MORPHOLOGY AND PATHOMORPHOLOGY

Angiogenesis after Transplantation of Auto- and Allogenic Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 4, pp. 442-447, April, 2010
Original article submitted September 15, 2009

Neoangiogenesis after transplantation of auto- and allogenic mononuclears and multipotent stromal cells from the bone marrow was studied on the model of inflammatory angiogenesis. Transplanted auto- and allogenic cells stimulate the formation of new blood vessels in the granulation tissue, this manifesting in an increase in the quantity and volume density of blood vessels. The most pronounced angiogenesis was observed after transplantation of allogenic mononuclears and multipotent stromal cells. It was associated with intense inflammatory infiltration, with less numerous and mature collagen fibers in the granulation tissue. Injection of allogenic cells led to stimulation and chronization of inflammation, infiltration with inflammatory and poorly differentiated cells, and more pronounced and lasting angiogenesis. However, neither auto-, nor allogenic transplanted labeled cells were detected in the walls of new blood vessels. Hence, it seems that bone marrow mononuclears and multipotent stromal cells stimulated angiogenesis mainly at the expense of production of angiogenic factors, and after transplantation of allogenic cells also by stimulating the inflammation.

Key Words: *granulation tissue; mononuclears; multipotent stromal cells; angiogenesis*

The development of new methods for the therapy of diseases associated with impaired blood supply to organs and tissues is an important problem. The strategy of therapeutic angiogenesis consists in restoration of blood supply to ischemic tissues by saturating them with endogenous and exogenous growth factors (recombinant proteins or genetic constructions), stem/progenitor cells (SPC) – endotheliocyte, smooth muscle cell, and pericyte precursors [12]. According to modern concepts, SPC transplantation stimulates angiogenesis by delivering growth factors and vascular wall cell precursors to ischemic tissues [7,12]. Neovascularization can be stimulated by various SPC types, for example, nonfractionated mononuclears, hemopoietic stem cells (HSC) [14], multipotent stromal cells (MSC) [11], endothelial cell precursors [4], and even fetal stem cells [9]. Cell transplantation leads to more intensive formation of new blood vessels. However, it remains not quite clear which cell type is the optimal transplant for angiogenesis stimulation.

Publications on the use of allogenic cells for angiogenesis stimulation do not provide sufficiently ample information. Importantly that allogenic cells carrying foreign antigens on their surface can cause, if transplanted, inflammatory reaction at the site of injection, which can serve as an additional mechanism stimulating neovascularization. We think that transplantation of allogenic cells can cause local inflam-

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mation without manifest alteration, that is, immediate exudation, migration, and proliferation of inflammatory cells and poorly differentiated precursor cells at the site of injection and thus stimulate angiogenesis. This mechanism of angiogenesis stimulation by transplantation of allogenic cells has never been discussed in literature. In order to verify this hypothesis, allogenic and autogenic mononuclears and bone marrow MSC were transplanted and their angiogenic potentials were compared.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (n=24; 180-200 g). Manipulations with laboratory animals were carried out in accordance with the bioethics philosophy and regulations of laboratory practice and corresponded to ethic standards presented in "International Recommendations on Biomedical Studies with the Use of Animals" (1985) and Order of the Ministry of Health of the Russian Federation No. 267 of June 19, 2006.

In order to induce the formation of granulation tissue, 1 ml suspension of fragmented hemostatic sponge (Spongostan, Ferrosan AG) in saline with and without cells was injected subcutaneously to ether-narcotized animals. This led after 7 days to the formation of granulation tissue around the transplant, in which angiogenesis could be evaluated qualitatively and quantitatively.

Each animal received (under ether narcosis) three transplantations along the dorsal midline at a distance of 3 cm from each other. The animals were divided into 6 groups, 4 rats and 12 objects for study per group. Group 1 rats (negative control) were injected with the hemostatic sponge suspension without cells. Group 2 (positive control) rats were injected with the hemostatic sponge suspension and 3×10^6 A204 human tumor (rhabdomyosarcoma) cells. Group 3 animals received injections of the hemostatic sponge suspension and 3×10^6 autogenic bone marrow mononuclears. Group 4 rats were injected with the hemostatic sponge suspension and 3×10^6 allogenic bone marrow mononuclears. Group 5 rats received the hemostatic sponge and 3×10^6 autogenic bone marrow MSC. Group 6 animals received the hemostatic sponge and 3×10^6 allogenic MSC.

Mononuclears and MSC were isolated as follows. Puncture exfusion of the bone marrow from the femoral and tibial bones through the knee joint cavity was carried out in animals narcotized with ether. Bone marrow mononuclears were isolated by the standard method in density gradient [5]. The MSC were isolated and cultured by the standard protocol [3]. The resultant cultures phenotypes were determined by specific positive (CD44, CD90, CD105) and negative (CD34, CD45) markers. The concentration of these cells in cultures was not lower than 80-90% in accordance with the cell culture passport. Functional activity of mesenchymal stromal cell culture was evaluated by capacity to directed differentiation into mesodermal cells (myogenesis, chondrogenesis, osteogenesis, adipogenesis) in standard media. Half of animals in each groups received cells labeled with PKH26 red fluorescent cell linker mini kit (Sigma) according to the instruction.

The rats were sacrificed 7 days after transplantation by ether overdosage. The material from animals injected with fluorescent labeled cells was cryofixed, cryosections were prepared, poststained with hematoxylin, and examined under a fluorescent microscope. The material from the rest animals was embedded in paraffin, sliced, stained after Mallory, and examined under a light microscope.

The following morphological parameters were evaluated in the granulation tissue around the transplant: number of blood vessels per mm^2, volume density, and the type of blood vessel by their wall thickness (arterioles, capillaries, venules). The composition of granulation tissue (cells, collagen fibrils, transplant, blood vessels) was analyzed by the dot counting method [1].

The means and standard deviations for absolute and percent parameters were evaluated. The values were compared by the Kruskal–Wallis rank unifactorial analysis of dispersions. Statistical analysis was carried out using SigmaPlot 11 software. The differences were considered significant at 5% confidence level.

RESULTS

Seven days after surgery, the site of transplantation was an oval compact formation colored pink to bright red (in accordance with the initial form of the transplant) with elastic glossy surface. Blood vessels growing from the periphery of these formations were easily discernible (Fig. 1, *a*, *b*).

Microscopic examination of histological preparations showed fragments of gelatin sponge forming compact accumulations in the center of this structure in all groups. A roll of loose fibrous connective tissue with numerous blood vessels formed at the transplant periphery, this tissue growing in places between the gelatin sponge particles. Pronounced cellular infiltration by round and spindle cells between the sponge particles was seen (Figs. 1, 2).

Transplantation of auto- and allogenic bone marrow mononuclears and MSC led to angiogenesis stimulation. The most effective stimulation of neovascularization was seen after allogenic cell transplantation (Fig. 1, *c*, *d*). The number and volume density of blood

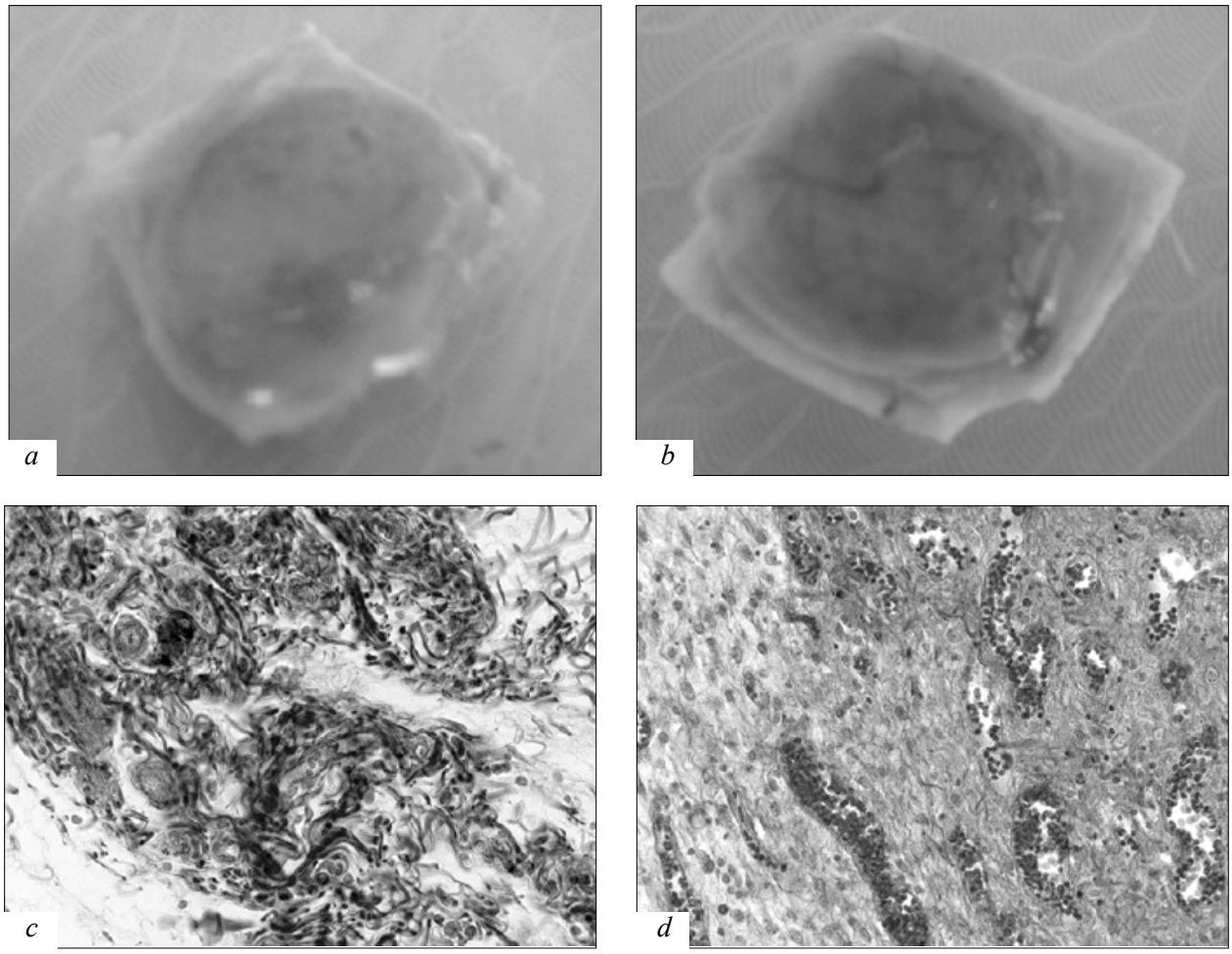

Fig. 1. Transplantation region (*a*, *b*) and granulation tissue (*c*, *d*) after 7 days in the negative control group (*a*, *c*) and after transplantation of allogenic mononuclears (*b*, *d*).

vessels were 301.1±132.9 vessels/mm^2 and 15.0±3.6% in the group injected with allogenic mononuclears and 299.6±101.3 vessels/mm^2 and 15.1±5.1%, respectively, in the group injected with allogenic MSC; this was significantly higher than in the negative control group (160.0±64.1 vessels/mm^2 and 4.3±2.0%; $p<0.05$). These values did not differ much from those in the positive control group (245.6±91.1 vessels/mm^2 and 13.5±4.8%, respectively; $p<0.05$). The quantity and volume density of blood vessels were 209.1±89.6 vessels/mm^2 and 8.3±2.8% after transplantation of autogenic mononuclears and 183.9±90.0 vessels/mm^2 and 14.0±4.9%, respectively, after transplantation of autogenic MSC, which was significantly higher ($p<0.05$) than in the negative control group. The volume density of blood vessels after transplantation of autogenic MSC was higher ($p<0.05$) than after autogenic mononuclears, while the numbers of vessels per mm^2 virtually did not differ; hence, new vessels in the autogenic MSC group were more mature.

The composition of granulation tissue was different in the groups (Table 1). Thicker and more numerous collagen fibers were found in the negative and positive control groups. The number of collagen fibers was significantly lower after transplantation of allogenic cells (maximally pronounced angiogenesis was seen in this group). The numbers of collagen fibrils

TABLE 1. Composition of the Granulation Tissue

Group	Collagen fibrils	Cells
Control	45.6±1.5	15.30±1.35
Autogenic mononuclears	37.2±1.7^{x}	17.40±1.64
Allogenic mononuclears	18.3±1.8^{*+}	26.90±2.24^{**++}
Autogenic MSC	27.00±4.12^{x}	27.20±2.45
Allogenic MSC	19.3±1.9^{*+}	26.20±2.13^{**++}
A204	42.1±2.7	16.40±2.24

Note. $^{*}p<0.001$, $^{**}p<0.05$ compared to the control; $^{+}p<0.001$, $^{++}p<0.05$ compared to A204; $^{x}p<0.01$ compared to allogenic MSC or allogenic mononuclears.

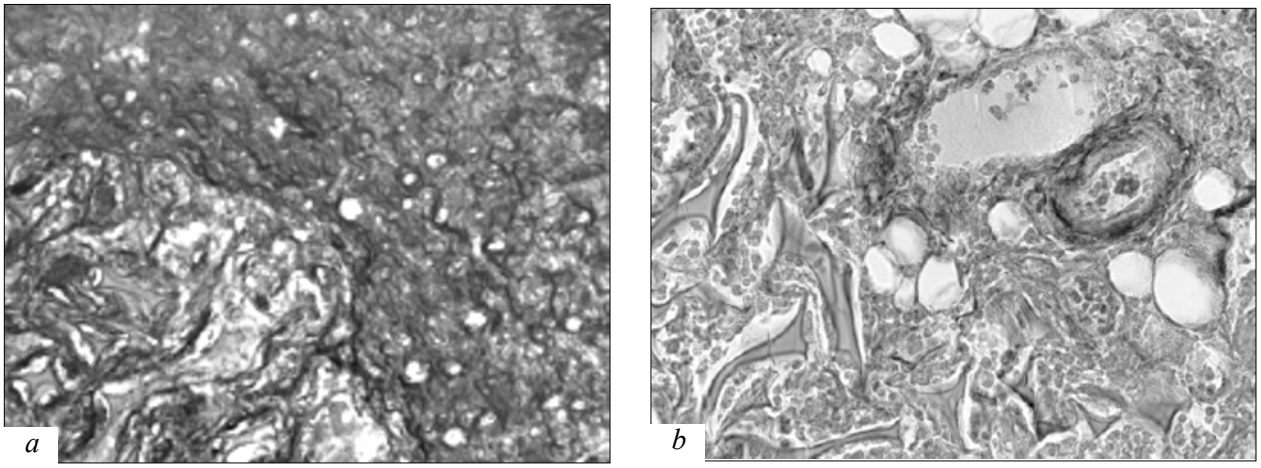

Fig. 2. Granulation tissue around the transplant 7 days after injection of allogenic mononuclears. Mallory's staining; ×10 (*a*), ×40 (*b*).

were significantly lower ($p<0.001$) after transplantation of autogenic mononuclears and MSC than after injection of allogenic cells.

The intensity of neovascularization and cellular infiltration formed a feedback relationship. The percentage of infiltrated cells in the granulation tissue was significantly higher in the allogenic mononuclears and MSC groups than in the positive and negative control groups. The highest percentage of cells in granulation tissue was found in the autogenic MSC group, presumably because of transplanted cells remaining in the transplant. Cell counts in the transplant were lower after injection of autogenic mononuclears: the cellular infiltration constituted just 17.4±5.2%. Presumably, some cells migrated to hemopoietic organs, as an appreciable fraction of mononuclears was presented by hemopoietic cells.

Qualitative composition of new blood vessels was different in the groups (Table 2). Arterioles and venules were more often detected in the granulation tissue of animals injected with allogenic cells. The new blood vessels in the negative control group were presented mainly by capillaries, their number being significantly higher than in the rest groups.

Fluorescent microscopy showed that labeled cells migrated from the transplant to the adjacent granulation tissue and were evenly distributed in it in all groups (Fig. 3). However, no data indicating differentiation of transplanted cells into endothelial cells of the inner vascular wall and to tunica media smooth muscle cells were obtained for any of the experimental groups. Labeled cells were rarely detected in the adventitial membrane; its borderline was blurred and fused with the adjacent loose fibrous connective tissue.

Transplantation of auto- and allogenic mononuclears and MSC led to angiogenesis stimulation. The number and volume density of vessels increased. No data indicating differentiation of transplanted cells into endotheliocytes or smooth muscle cells were obtained, because no labeled cells were detected in the intima and media of new vessels. It seems, that the synthesis

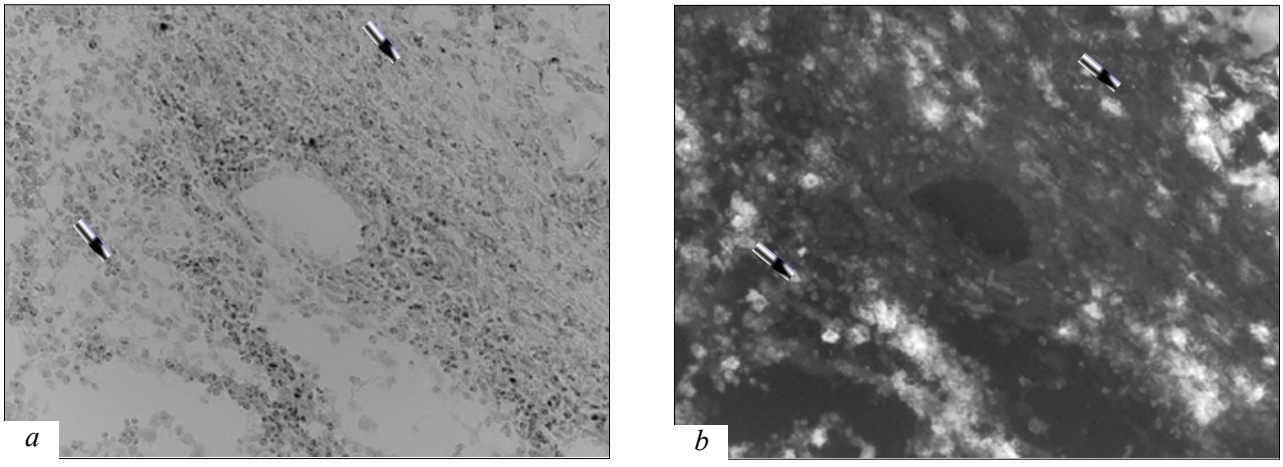

Fig. 3. Location of transplanted labeled cells (arrows). *a*) light microscopy; *b*) fluorescent microscopy.

TABLE 2. Qualitative Composition of New Blood Vessels

Group	Capillaries	Venules	Arterioles
Control	51.0±5.0	28.0±4.5	21.0±4.1
Autogenic mononuclears	32.0±4.7*	45.0±5.0*+	23.0±4.2
Allogenic mononuclears	36.0±4.8*	30.0±4.6	34.0±4.7*
Autogenic MSC	31.0±4.6*	46.0±5.0*+	23.0±4.2
Allogenic MSC	26.0±4.4*	48.0±5.0*+	26.0±4.4
A204	38.0±4.9	34.0±4.7	28.0±4.5

Note. $p<0.05$ compared to: *negative control, +positive control.

and release of angiogenic factors, such as VEGF, Ang1 and 2, FGF, PDGF, *etc.* is the main mechanism of angiogenesis stimulation after transplantation of autogenic and allogenic bone marrow cells [8]. Injection of autogenic MSC leads to the formation of granulation tissue with a higher volume density than after injection of autogenic mononuclears, the number of blood vessels being the same. Angiogenesis was maximally pronounced after transplantation of allogenic cells, when granulation tissues had the higher cell counts and less numerous and mature collagen fibers. Transplantation led to the development of inflammatory reaction and formation of granulation tissue, while injection of cells, primarily allogenic, led to stimulation and chronic transformation of inflammation, cellular infiltration, and more pronounced and longer angiogenesis.

At present, the relationship between inflammation and angiogenesis is obvious [10,13]. Angiogenesis in tissue ischemia is an obligatory component of inflammatory process. Blood vessels (granulation tissue) form during the productive stage of inflammation along with other reparative processes. Angiogenic growth factors, for example, angiopoietin II, regulating angiogenesis, are potent proinflammatory factors [6]. Endotheliocytes, smooth muscle cells, and pericytes are directly involved in inflammatory reactions. Hence, angiogenesis process can be modulated indirectly through modulation of various inflammation stages (alteration, exudation, and reparation).

For example, laser revascularization is used to restore myocardial circulation in coronary disease [2]. This method consists in the following: microinjuries are inflicted, not leading to impairment of myocardial function, but causing local inflammatory reactions and effectively stimulating angiogenesis. Transplantation of allogenic cells also causes an inflammatory reaction, but without manifest alteration stage, that is, direct exudation, migration, and proliferation of inflammatory cells and poorly differentiated precursor cells at the site of injection; hence, angiogenesis is stimulated, which is not the case with autogenic cells not eliminated by the immune system.

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