Use of Human $VEGF_{165}$ Gene for Therapeutic Angiogenesis in Coronary Patients: First Results

L. A. Bokeriya, E. Z. Golukhova, M. V. Eremeeva, I. P. Aslanidi, V. Yu. Merzlyakov, G. P. Georgiev*, S. L. Kiselev*, I. I. Berishvili, M. N. Vakhromeeva, R. A. Serov, T. V. Artyukhina, Yu. S. Basarab, E. S. Polyakova, and M. A. Lukashkin

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 3, pp. 123-130, September, 2005
Original article submitted May 21, 2005

The paper presents the first results of therapeutic angiogenesis in clinical cardiosurgery: human $VEGF_{165}$ gene transplantation to coronary patients. The use of this therapeutic method is particularly effective in patients with inoperable cardiovascular injuries, *i.e*. patients with the most severe condition, in whom treatment remains little effective at the modern level of cardiosurgery development.

Key Words: *angiogenesis; gene therapy; VEGF; coronary disease*

Angiogenesis in chronic myocardial ischemia is the first compensatory mechanism of collateral bloodflow increase. However, the angiogenic process is often insufficient for complete normalization of the bloodflow in ischemic tissues and for disappearance of angina pectoris symptoms. Inadequate local production of cytokines and other angiogenic factors or reduced sensitivity of atherosclerotic endothelium to growth factors make adequate tissue reperfusion impossible [9,14]. The new strategy, called therapeutic angiogenesis, is based on the concept of additional injection of growth factors (or their genes) for stimulation of vascular growth in ischemic areas.

Three processes leading to vascular growth are distinguished: angiogenesis, arteriogenesis, and vasculogenesis. Angiogenesis is studied best of all. The term **angiogenesis** (the growth of new capillary network from existing and functioning vessels) was introduced by A. T. Hertig in 1935 (he studied the formation of new vessels in the placenta). J. Folkman in 1971 described the neovascularization process associated with the growth of vascular tumors and theoretically outlined the new trends of therapy (antiangiogenic therapy) [5]. **Angiogenesis** is the formation of blood vessels consisting of a single layer of endothelial cells (without smooth-muscle layer). **Vasculogenesis** (formation of primary vessels from angioblasts) is characterized by the development of new vascular structures from morphologically undifferentiated mesenchymal (stem) cells, *e. g.*, at the early stages of embryogenesis. **Arteriogenesis** is the formation of large vessels, when not only endothelial, but also smooth-muscle cells migrate and proliferate; the vessels acquire vasomotor characteristics and elasticity essential for adaptation to changing requirements of blood supply to tissues. The arteries are thus formed, possessing completely developed tunica media and smooth-muscle wall. Smooth-muscle cells and pericytes ensure structural integration of vascular wall (due to the formation of the extracellular matrix), provide hemostasis, regulate vascular permeability, and create the blood-tissue barrier (partially retaining plasma molecules penetrating through the endothelium), and are responsible for vascular remodeling and plasticity (smooth-muscle cells make the vessels more resistant to regression).

Transluminal balloon angioplasty and aortocoronary shunting now became "the golden standard" in the treatment of coronary disease. The efficiency of

A. N. Bakulev Institute of Cardiovascular Surgery, Russian Academy of Medical Sciences; *Institute of Gene Biology, Russian Academy of Sciences, Moscow

0007-4888/05/1401-0106

these procedures is limited by the development of vascular restenosis and occlusion of the shunts. Moreover, complete revascularization of the myocardium cannot be attained by these procedures in more than 15% patients for various reasons [6,11]. According to other reports, complete revascularization after aortocoronary bypass surgery cannot be attained in 37% patients [8]. In the USA intervention procedures are impossible in 100,000-200,000 patients annually because of diffuse involvement of cardiac vessels [12]. Failure of revascularization of even one ischemic area in the myocardium deteriorates the treatment results (decreasing the survival rate and leading to a relapse of angina pectoris). These patients require an alternative revascularization strategy, and therapeutic angiogenesis can become this strategy.

VEGF (vascular endothelial growth factor) is a mitogen of vascular endothelial cells. It was isolated from arteries, veins, and lymph vessels and exhibited no mitogenic effect on other cell types [4]. Studies carried out at several laboratories proved the leading role of VEGF in the pathological angiogenesis during the development of tumors and ischemic diseases of the retina and demonstrated the possibility of VEGF-induced angiogenesis in models of coronary and peripheral ischemia in animals [5]. VEGF is a polypeptide with a molecular weight of 45 kDa; it has 4 isoforms containing 206, 189, 165, or 121 amino acid residues ($VEGF_{165}$ is the predominant isoform). Several members of the VEGF family were recently identified: placental growth factor, VEGF-B, VEGF-C, and VEGF-D. More than 15-year preclinical studies formed the basis for clinical studies of neoangiogenesis potentialities. Despite effective use of angiogenic factors for restoration of the bloodflow in ischemic tissues of mice, dogs, and swine, transition to clinical practice was more difficult. The aim of phase I of clinical trials was to prove the safety of therapeutic angiogenesis and evaluate its clinical efficiency. Phase I of clinical trials included preparation to phases II and III of randomized double blind placebo-controlled studies (Table 1). Three first studies (VIVA, FIRST, Hep-Alg) were devoted to protein therapy of coronary disease; the possibility of angiogenesis in lower limb ischemia was evaluated in the TRAFFIC study; and three last studies (AGENT, $VEGF_2$, KAT) investigated the potentialities of gene therapy for stimulation of neoangiogenesis in coronary patients.

A new preparation Angiostimulin based on a plasmid construction containing human $VEGF_{165}$ gene (Fig. 1) was created at Institute of Gene Biology in collaboration with A. N. Bakulev Institute of Cardiovascular Surgery. After toxicological trials the drug was recommended for clinical use in 2002. Experimental and clinical studies of the drug efficiency are carried out at Department of Noninvasive Arrhythmology (Head: Prof. E. Z. Golukhova) of A. N. Bakulev Institute since October 2002.

Experimental study. A model of peripheral chronic ischemia of rat skeletal muscle was created for evaluating the efficiency of angiogenic preparations. On day 10 after ligation of the femoral artery, VEGF gene, bFGF (basic fibroblast growth factor) gene in doses of 250, 500, and 750 μg/animal or normal saline were injected into the ischemic area. The effect of the drug was evaluated on day 30 postinjection. The drug was considered effective, if light microscopy of experimental sections of rat skeletal muscle showed a significant increase in the density of capillary network in comparison with its initial density (before drug injection; Fig. 2). The density of the capillary network was characterized as the median number of open capillaries per at least 10 visual fields in each preparation (Fig. 3). We detected a statistically significant increase

TABLE 1. Results of Randomized Studies of Angiogenic Factors in Cardiovascular Surgery

Study	Phase	*n*	Drug	Injection	Result
VIVA	II	178	$VEGF_{165}$ protein	Intravenous, intracoronary	Insignificant, trend to improvement in the high-dose group by month 4
FIRST	II	337	FGF_2 protein	Intracoronary	Alleviation of symptoms by day 90, insignificant changes by day 180
Hep-Alg	I/II	24	FGF_2 protein in polymer (slowly released)	Intramyocardial	Improvement by day 90, result persisted after 3 years
TRAFFIC	II	190	FGF_2 protein	Intraarterial	Improvement by day 90, insignificant changes on day 180
AGENT	I/II	79	Ad-FGF_4	Intracoronary	Trend to improvement
$VEGF_2$	I/I	19	Plasmid $VEGF_2$	Intramyocardial	Alleviation of symptoms
KAT	II	103	Protein, adenoviral or plasmid $VEGF_{165}$	Intracoronary	Improved perfusion in Ad-VEGF group

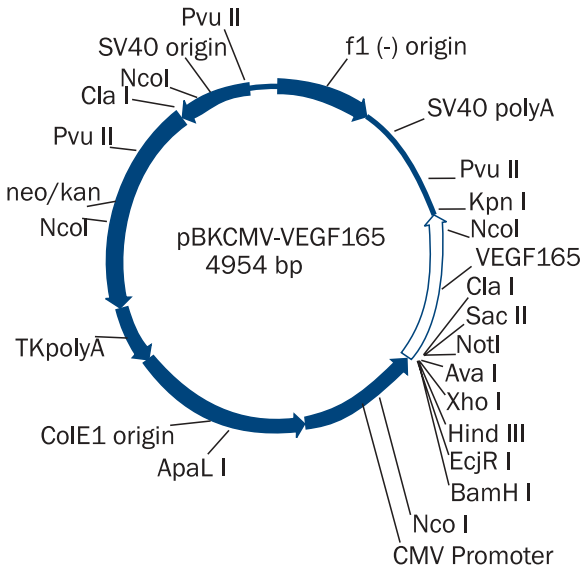

Fig. 1. $VEGF_{165}$ DNA plasmid construction (Angiostimulin drug).

in the density of capillaries 30 days after injection of VEGF gene (irrespective of the dose) but not after bFGF gene and normal saline (control group).

Clinical study. Angiostimulin (1000 μg) was injected into the myocardial zone requiring stimulation of neoangiogenesis at the final stage of the surgery (Fig. 4). The drug dose of 1 ml was divided into 4-6 intramyocardial injections. The site of injection was determined on the basis of angiocoronarography, scintigraphy, and positron emission tomography as an area of ischemic, but viable myocardium which could not be revascularized by surgical methods for technological causes (poor distal vascular bed, diffuse involvement, little diameters of the artery).

Staring from October 2002 until April 2005 a total of 29 patients were treated in the Noninvasive Arrhythmology Department by the method of therapeutic angiogenesis (angiostimulin injections) in combination with operations for myocardial revascularization.

Criteria for inclusion into the study: indications for aortocoronary bypass surgery and the presence of a zone of ischemic, but viable myocardium (according to scintigraphy and positron emission tomography findings), supplied with the blood via the coronary artery acknowledged unfit for bypass surgery (according to coronarography findings or results of intraoperative revision).

Criteria for exclusion from the study group: decreased left-ventricular ejection fraction (below 20%); a history of malignant tumors; proliferative retinopathy; plasma creatinine level ≥2.5 mg/dl; concomitant valvular disease.

The drug was injected to 9 patients during aortocoronary shunting, to 5 during transmyocardial laser revascularization (TMLR), to 2 during mini-invasive revascularization of the myocardium (MIRM), to 7 during MIRM+TMLR, and to 6 during aortocoronary shunting+TMLR. Coronary disease was diagnosed in all patients at admission on the basis of complaints, case history, clinical picture of angina pectoris, and additional methods of examination. The mean age of patients was 51.6±7.5 years. The majority (83%) of patients were referred to angina functional classes III and IV according to classification of Canadian Cardiovascular Society (CCS). Twenty-four (83%) patients had a history of myocardial infarction, 10 (34.5%) of these had 2 and more infarctions. The mean duration of coronary disease was 58.5 months. The mean period elapsed after the last myocardial infarction was 18.1 months. In 11 (38%) patients ECG showed *Q* wave, in 3 patients left-ventricular fibromuscular aneurysm was diagnosed. All patients were subjected

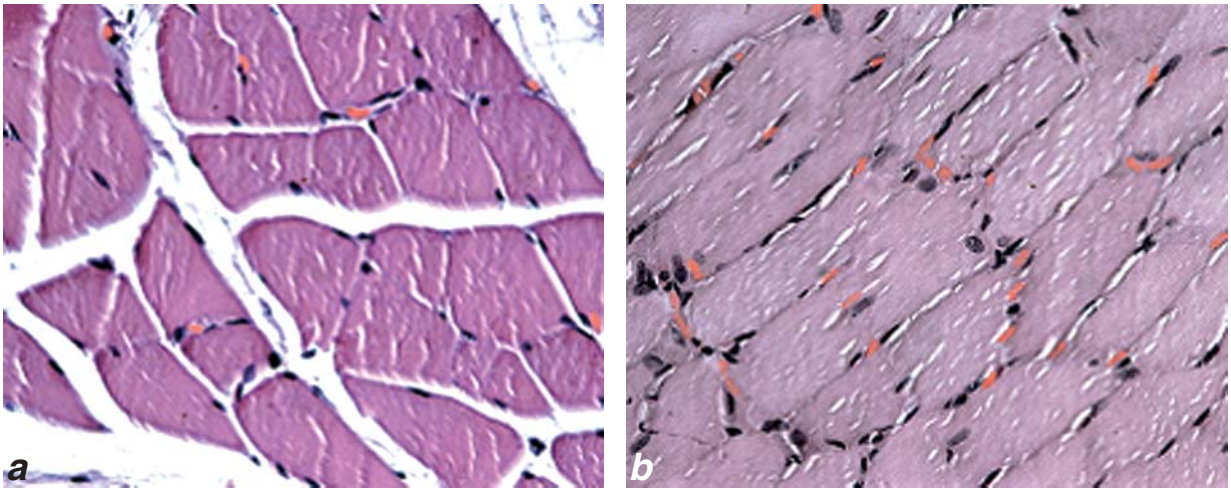

Fig. 2. Light microscopy of rat skeletal muscle before (*a*) and 30 days after (*b*) injection of VEGF gene. Increased number of open capillaries in visual field one month after drug injection.

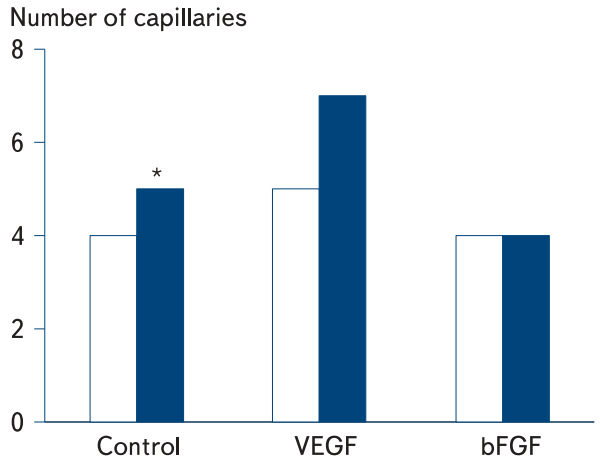

Fig. 3. Dynamics of capillary network density in experimental animals treated with VEGF, bFGF, and in controls. Light bars: before injection; dark bars: after injection. *$p<0.05$ compared to the value before injection.

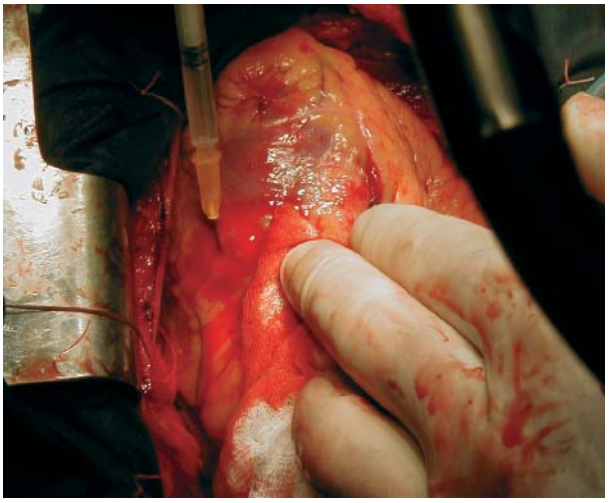

Fig. 4. Injection of angiostimulin (intraoperative view) into the anterior left-ventricular myocardial wall.

to cardiosurgery for the first time. CO_2 laser was used in all cases with laser revascularization. An average of 34.8±3.6 channels were created during one isolated TMLR operation. If TMLR was combined with direct revascularization of the myocardium, this value was 13.1±3.6 channels. Laser perforations were carried out after restoration of cardiac activity under conditions of parallel perfusion before heparin neutralization. Transmural penetration was tested by transesophageal EchoCG or by pulsed blood jet from the site of laser exposure. Bleeding was stopped by pressing a gauze tupffer. Laser perforations in isolated TMLR were performed on a working heart. Semiconductor laser was used in 3 patients (together with CO_2 laser); the mean number of perforations was 12.

MIRM was carried out using the method of pump coronary artery bypass grafting (OPCAB) through median sternotomy without artificial circulation in all cases. Myocardial stabilization system Octopus-3 and Starfish system (heart verticalizer; Medtronic) were used during surgery for exposure of the posterior and lateral left-ventricular myocardial walls during manipulations of the working heart. A total of 72 anastomoses with coronary arteries (3 per patient, on average) were carried out. Isolated left internal thoracic artery was used in 21 (88%) patients, radial artery in 11 (46%), and major subcutaneous vein in 22 (92%) cases. Operations with at least one arterial bypass were carried out in 22 (92%) patients; 2 and more arterial conduits were used for revascularization in 11 (46%) patients.

The following methods of examination were used in the study: ECG, EchoCG with evaluation of left-ventricular myocardial contractility by 16-segmentary model, treadmill stress test, coronarography, ECG-

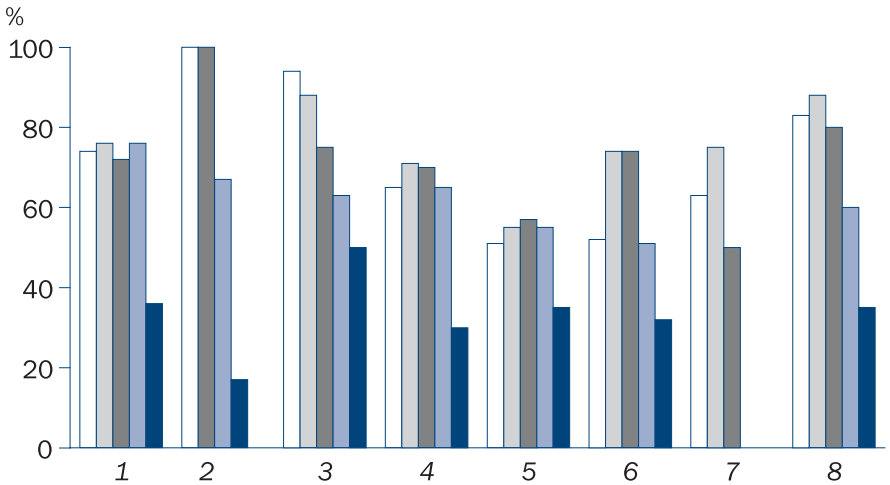

Fig. 5. Dynamics of quality of life in all patients during 1 year after surgery according to SF-36 questionnaire. *1*) mental health; *2*) role limitations because of emotional problems; *3*) social functioning; *4*) energy/viability; *5*) total attitude to health status; *6*) physical pain; *7*) role limitations because of physical problems; *8*) physical functions. Light bars: 1 year; light-gray bars: 6-8 months; dark gray bars: 2-4 months; blue bars: up to 1 month; navy-blue bars: initial level.

synchronized ^{99m}Tc single-photon emission computer-aided tomography of the myocardium (synchro-SPECT), positron emission tomography of the myocardium with fluorodeoxyglucose (^{18}F-FDG), and evaluation of quality of life using SF-36 questionnaire.

RESULTS

Twenty-four (83%) of 29 patients initially had angina functional class III-IV; after the intervention the majority (21 patients, 84%) were referred to functional classes I and II. The average functional class of angina pectoris (CCS) was 3.1 initially and 1.7 after 6-12 months. The need in oral nitroglycerin for arresting angina pectoris attacks decreased from 11 to 0.7 tablets daily. Quality of life improved by all scales of SF-36 questionnaire (Fig. 5).

In order to detect the direct effect of angiostimulin on the increment of myocardial perfusion, all left-ventricular segments were classified depending on the type of therapy, using the scheme developed at Institute of Cardiovascular Surgery [1], according to which scintigraphy and coronarography findings were compared and the protocol of the operation was presented. The segments were classified depending on the initial accumulation of the radiopharmaceutrical into normally perfused (accumulation >75%), with moderately reduced perfusion (51-74%), with significant reduction of perfusion (30-50%), and with pronounced reduction (<30%).

Segment-by-segment evaluation depending on the type of treatment of the myocardium showed a significant improvement of perfusion in segments exposed to CO_2 laser revascularization and injection of angiostimulin (TMLR+VEGF) and in the adjacent segments starting from months 2-4 after surgery (Fig. 6, *a*, *b*). Evaluation of myocardial blood supply in patients subjected to isolated CO_2 laser revascularization (TMLR; no gene injection) showed significant improvement of perfusion only by month 6 after surgery in treated segments.

Hence, angiogenesis leading to improvement of blood supply in the treated myocardial segments and maintaining perfusion in the adjacent segments was initiated in myocardial zones treated using a combination of CO_2 laser revascularization with injections of angiostimulin. This process manifests under conditions of myocardial ischemia and is less pronounced after its disappearance (after shunting of this myocardial zone).

Our results are in line with the opinions of many scientists on higher activity of angiogenesis after combined treatment with TMLR and angiogenic factors and a less pronounced effect of monotherapy with angiogenic factor [10]. M. P. Pelletier *et al.* carried out combined operations on rats [13]. They perforated the myocardium with needles and in parallel with this, injected vascular growth factors. Angiogenesis was more pronounced after combination of these methods. G. Lutter *et al.* in experiments on swine studied the effects of $VEGF_{121}$, TMLR, and their combination on the myocardium on a model of chronic ischemia and found that combined effect of laser exposure and angiogenic factor most pronouncedly increased the density of capillary network and regional bloodflow [10]. Some studies showed that TMLR induced the growth of new vessels [7,16]. These and other studies [11] demonstrated obvious angiogenesis after TMLR. Many scientists came to a conclusion that if angiogenesis was the leading factor determining the efficiency of TMLR, incorporation of angiogenic factors together with TMLR seemed to stimulate the regional bloodflow and promoted an improvement of patients' clinical status.

No doubt, these first observations and experience gained in the use of this drug are not yet extensive. Studies of angiogenesis were started almost 30 years ago on the basis on the hypothesis about the relationship between tumor development and vascularization. At first sight, the strategic goal of research programs in this direction seems straight-line: the factors inducing revascularization in preclinical models were identified and proposed for clinical testing; their efficiency and safety were demonstrated. However, the initial series of double-blind randomized trials (Table 1) with VEGF and FGF cannot unambiguously indicate their therapeutic efficiency. Some data indicate positive results, but the results of the trials in general are unsatisfactory. The reasons for this can be as follows.

First, young and healthy animals are mainly used as experimental models; the biology of these animals can differ from that of elderly patients with pronounced atherosclerosis. The mechanisms of natural revascularization in these patients are inadequate and can be particularly resistant to growth factor therapy. Many studies noted high variability of patients' reactions to therapy with angiogenic factors, which indicates insufficiency of our data on the biological factors which can be essential for patients' response to angiogenesis stimulation. Presumably, the severity of symptomatic manifestations of the disease determines the intensity of the response: the improvement due to therapy is more pronounced in patients with more pronounced symptoms. Second, the tested agents more effectively initiate angiogenesis (capillary growth) than the growth and development of large vessels (arteriogenesis). Hence, they can be unable to provide rapid and considerable bloodflow increase during developing ischemia.

Single injection of the recombinant factor cannot produce sufficient positive angiogenic effect. Tempo-

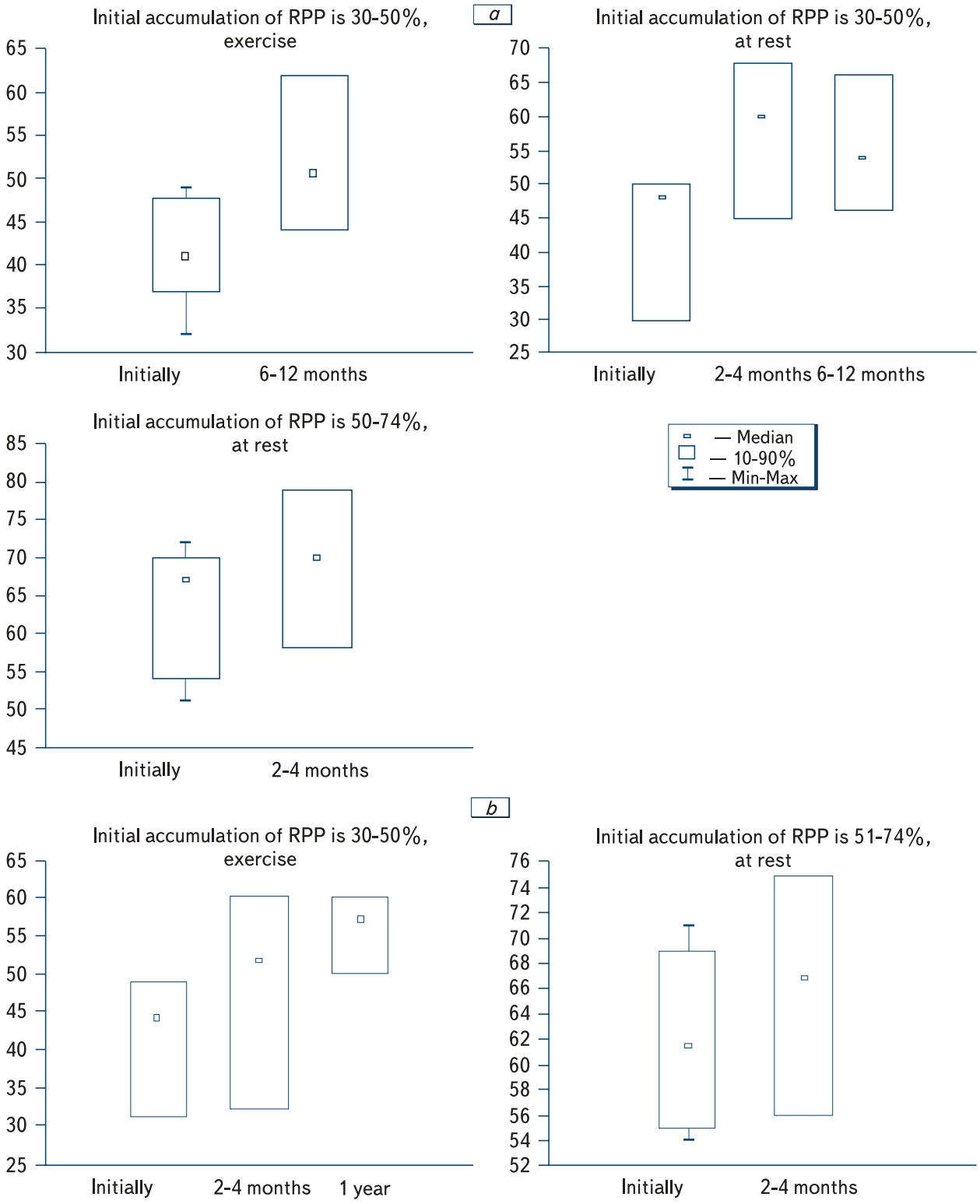

Fig. 6. Diagrams presenting significant changes in the accumulation of radiopharmaceutical (RPP; %) in TMLR+VEGF segments (*a*) and adjacent segments (*b*) during exercise and at rest.

rary favorable effect from a single injection was observed in some experimental models, but absolute improvement was negligible, while the life span of newly formed vessels was unknown. Some authors reported better results of therapy with a combination of angiogenic factors compared to monotherapy. On the other hand, gene therapy (in contrast to protein therapy) presumably modified the agent pharmaco-

kinetics, which, in turn, can be the cause of unsatisfactory results.

Normal (physiological) angiogenesis is the final result of cooperation of several angiogenic factors acting simultaneously or in succession. Some of these factors induce proliferation and migration of endothelial cells (VEGF), others stimulate the growth of smooth-muscle layer around new vessels, transforming the new blood vessels into mature arterioles (Ang-1, platelet-derived growth factor BB). Presumably, a combination of several agents, but not monotherapy, is needed for effective revascularization [15].

Hence, a significant angiogenic effect of angiostimulin ($VEGF_{165}$ gene) was observed on a model of chronic ischemia of the rat skeletal muscle. Trials of bFGF gene-based drug showed no appreciable effect.

The first experience of clinical use of angiostimulin indicates its safety. No side effects and complications were detected during examination of patients in the early and delayed postoperative period. Injection of the drug was associated with solitary extrasystole during the injection (during operation on working heart).

Segment-by-segment analysis of myocardial blood supply showed a significant increase in perfusion during the periods from 2-4 months to 1 year postoperation in the segments injected with angiostimulin and exposed to CO_2 laser TMLR. Isolated exposure to CO_2 laser TMLR significantly increased perfusion only 6 months after surgery. The use of angiostimulin injections alone during aortocoronary bypass surgery did appreciably increased perfusion during the periods of 2-4 months to 1 year postoperation. Increased perfusion after combined use of CO_2 laser TMLR and angiostimulin was observed under conditions of myocardial ischemia, but not in the shunted segments, where ischemia disappeared after surgery.

The first results of successful experimental studies and clinical trials of angiogenesis stimulation in coronary patients give birth to hope that this treatment modality will soon be used alone or in combination with other methods in the treatment of coronary disease.

REFERENCES

1. L. A. Bokeriya, I. I. Berishvili, I. P. Aslanidi, and M. N. Vakhromeeva, *Transmyocardial Laser Revascularization: Perfusion, Function, and Metabolism of the Myocardium* [in Russian], Moscow (2004).
2. L. A. Bokeriya, G. P. Georgiev, E. Z. Golukhova, *et al.*, *Byull. Nauch. Tsentra Serdechno-Sosudistoi Khirurgii*, **5**, No. 3, 19-38 (2004).
3. L. A. Bokeriya, G. P. Georgiev, E. Z. Golukhova, *et al.*, *Ibid.*, No. 7, 70-91.
4. N. Ferrara and T. Davis-Smyth, *Endocr. Rev.*, **18**, 4-25 (1997).
5. J. Folkman, *N. Engl. J. Med.*, **285**, No. 21, 1182-1186 (1971).
6. E. L. Jones, J. M. Craver, R. A. Guyton, *et al.*, *Am. J. Cardiol.*, **51**, 7-12 (1983).
7. T. Kohmoto, C. De Rosa, N. Yamamoto, *et al.*, *Ann. Thorac. Surg.*, **65**, 1360-1367 (1998).
8. D. C. Levin, C. F. Beckmann, T. A. Sos, and K. Sniderman, *Radiology*, **142**, 317-332 (1982).
9. D. W. Losordo, P. R. Vale, and J. M. Isner, *Am. Heart J.*, **138**, S132-S141 (1999).
10. G. Lutter, T. Attmann, C. Heilmann, *et al.*, *Eur. J. Cardiothorac. Surg.*, **22**, No. 5, 753-761 (2002).
11. C. A. Mack, S. R. Patel, and T. K. Rosengart, *J. Clin. Laser Med. Surg.*, **15**, 275-279 (1997).
12. D. Mukherjee, D. L. Bhatt, M. T. Roe, *et al.*, *Am. J. Cardiol.*, **84**, 598-600 (1999).
13. M. P. Pelletier, A. Giaid, and S. Siwaraman, *Ann. Thorac. Surg.*, **66**, 12-18 (1998).
14. W. Schaper and W. Ito, *Circ. Res.*, **79**, 911-919 (1996).
15. M. Simons and J. A. Ware, *Natl. Rev. Drug Discov.*, **2**, 863-872 (2003).
16. T. Spanier, C. R. Smith, and D. Burkoff, *J. Clin. Laser Med. Surg.*, **15**, 269-273 (1997).
17. N. Yamamoto, T. Kohmoto, A. Gu., *et al.*, *J. Am. Coll. Cardiol.*, **31**, 1426-1433 (1998).