Biocompatibility of Tissue Engineering Constructions from Porous Polylactide Carriers Obtained by the Method of Selective Laser Sintering and Bone Marrow-Derived Multipotent Stromal Cells

T. B. Bukharova[1,3], E. N. Antonov[2], V. K. Popov[2], T. Kh. Fatkhudinov[1,4], A. V. Popova[2], A. V. Volkov[1,4], S. A. Bochkova[2], V. N. Bagratashvili[2], and D. V. Gol'dshtein[1,3]

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We studied the biocompatibility of porous polylactide carrier matrices obtained by means of surface selective laser sintering. Carrier matrices had no cytotoxic activity, but maintained adhesion and proliferation of cells. Subcutaneous transplantation of tissue engineering constructions from these carriers and bone marrow-derived multipotent stromal cells did not cause the inflammatory response and pathological changes in rats. The conditions for organotypic regeneration were provided at the site of transplantation (high degree of blood supply and considerable amount of immature precursor cells).

Key Words: *tissue engineering construction; polylactide carriers; selective laser sintering; multipotent stromal cells; regeneration*

The development of tissue engineering constructs (TEC) from three-dimensional (3D) biodegradable matrices, which are resorbed *in vivo* with the repair of the damaged, is one of the main trends in modern tissue engineering. The matrices are prepared from various materials (ceramics, bioactive glass, *etc.*). Bioresorbed polymers hold much promise in this respect [6]. There is a variety of methods and technologies for the preparation of polymeric matrices (*e.g.*, rapid prototyping). They are based on layer-by-layer preparation of 3D structures of random shape and internal architecture [4]. The use of these structures allows us to perform a wide-range fine regulation of the porosity and architectonics of the matrices. Moreover, the composition of the constituent materials can vary under these conditions. Modern computer technologies allow preparing structures with reproducible characteristics. Several years ago, we developed a new method of surface selective laser sintering (SSLS). It serves as a specific general technique of rapid prototyping. This rapid method provides high accuracy in the preparation of carriers. The proposed method allows us to synthesize matrices with specific internal structure from thermally unstable materials (*e.g.*, various bioresorbed polymers). It should be emphasized that the physicochemical properties of materials remain unchanged under these conditions [1].

Bone marrow-derived mesodermal multipotent stromal cells (MSC) possess high proliferative activity and differentiation potential. They hold much promise in cell therapy for reparation of damaged tissues or organs [5,8]. MSC provide inductive potential of constructions. The stimulation of regenerative processes

[1]ReMeTeks Closed Corporation, Moscow; [2]Institute for Problems of Laser and Information Technologies, Russian Academy of Sciences, Troitsk; [3]Medical Genetic Research Center; [4]Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** bukharova-rmt@yandex.ru. T. B. Bukharova

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by MSC is associated with the release of biologically active factors and, probably, with replacement of lost cells. Mobilization of precultured MSC on a biocompatible carrier provide targeted transport, high concentration of cells in the site of injection, good survival of transplanted cells, and genotyped differentiation.

Porous 3D structure with a system of open interconnected pores is an optimal configuration of a TEC carrier. This structure provides sufficient circulation of the culture medium in the carrier volume [7]. This spatial configuration provides the highest degree of cell inoculation in the carrier and improves cell survival in the matrix. Moreover, this structure provides favorable conditions for the growth of blood vessels and migration of recipient's cells. The composition and strength of the synthetic matrix can vary during SSLS (due to different degree of sintering) [1], which allows us to regulate the time of TEC resorption in tissues [9].

TEC was prepared from polylactide matrices (lactic acid polymer). Polymers from lactic and glycolic acids and copolymers were extensively studied. These safe structures were approved for medical practice. Degradation products (carbon dioxide and water) are nontoxic. They are eliminated from the organism by common metabolic pathways [3].

This work was designed to study biocompatibility of polylactide carriers obtained by means of SSLS. We developed a new method for the preparation of TEC with these carriers by using bone marrow-derived MSC. An experimental study was performed to evaluate the type of reparative processes and rate of polymer resorption.

MATERIALS AND METHODS

Carrier matrices were prepared from polylactide (Poly(D,L)-lactic acid, molecular weight 83 kDa; Alkermes) by the method of SSLS [1]. The powder of polylactide (particle size ~200 μ) was sintered layer-by-layer on a SLS-100 selective laser sintering device (Institute for Problems of Laser and Information Technologies). A single-mode fiber laser (IRE-Polyus) was used as the source of radiation. The emission wavelength was 1.06 μ. The power did not exceed 10 W. A sensitizing agent carbon (0.1 wt %, particle size ~100 nm) was added to the powder of polylactide.

The matrices appeared as discs (diameter 12 mm, height 4 mm) with a 3D cell structure (Fig. 1). The size of cells was 0.6×0.6 mm. The width of sintered tracks was ~0.8 mm. The surface of sintered powder was scanned with a laser beam (20 cm/sec).

We prepared two types of matrices, which differed by the intensity of laser radiation. To change the intensity, laser beam diameters in the surface of sintered powder were 125 and 800 μ. The intensity of radiation was adjusted to obtain the same width of sintered tracks. In the first case, the width of sintered tracks was 1.5-fold greater than in the second case.

Experiments were performed with MSC culture from human bone marrow. The fraction of MSC was isolated by the standard method [2]. MSC were cultured in DMEM/F12 1:1 medium, which contained 2 mM L-glutamine (PAA Laboratories), 10% FBS (HyClone-Perbio), and 0.5 mg/ml ammonia (Sintez). Culturing was conducted under standard conditions (37°C, 5% CO_2, saturation humidity).

Immunophenotyping of the cell culture was performed on a FACS Caliber flow cytofluorometer (BD Biosciences). We revealed that 60-90% cells express stromal markers CD90 and CD105, but were negative for CD34. Functional activity of MSC was evaluated from the ability for directed differentiation into mesodermal lines (myogenesis, chondrogenesis, osteogenesis, and adipogenesis) in inductive media.

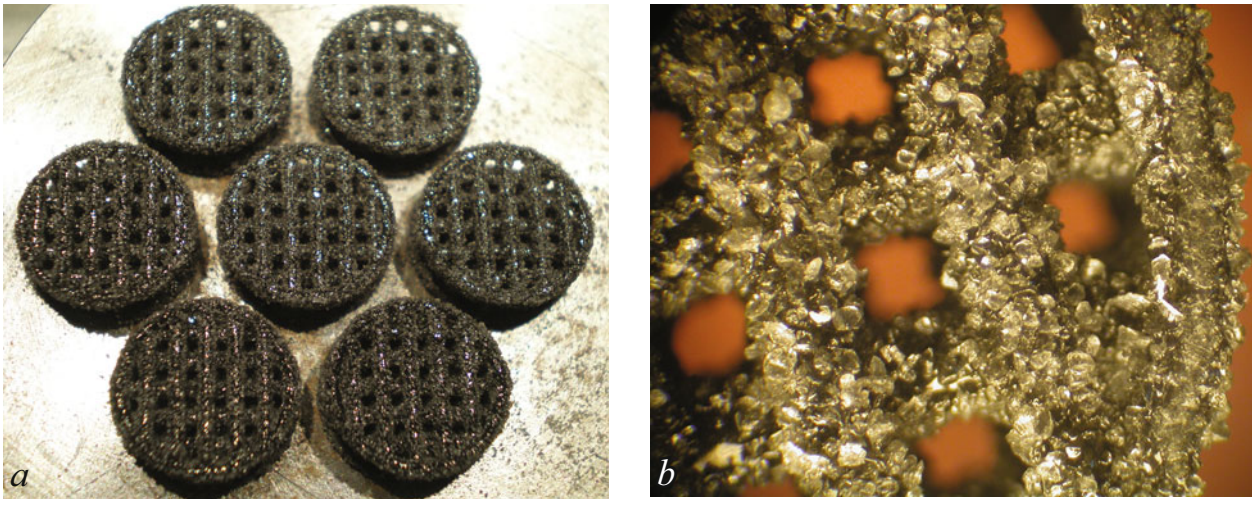

Fig. 1. Carrier matrices; preparation by the method of SSLS. Magnification: (*a*) ×2; (*b*) ×15.

Cytotoxicity of study samples was estimated in the MTT test. MSC were placed in 96-well plates. The density of inoculation was 5000-10,000 cells per well. One day after adhesion, the samples were put in wells for 1 and 7 days. Control wells contained no cells. Methyl tetrazolium tetrabromide (MTT; PanEco) was added in a concentration of 0.5 mg/ml. Incubation was performed at 37°C for 2 h. The degree of staining was evaluated visually. The reaction was stopped by removal of MTT-containing medium. Formazan was eluted with DMSO (PanEco). Formazan absorption was calculated. The baseline value at 620 nm was subtracted from optical density of the eluate at 540 nm. The measurements were performed using a Fluoroscan Ascent photometer (Labsystems).

The cell suspension was prepared. MSC were removed from Petri dishes with Versen solution and 30% trypsin (PanEco) and centrifuged at 1100 rpm and 18°C for 10 min. The pellet was resuspended in the culture medium.

The matrixes were sterilized and put in 24-well plates (Nunclon). They were covered with the suspension, which contained 2-3 million cells per ml medium (100 µl suspension per sample). Cell adhesion to the wall of pores was induced by incubation of the cell suspension for 60 min under standard conditions. The culture medium was added. After 1 day, the samples of cells were put in other plates and cultured under standard conditions. The growth medium was replaced at 2-day intervals.

Before transplantation, TIC was washed 3 times with physiological saline (PanEco) at room temperature.

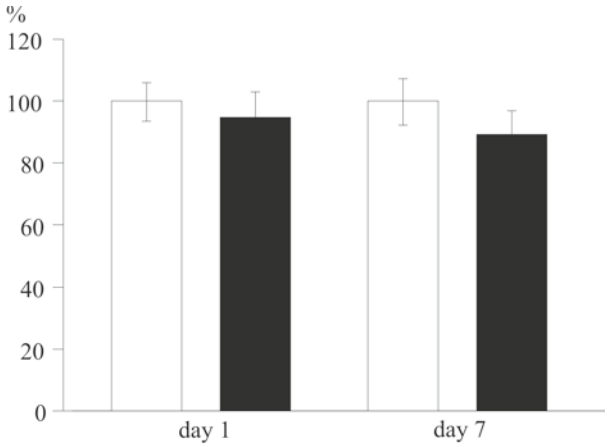

Fig. 2. MTT test: relative optical density. Light bars: control, dark bars, experiment.

The degree of cell adhesion and density of sample inoculation with MSC were evaluated by scanning electron microscopy (SEM) and light microscopy. The study was performed with a vital fluorescent marker PKH26 (red fluorescent cell linker kit, Sigma).

For SEM, the samples were washed with PBS, fixed with 2.5% glutaraldehyde (Pancreac) for 24 h, dehydrated, and dried with hexamethyldisilazane. Hexamethyldisilazane provides preservation of the polymer structure. The samples were covered with a layer of gold (10 nm) in an IB 3 device (Eiko) and examined under an S-570 scanning electron microscope (Hitachi) at accelerating voltage of 15 kV.

Vital labeling of cells was performed with a membrane fluorescent marker PKH26. PKH26 kit includes

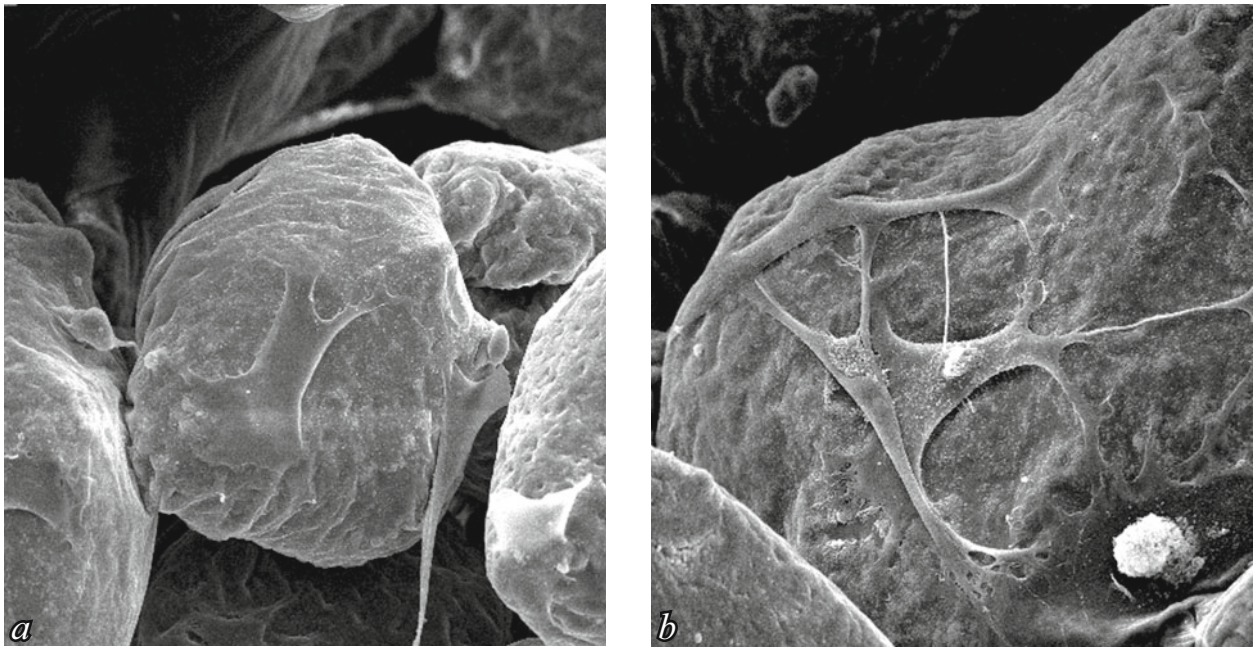

Fig. 3. MSC adhesion on the surface of polalactide carriers after 3-day culturing (SEM, ×600). (*a*) individual cells; (*b*) clusters of cells.

the stock solution of PKH26 and solvent C. Before staining, the cell suspension was washed with a serum-free medium and centrifuged at 1100 rpm for 5 min. The pellet was resuspended in solvent C. An equivalent volume of 4×10^{-6} PKH26 was added. Incubation was performed for 5 min. Staining was stopped by adding 1% BSA (PanEco). The samples were examined under an Olympus CK 40 inverted light microscope equipped with an Olympus camedia C-5050 hardware-software complex (Olympus).

Experiments were performed on adult male outbred rats weighing 180-200 g. TEC was implanted subcutaneously on the back of animals to study grafting, neoangiogenesis, and invasion of TEC with recipient tissue. The hair on the back was removed, the skin on the withers and 4 cm caudally was cut, the fasciae were bluntly drawn apart, and TEC was inserted into both beds. The animals were euthanized by ether overdose on days 30 and 90.

Tissue samples were fixed in 10% neutral formalin. Tissue fragments were embedded into paraffin. Serial sections were prepared by the standard method. The sections were stained by the Mallory method and with hematoxylin and eosin. The samples were examined under an Axioplan-2 microscope (Zeiss).

RESULTS

MTT test showed that the test samples produced no toxic effects on MSC (Fig. 2). This conclusion was derived from the absence of statistically significant differences between the treatment group and control (without sample).

On day 3 of culturing, the cells were spread over the surface of the matrix and closely adhered to it (Fig. 3). Each cell was mainly located on an individual granule of polylactide. Some cells had processes that were attached to the adjacent granules (Fig. 3, *a*). The cells were regularly spread over the surface of the carrier. Some cells were arranged in clusters (Fig. 3, *b*).

The density of cells was evaluated from microphotographs of fluorescently labeled cells (Fig. 4). On day 3 of culturing, the cells were found on the surface and in the pores of the carrier. They were located sepa-

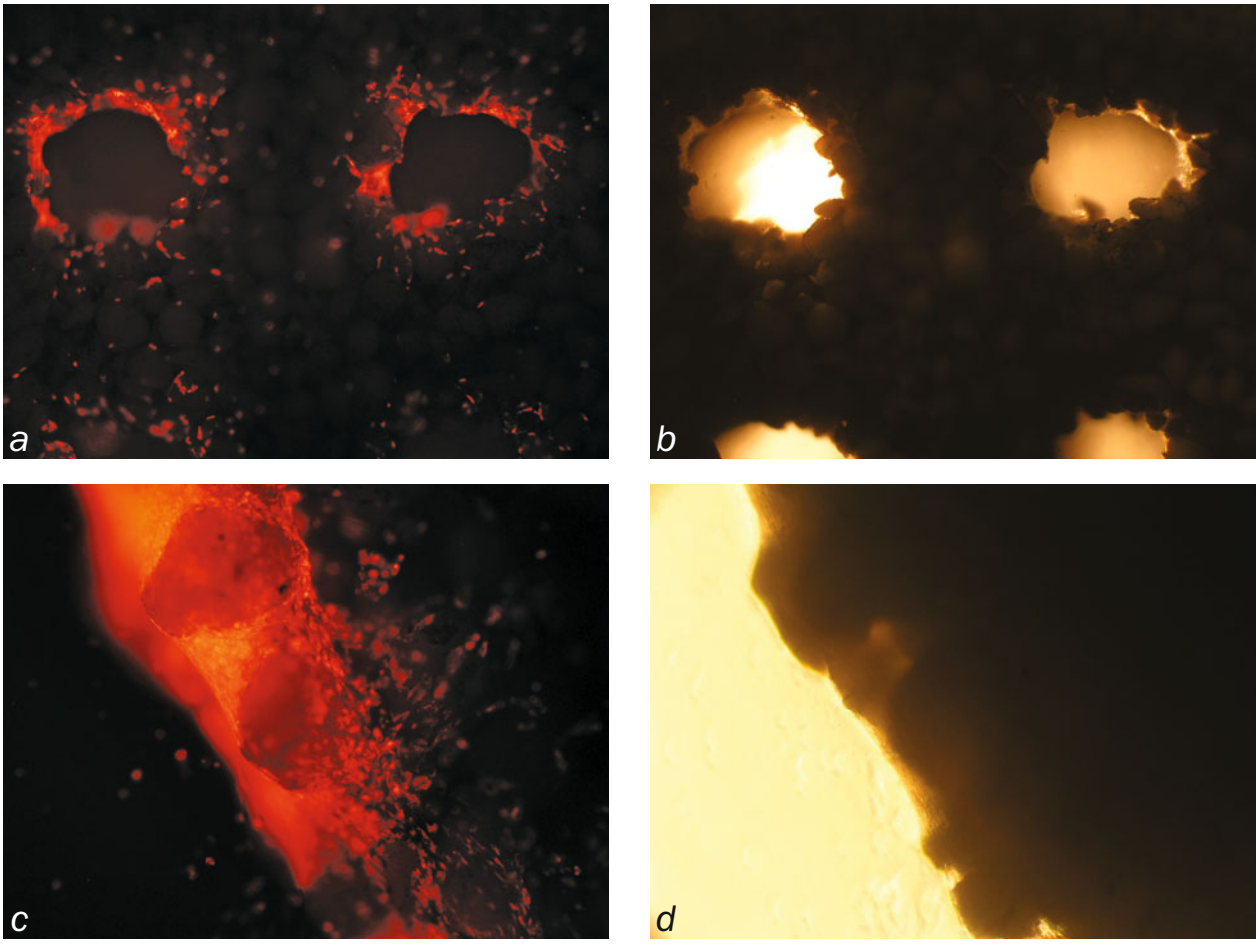

Fig. 4. Density of SMC on polylactide carriers after culturing for 3 (*a*, *b*) and 6 days (*c*, *d*; light microscopy, ×40). (*a*, *c*) PKH26-labeled cells; (*b*, *d*) non-labeled cells.

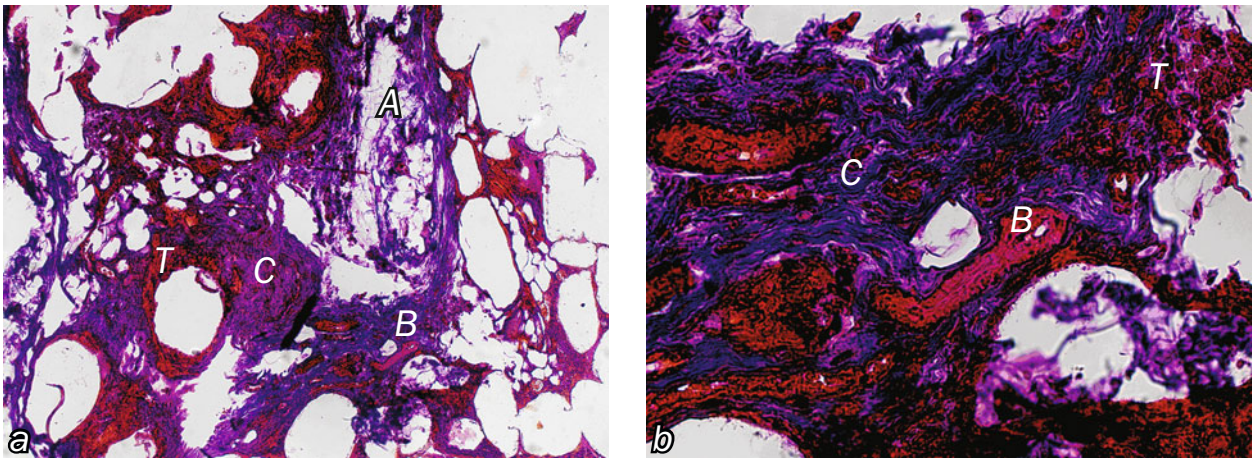

Fig. 5. Transplantation, day 30 (Mallory's staining). Here and in Fig. 6: magnification: (*a*) ×10; (*b*) ×40. *T*, transplant; *C*, connective tissue; *A*, adipose tissue; *B*, blood vessel.

rately or arranged in groups. After 6-day culturing, the cells were arranged in dense clusters and formed a multilayer structure. The density was highest at the surface of the carrier (most favorable conditions).

TEC was a porous polylactide carrier of the standard size. It was populated with bone marrow-derived allogeneic MSC (culturing for 1-3 passages). The carrier included 200,000-300,000 immobilized cells. The cells were incubated on the carrier for 7 days. The optimal time of incubation was selected empirically. Cell viability was at least 85%. Immunophenotyping showed that the expression of stromal markers CD90 and CD105 is typical of 60-90% cells. CD34 expression was not detected. TEC was transported in glass flasks with 5 ml physiological saline at 25-37°C for not more than 3 h. The glasses were not shaken.

The animals were euthanized on day 30 after surgery. A wide strip of the skin was excised on the back of animals. The localization of cell transplant was visualized in the subdermal area. The site of transplantation was an oval compact structure, which fit the initial shape of TEC. It had a grey color and bright elastic surface. The growth of blood vessels was well pronounced in the peripheral region of these structures. The signs of granulation were observed in some samples, which is typical of active angiogenesis.

Histological study revealed the presence of TEC particles in samples. It was fragmented after the delivery through a series of alcohols and chloroform (partial dissolution of lipids). The peripheral region of the transplant was surrounded by a thin wall of loose fibrous connective tissue. This tissue grew into the pores of TEC. Loose fibrous connective tissue that surrounded and invaded the structure of TEC had a granular nature. It included a considerable number of blood vessels with different diameters. The tissue was strongly infiltrated with immature fibroblast-like cells. These signs reflect the process of tissue formation around and inside TEC (Fig. 5).

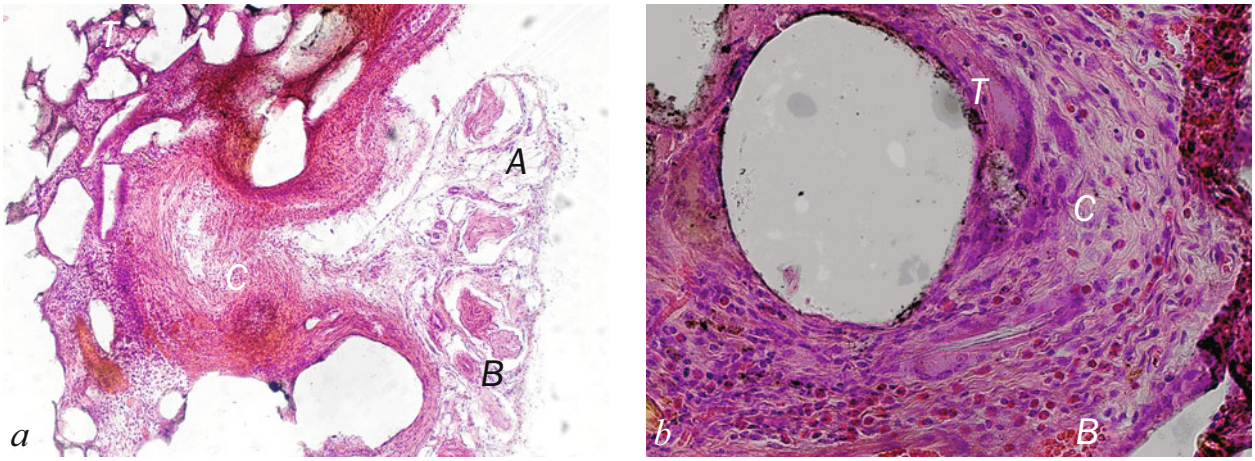

Fig. 6. Transplantation, day 90 (hematoxylin and eosin staining).

Granulation tissue was presented by loose fibrous connective tissue with a large number of vessels (primarily capillaries). The tissue was infiltrated with inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and erythrocytes) and germ cells for connective tissue regeneration (immature fibroblasts, adventitial cells, and pericytes). The latter cells had a spindle shape, light cytoplasm, and large light nucleus. Some regions of connective tissue were characterized by a more regular and dense arrangement of collagen fibers. Adipose tissue regions were identified.

On day 30, the vessels were mainly presented by capillaries with a narrow lumen. The wall of capillaries consisted of endothelial cells, basal membrane of these cells, and pericytes. Additional layers of the wall in other vessels were formed from smooth muscle cells and connective tissue cells. Large venules and arterioles were rarely found.

On day 90, the site of transplantation was an oval compact structure, which fit the initial shape of TEC. However, the structure had a smaller size. The initial diameter of TEC or carrier matrix was 12 mm. On day 30 after subcutaneous implantation, the size of this transplant and connective tissue capsule was 14±2 mm (p>0.05). On day 90, the size of the transplant and capsule in both groups was 8±5 mm. The decrease in the size of the transplant reflects slow resorption of the carrier matrix. No differences were found in the size of carriers after treatment with laser beams of various diameters.

Histological study on day 90 showed that small particles of TEC are covered by massive layers of the connective tissue (Fig. 6). The peripheral region of the transplant was surrounded by a wall of loose fibrous connective tissue. This tissue grew into the pores of TEC. The connective tissue was characterized by the presence of thick oriented bundles of collagen fibers, lower number of cells, and smaller amount of blood vessels. They were mainly presented by large blood vessels with a thick wall (arterioles).

Our results indicate that carrier matrices from polylactic acid (technique of SSLS) have no cytotoxic activity, but maintain the adhesion of cells. The 3D porous structure of the carrier provides an adequate supply of oxygen and nutrient substances to the cells. This is observed at the surface of study samples and inner wall of the pores. Pathological signs, severe inflammatory infiltration, neoplastic processes, and other changes in surrounding tissues were not found after subcutaneous transplantation of TEC from polylactide carriers and MSC. The general state of animals was satisfactory in various periods of the study. Granulation tissue with a large number of blood vessels and immature proliferating cells was formed inside and around the transplant. Transplantation of TEC from a carrier of polylactic acid and bone marrow-derived MSC to the abnormal tissue or organ provides the conditions for organotypic regeneration (high degree of blood supply and considerable amount of immature precursor cells). The observed changes and paracrine influences of the microenvironment contribute to regeneration of lost tissues.

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