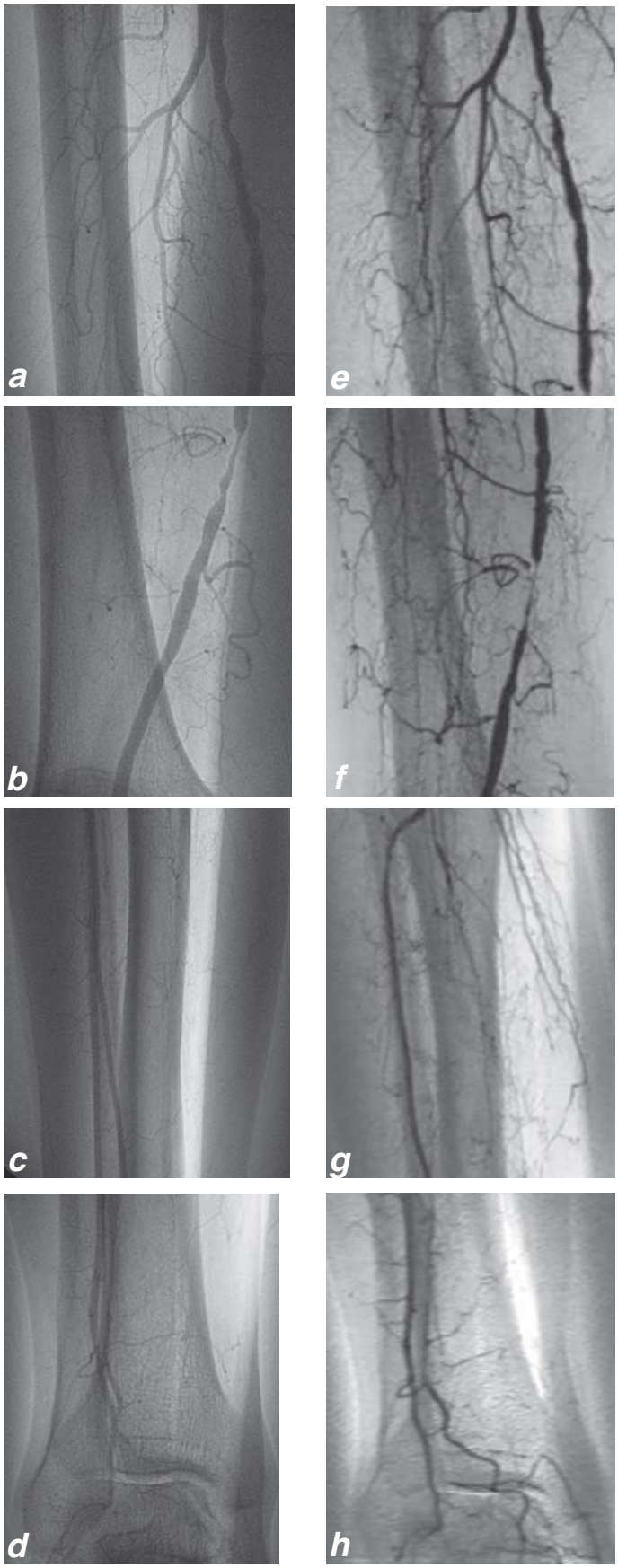

Fig. 5. Patient B, 76 years. Angiography of the lower extremity before (*a-d*) and 3 months (*e-h*) after angiostimulin treatment.

irrespective of implantation route. Activity of the endothelium increased 1 month after intraarterial and 3 months after intramuscular administration of angiostimulin.

Activation of the endothelium after treatment with angiogenesis stimulators was associated with improvement of hemostasis parameters without concomitant infusion therapy and changes in antiaggregation therapy. After 1 month, some parameters of hemostasis, in particular, international normalized ratio and activated partial thromboplastin time increased. This indirectly suggests the appearance of newly formed endothelium producing a complex of natural anticoagulants and substances responsible for its athrombogenicity [2,15], which improves rheological parameters of the blood: after 6 months, blood viscosity in patients of both groups significantly decreased due to a sharp decrease in fibrinogen concentration (Fig. 3).

The dynamics of the mean index of malleolar pressure (IMP) at rest according to Doppler ultrasound study is presented in Table 2. After 1 month, IMP increased by 0.1 in 40% patients in group 1 and in 33% patients in group 2. An appreciable increase in IMP was noted in both groups after 6 months. The mean IMP increment in group 1 surpassed that in group 2 (0.18 and 0.1, respectively. The delayed increase in this parameter can be explained by stabilization (arterialization) of the formed capillaries.

According to the treadmill test, the duration pain-free and maximum walking significantly increased throughout the observation period (maximum increase was observed after 1 month).

The data of noninvasive methods were confirmed by angiography. Three months after injection of angiogenesis stimulators, new collateral vessels appeared in all patients (compared to previous angiography). On average, the increase in the collateral network in group 1 patients was +2.28±0.75 and in group 2 patients +2.0±0.45 [14]. In patients of both groups examined in a delayed period (after 1-1.5 years) angiography revealed not only stability of newly formed collateral arteries, but also increase in their diameter (Fig. 4, 5).

Clinical improvement considerably affected patient's quality of life, but their physical activity (SF-36 questionnaire) did not return to normal due to severe concomitant disease without appreciable improvement (+3 according to Rutherford scale) after treatment.

Thus, clinical efficiency of angiogenesis stimulators was most pronounced after 3 months in both groups irrespective of the stimulator and the route of its administration, therefore the question on the need of repeated treatment can be solved after 3 months. The positive dynamics of the clinical state of the extremities in patients of both groups consisted in moderate improvement (+2 according to Rutherford scale), therefore isolated application of this method is insufficient for the treatment of patients with critical ischemia. When planning combined therapy with application of angiogenesis stimulators, their antithrombotic effect should be taken into account.

This effect was more pronounced in patients receiving autologous endothelioblast precursor cells, than in patients treated with angiostimulin. However, cell therapy is not indicated for individuals above 51-60 years, for patients with concomitant diseases affecting bone marrow composition and potencies of precursor cells, and for patients with a history of cancer diseases due to the risk of dissemination.

We found no significant difference between different administration routes, but judging from the data of transcutaneous monitoring of oxygen pressure we can conclude that intramuscular injections are more preferable, probably because more targeted delivery of the angiogenesis stimulator to ischemic muscles.

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