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Effect of Endothelial Growth Factor on Postinfarction Remodeling of Rat Myocardium

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The reparative processes in the myocardium were studied during the postinfarction period after intracardiac injection of vascular endothelial growth factor. The cytoprotective and mitogenic effects of increased concentration of vascular endothelial growth factor on cardiomyocytes were revealed: viable muscle cells were present in the myocardial necrotic zone over 3 days, while cardiomyocytes in the periinfarction zone exhibited mitotic activity of within 7 days after infarction modeling. One of the main morphogenetic effects of high concentrations of vascular endothelial growth factor is stimulation of angiogenesis in all zones of the infarcted myocardium. On the other hand, injection of exogenous vascular endothelial growth factor stimulated collagen formation processes in the infarction zone (a 44% increase of the volume of collagen fibers) and formation of cardiosclerosis foci in neoangiogenesis zones in the intact myocardium, which is an unfavorable side effect of high concentrations of vascular endothelial growth factor.

Key Words: *myocardial infarction; vascular endothelial growth factor; morphology; morphometry*

Study of regulation of reparative processes in the myocardium during the postinfarction period is essential for the development of new approaches to stimulation of the regeneratory reactions of cardiomyocytes and other myocardial cell populations. One of the key stages in myocardial reparation is restoration of the coronary blood vessels distally from the site of occlusion. Various approaches based on the use of cell technologies and cytokine therapy [14] are used for this purpose in modern clinical and experimental studies.

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By the present time, the vascular endothelial growth factor (VEGF) is regarded as one of the most potent regulators of neoangiogenesis and vasculogenesis processes [5,11,15]. According to modern ideas, VEGF initiates the angiogenesis processes during different periods of embryonic and postnatal development and induces neoangiogenesis, the initial stage of myocardial remodeling during the postinfarction period [1,8]. Among the main physiological effects of VEGF are mitogenic and cytoprotective effects towards vascular endotheliocytes, the permeabilizing and chemotaxic effects [6,11]. In physiological concentrations, VEGF is essential for survival of the endothelium, while its shortage can promote apoptosis of endothelial cells [7].

The expression of VEGF in the peripheral vessels in ischemia of the limbs was studied in numerous

clinical and experimental works [2,9,10]. However, the effect of VEGF on the dynamics of changes in cell populations of the postinfarction myocardium and possible effects of changed concentrations of this factor on the morphogenesis of reparative processes remain unclear.

We studied the effects of high concentrations of VEGF on the course of reparative processes in the myocardium during experimental infarction.

MATERIALS AND METHODS

The study was carried out on 125 female Wistar rats (200-250 g) aged 9 months. Experimental animals were randomly divided into 3 groups. In group 1 (n=45), myocardial infarction (MI) was induced; in group 2 (n=40), MI was induced and VEGF was injected; and group 3 (n=40) rats were sham-operated in order to rule out the effects of surgical intervention on the studied parameters. Myocardial infarction was induced in groups 1 and 2 by diathermocoagulation of the pericone interventricular artery [1]. Group 2 animals were injected with a single dose (100 ng) of VEGF into the left-ventricular cavity 1.5 h after MI simulation. The animals of groups 1 and 3 were injected with 0.85% NaCl (0.1 ml) during the same period. The animals were sacrificed 2, 6, 12 h, 1, 3, 7, 14, and 30 days after MI simulation. All surgical interventions were carried out under aseptic conditions. Experiments were carried out in accordance with regulations of the European Convention for Animal Protection (Strasbourg, 1986).

The type and severity of structural changes in the myocardium under conditions of the natural course of infarction and of modified VEGF level were evaluated by morphological and morphometric analyses. The hearts of the experimental animals were fixed in 10% neutral formalin, embedded in paraffin, the sections were stained with hematoxylin and eosin and after van Gieson. Paraffin sections were examined under a universal Leica DM4000B light microscope. Microphotographs were taken with Leica DFC320 digital camera and Leica Qwin V3 software. Morphometric study of the myocardium was carried out using ImageJ software with a set of modules for medical morphometry. The cardiomyocyte and capillary sections per unit of section area (13,200 μ^2) were counted at ×600. Fibrosis severity was evaluated by changes in the volume density of collagen fibers by a previously developed method [3].

Plasma concentrations of VEGF were measured by EIA using R&D Systems kits. The measurements were carried out on an ELX800 enzyme immunoassay analyzer (BioTek).

Statistical processing of the results included calculation of the means, errors in the means, and comparison of the data by Student's test. The level of significance for statistical analysis was 0.05.

RESULTS

Study of the dynamics of serum VEGF concentrations in sham-operated animals showed their slight elevation 2 h after the MI induction, after which this parameter decreased (Table 1). This dynamics is in line with general opinion according to which the level of VEGF increases in response to any damage of muscle tissue. Under conditions of natural MI course (group 1), the concentration of VEGF was elevated during the period from 6 h until 3 days after MI creation. The peaks of VEGF were observed after 6 and 12 h: by 7.7 and 9.7 times, respectively, compared to sham-operated animals (p<0.05).

Injection of exogenous VEGF (group 2) led to a significant increase in its serum concentration as soon as 2 h after MI induction and was almost 18-fold

TABLE 1. Changes in Serum VEGF Concentrations during the Postinfarction Period (pg/ml; $M\pm m$)

Period after intervention	MI	MI+VEGF	Sham-operated animals
2 h	41.16±3.23	723.37±66.50*	46.93±2.78
6 h	356.69±108.06+	1036.745±19.090*	46.10±8.69
12 h	283.91±23.68+	33.46±17.34*	29.38±7.22
1 day	70.22±11.03	99.61±79.61	35.07±6.80
3 day	80.10±5.84+	57.53±28.33	29.11±8.84
7 day	40.25±8.18	41.14±7.38	14.39±5.47
14 day	19.06±2.98	32.47±0.18	21.29±10.93
30 day	21.80±7.95	18.09±5.21	17.56±9.11

Note. p<0.05 compared to +sham-operated animals; *animals with MI receiving no VEGF.

higher than during the natural course of the process. The concentration of VEGF reached the maximum in both groups after 6 h, being 3-fold higher after injection of exogenous VEGF (Table 1). By 12 h postoperation, the concentration of VEGF in group 2 decreased significantly in comparison with group 1, which could be caused by reduction of VEGF production by the negative feedback mechanism.

During the natural course of the pathological process (group 1), the changes characteristic of MI were observed in the necrotic focus just after 2 h: uneven intensification of cardiomyocyte staining and their necrobiosis (disappearance of cross-striation and lumpy degradation of myofibrils; Fig. 1, *a*). This was paralleled by degenerative and necrobiotic changes in endotheliocytes. Desquamation of endothelial cells into the vascular lumen was seen in some cases.

Granulation tissue in the MI zone formed by day 7 in the group with the natural course of the reparative process. New vessels containing virtually no blood cells were present in the MI focus and in the marginal zone (Fig. 1, *b*). Presumably, the absence of formed elements of the blood in these vessels indicated that they were not connected to intramural arteries. The new vessels formed from the "intact" myocardial zone towards the necrotic focus by growing into the infarcted zone. The peak of angiogenesis was observed on day 3 after MI simulation. Later (during the formation of connective tissue cicatrix and its maturation) the number of capillaries gradually decreased.

Importantly, no viable cardiomyocytes were detected in the infarcted zone by the end of day 1. After 7 days, cardiomyocyte hypertrophy and degenerative changes were seen in the marginal zone; no mitotic division of myocytes was detected. Starting from day 7, granulation tissue was gradually replaced with fibroblasts and the formation of connective tissue cicatrix progressed (Fig. 1, *c*) and was completed by day 30. This was paralleled by moderate edema of the resultant connective tissue cicatrix; in some cases mainly perivascular slight leukocyte infiltration was observed (Fig. 1, *d*).

Interstitial edema was seen in the interphase zone between the necrotic and "intact" myocardium during

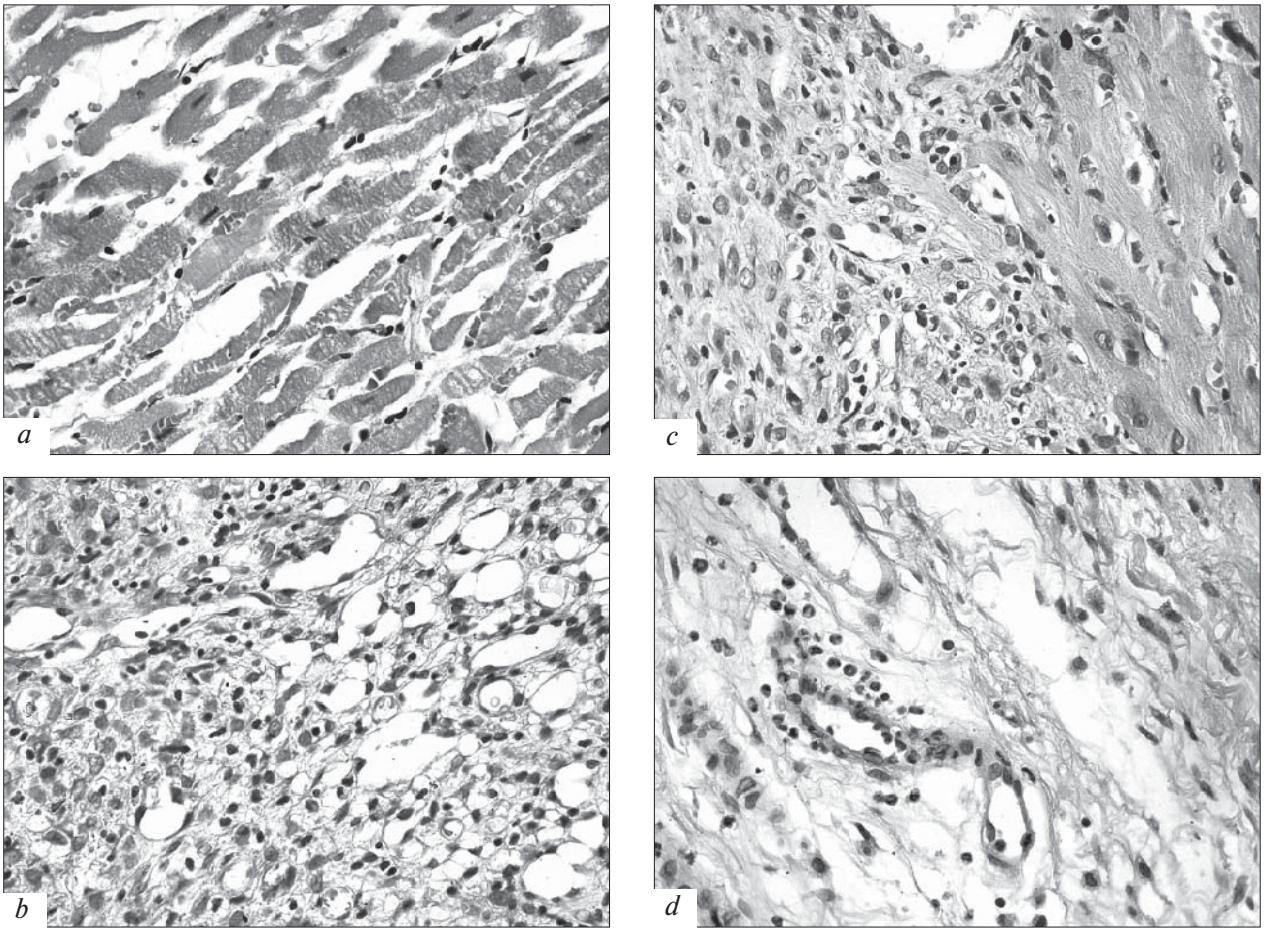

Fig. 1. Morphological changes in rat myocardium during experimental MI. Hematoxylin and eosin staining, ×400. *a*) lumpy degradation of myofibrils in cardiomyocytes in MI zone after 2 h; *b*) granulation tissue in MI zone after 7 days; *c*) solitary vessels in the marginal zone after 7 days; *d*) minor leukocytic infiltration of connective tissue cicatrix after 30 days.

the early (2-6 h) period in group 1 animals. Cardiomyocytes with ischemic changes, presenting by vacuolar degeneration or myocytolysis, were often seen at the periphery of the necrotic zone. Some myofibrils were fragmented; cross striation was lost in many of them.

Capillary network decreased significantly in the marginal zone as a result of destruction. Vascular dilatation and stasis were seen in the zone of transition from intact myocardium to necrotic focus. Marginal stasis of leukocytes in vascular lumen was seen. The peak of neutrophilic infiltration in the marginal zone was observed 12 h after simulation of infarction.

Moderate interstitial edema developed in the intact myocardial zone after 2-6 h of infarction development. It leveled after 24 h. Intravascular rheological disorders, consisting in the development of erythrocyte stasis, were noted. The vessels in the microcirculatory network were plethoric to a different degree. Moderate degenerative changes in some cardiomyocytes were observed later. By the end of the experiment, moderate diffuse mononuclear infiltration of interstitial tissue was observed.

Injection of exogenous VEGF promoted longer retention of viable cardiomyocytes in the necrotic zone (up to 3 days after MI simulation; Fig. 2, *a*). Cardiomyocytes in the necrotic zone were mainly dissociated. Growth of myofibrils into granulation tissue was seen in the marginal zone. Mitotic activity of cardiomyocytes in the marginal zone and in the intact myocardium was recorded over 7 days (Fig. 2, *b-d*).

Quantitative evaluation of cardiomyocyte population showed their 10-60% greater number per unit of area during day 1 in all zones of the postinfarction myocardium after intracardiac injection of VEGF in comparison with the natural course of the process. Significant differences were detected 12 and 24 h after MI induction. No viable cardiomyocytes were detected after 12 h in group 1, while in group 2 cardiomyocytes were detected in the detritus after 12 and 24 h (7±3 and 11±4, respectively; $p<0.05$). The differences became less pronounced starting from day 7 of the experiment and disappeared after 30 days.

The increase in the number of cardiomyocytes per unit of area after injection of exogenous VEGF during the first 7 days of the experiment can be explained by stimulation of the cytoprotection system and mitogenic activity of cardiomyocytes. Since cardiomyocytes express VEGF receptors (VEGFR-1 and VEGFR-2) under certain conditions [13], both direct and indirect effects of VEGF on stimulation of cellular and intracellular regeneration of cardiomyocytes are possible [12]. The cytoprotective effect of VEGF towards cardiomyocytes in ischemia can be realized through paracrine activation of the phosphatidyl inositol-3-kinase chain [4]. Importantly, the cytoprotective and mitogenic effects of exogenous VEGF were detected only when its blood concentration was high.

One of the main morphogenetic effects of high VEGF concentrations was stimulation of angiogenesis in all zones of infarcted myocardium: throughout the entire experiment in the periinfarction zone and in the intact myocardium and on days 7-30 in the necrotic zone (cicatrix; Fig. 2, *e*). The number of capillary profiles in the marginal zone of group 2 animals was significantly higher than in group 1 after 7 days (group 1: 86±11; group 2: 128±9, $p<0.05$) and after 14 days (group 1: 75±7, group 2: 128±17, $p<0.05$). Significant difference in the necrotic zone was recorded 14 days after MI simulation (group 1: 81±3, group 2: 137±16, $p<0.05$). These changes were caused by proliferation of endothelial cells. It is noteworthy that blood cells were present in new vessels. Stimulation of angiogenesis promoted vascularization of the connective tissue cicatrix, formed after 30 days; its blood vessels also contained blood cells.

In group 2, infiltration of the necrotic zone with neutrophils and macrophages was more pronounced. Neutrophilic infiltration of the necrotic zone started as early as 2 h after MI induction and lasted till the end of the first 24 h, when the neutrophilic reaction was the most pronounced (3-fold higher than in group 1). The maximum intensity of macrophagic reaction was observed after 7 days. The peak of lymphocytic infiltration was recorded 24 h after induction of MI. The formation of granulation tissue in the necrotic zone in group 2 started from day 3 postoperation (similarly as in group 1).

Infiltration of the marginal zone by neutrophils, macrophages, and lymphocytes was more pronounced in group 2 than in group 1. Neutrophilic infiltration was recorded from 2 to 24 h postoperation, being the maximum after 12 and 24 h. Macrophagic reaction was more intense than in group 1, particularly after 7 days. In contrast to group 1, fibroblast cells in group 2 appeared in the marginal zone just 6 h after MI induction, their count increasing from day 3.

The effect of modified concentration of VEGF on fibroblast hyperplasia in MI was paralleled by more intense collagen formation in the left-ventricular wall. In group 1 animals, the formation of collagen fibers in the infarction zone was recorded from day 3 postoperation and lasted until the end of observation. The maximum volume density of collagen fibers was recorded 30 days after MI simulation (41.7±1.0%). After injection of exogenous VEGF the level of collagen formation in the necrotic zone was significantly higher than in group 1: on day 30 postoperation the volume of collagen fibers reached 60.23±4.70%, that is, increased by 44% ($p<0.05$).

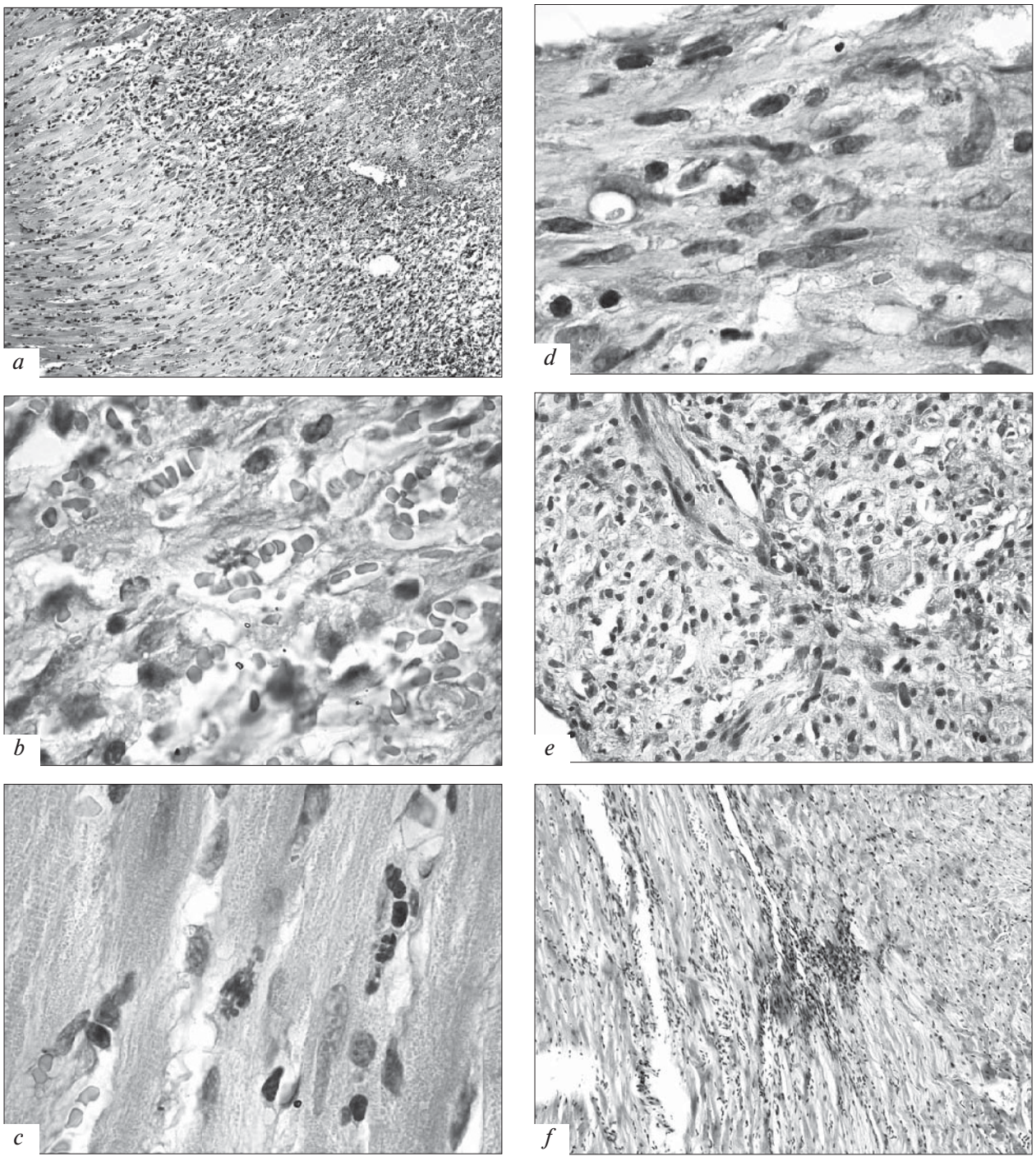

Fig. 2. Morphogenesis of reparative processes in the myocardium during the postinfarction period after intracardiac injection of VEGF. Hematoxylin and eosin staining. *a*) viable cardiomyocytes in MI zone and marginal zone after 3 days, ×100; *b*) mitotic activity of cardiomyocytes in the marginal zone after 3 days, ×1000; *c*) mitotic activity of cardiomyocytes in "intact" zone after 3 days, ×1000; *d*) mitotic activity of cardiomyocytes in the marginal zone after 7 days, ×1000; *e*) intense formation of blood vessels in MI zone after 7 days, ×400; *f*) focal cardiosclerosis in "intact" myocardium after 30 days, ×100.

In group 1, the volume of collagen fibers in the marginal zone increased significantly by day 7 postoperation. Later this parameter continued to increase, reaching the maximum values by day 30. In group 2, the dynamics of the level of collagen fibers in the marginal zone was similar. The volume of collagen fibers increased 3 days after MI simulation. By day 30, collagen formation in the marginal zone was more intense in comparison with the earlier periods. However, the difference in comparison with group 1 was negligible.

According to light microscopy data, 40% animals in group 2 developed focal cardiosclerosis in intact myocardial zone by the end of the period of observation (Fig. 2, *f*). We regarded the small focal sclerotic process in the intact myocardium as an unfavorable side effect of high concentrations of VEGF. These changes could be caused by earlier and more intense attraction of fibroblasts into the focus of lesions and by their more pronounced synthetic activity, which manifested by greater volume density of collagen fibers.

Hence, the effect of intracardiac injection of VEGF on the postinfarction remodeling of the myocardium is determined by several morphogenetic events: stimulation of the cytoprotection system and the cardiomyocyte mitogenic activity; more intense angiogenesis in all the studied myocardial zones; prolonged and more intense leukocytic infiltration of the necrotic zone; more intense collagen formation in the necrotic zone and formation of cardiosclerosis foci in the intact myocardium. Intracardiac injection of VEGF in the above dose stimulated not only angiogenesis, but also cardiomyogenesis during the postinfarction period. However, the detected side effects (more intense collagen formation and development of focal cardiosclerosis) necessitate more precise definition of conditions for the use of VEGF for correction of organ and tissue ischemia.

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