*Elasto*PHB® Membrane Systems with Immobilized Bone Marrow Stromal Cells Optimize Conditions for Regeneration of Damaged Tissue

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The effects of autologous bone marrow stromal cells immobilized on *Elasto*PHB^R membranes on reparative processes were studied on a model of rat skeletal muscle injury. Bone marrow stromal cells inhibited substitute (sclerosing) regeneration and activated reparative (reconstructive) regeneration of tissues.

Key Words: *bone marrow stromal cells; ElastoPHB^R membrane; regeneration*

Cell transplantation now becomes a potent instrument in the treatment of diseases associated with local injuries to the locomotor system tissues (bone, cartilaginous, and muscular [17,16]), skin [5], *etc.* An important problem is the development of carriers, temporary polymeric biodegraded two-dimensional (membranes) or three-dimensional (sponges, gels) matrices (carcasses), delivering viable cells into the pathological foci, keeping them there, and optimizing conditions of their therapeutic effect. At the stage of culturing these carriers can create conditions for the formation of structures ensuring optimal level of cell functioning. The requirements to implanted matrices are controlled time of their biodegradation, high degree of biocompatibility at the protein and cellular levels, absence of toxicity of the carrier and products of its biodegradation, capacity to pore formation during contact with biological media, and sufficient strength to endure surgical manipulations [8].

Membranes based on polymers and copolymers of chemical (lactic and glycolic acid copolymers) and natural (including bacterial) origin (chytosan, hyaluronic acid esters, alginate, collagen, silk fibroins, polyhydroxybutyrate (PHB) and its copolymers) were studied as biomaterials for organization of cell growth and differentiation to be used for wound and burn healing, for preventing the formation of postoperative adhesions, for temporary replacement of damaged or lost tissues (*e.g.*, bone tissue) [13,18-20,24].

Polyesters of bacterial origin now occupy a special place among biodegraded materials: PHB, polyoxyoctanoate, β-hydroxybutyrate and β-hydroxyvalerate copolymer (PHB-co-PHV), which possess all properties of implanted matrices.

Unfortunately, these homopolymers and the copolymer are not sufficiently strong and elastic; they are hydrophobic, which essentially limits their use. In order to improve the hydrophilic and elastic characteristics and thus improve the membrane compatibility with cells and tissues, *Elasto*PHB® biodegraded material was created at Center for Studies of Biomaterials, Institute of Transplantology and Artificial Organs [14]. This material contains high-molecular hydrophilic plastisizer, improving hydrophilic and elastic properties of the membrane. Incorporation of high-molecular hydrophilic plastisizer in the composition of PHB-co-PHV bacterial copolymer appreciably improved its mechanical characteristics without deteriorating biocompatibility of the biopolymer. *Elasto*PHB® samples exhibit no local irritating, sensitizing, and

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toxic effects, they are hemocompatible, apyrogenic, can be sterilized, and meet all requirements to materials intended for long contact with tissues [6,8].

We studied the possibility of using *Elasto*PHB® biodegraded membranes for immobilization and delivery of bone marrow stromal cells into the focus of injury and evaluated the effects of this bioconstruction on reparative processes in damaged tissues on a model of skeletal muscle damage.

MATERIALS AND METHODS

The study was carried out on 8 Wistar rats (250-300 g). All manipulations were performed under intraperitoneal narcosis (ketamine, 50 mg/kg, and xylasoline, 10 mg/kg). The animals were divided into 4 groups. Group 1 rats (n=2) were implanted *Elasto*PHB® membrane onto intact thoracic muscles on both sides. In group 2 the membranes were implanted onto damaged (resected) areas of thoracic muscles on both sides from the sternum (n=2). In groups 3 and 4 (2 animals per group) the membranes with immobilized bone marrow cells were implanted on damaged thoracic muscles to the right and left from the sternum so that the cells contacted with the wound surface. The difference between groups 3 and 4 was as follows: in group 4 stromal cells during culturing were additionally treated with 5-azacytidine for demethylation of some DNA sites responsible for cell differentiation.

Stromal cells were derived from bone marrow mononuclear fraction of rat femur. The mononuclear fraction was isolated using solution for erythrocyte lysis [4]. The mononuclear fraction was transferred to Petri dishes (100,000 cells/cm^2) in IMDM (Sigma) with 10% FCS (Hy-Clone) and cultured at 37°C, 5% CO_2, and 95% humidity. After 48 h the medium was completely replaced and nonadherent cells were discarded. After 10 days of culturing (100,000 cells/cm^2) adherent cells were transferred to 6-well plates. Each well contained a 1.5 cm^2 membrane coated with fibronectin (Sigma) fixed on the bottom. The cells were cultured for 7 days. Before transplantation the membranes were washed in Hanks' solution. Cell cultures for group 4 were treated with 5-azacytidine, which was added to the growth medium in a concentration of 6 μmol/liter for 24 h.

Substrate for cell immobilization was sterile samples of *Elasto*PHB® membranes (rectangular 0.5×1.0 cm membranes 70±5 μ thick) made under laboratory conditions in Center for Studies of Biomaterials served as. The *Elasto*PHB® components were PHB-co-PHV copolymer with 15-30% hydroxyvalerate (M=295-360 kDa, 50-60% crystallinity, from Institute of Biophysics, Siberian Division of Russian Academy of Sciences, Krasnoyarsk) and high-molecular-weight hydrophilic plastisizer improving the hydrophilic and elastic characteristics of the material [6].

Transplantation. Thoracic muscles of narcotized animals were exposed. Surface muscles were bluntly detached along the fascia. In group 1 animals one membrane was sutured to the surface of intact muscles on each side. In other groups a deeper muscle fragment (0.5×0.5×0.2 cm) was dissected from each side. After hemostasis the membrane was sutured (by interrupted suture) to the surface of damaged muscle. In group 2 the membrane contained no immobilized cells, while in groups 3 and 4 membranes with immobilized autologous bone marrow stromal cells were used. The transplants were covered with surface muscles in all experimental groups.

Biopsy specimens were collected 4 weeks after transplantation. Sections were stained with hematoxylin and eosin. The type and degree of inflammatory and reparative reaction in the damaged and intact muscle tissues in zones adjacent to the membrane were evaluated. Chloroform used for the treatment of histological preparations dissolved the membranes and fissure-like cavities were seen at sites of their location.

Identification of transplanted cells in biopsy specimens was performed using recombinant deleted adenovirus containing *E. coli* LacZ gene producing bacterial β-galactosidase in live cells. The virus was added to the culture medium 24 h before transplantation for stromal cell infection. The survival of bone marrow stromal cells after transplantation was evaluated on cryostate sections of biopsy specimens; the sections were immunohistochemically stained for β-galactosidase activity product after incubation with X-Gal substrate [13].

RESULTS

Stromal cells seeded onto fibronectin-coated plastic with membranes flattened and proliferated forming after 7 days a 80-95% monolayer on membrane-free plastic areas and a 60-70% monolayer of stromal cells on the membranes (Fig. 1). No morphological differences and differences in proliferative activities of cells treated and not treated with 5-azacytidine were detected.

The membranes were sufficiently strong and elastic: damaged muscles could be easily wrapped in them and the membranes were tightly fixed to intact tissues by interrupted suture with a 8/0 thread. In none animal pyonecrotic complications developed in the postoperative wounds.

Immobilized cells rapidly left the membrane [16]. The data of the present study confirm that these cells are highly tropic to native tissues: 1 month after trans-

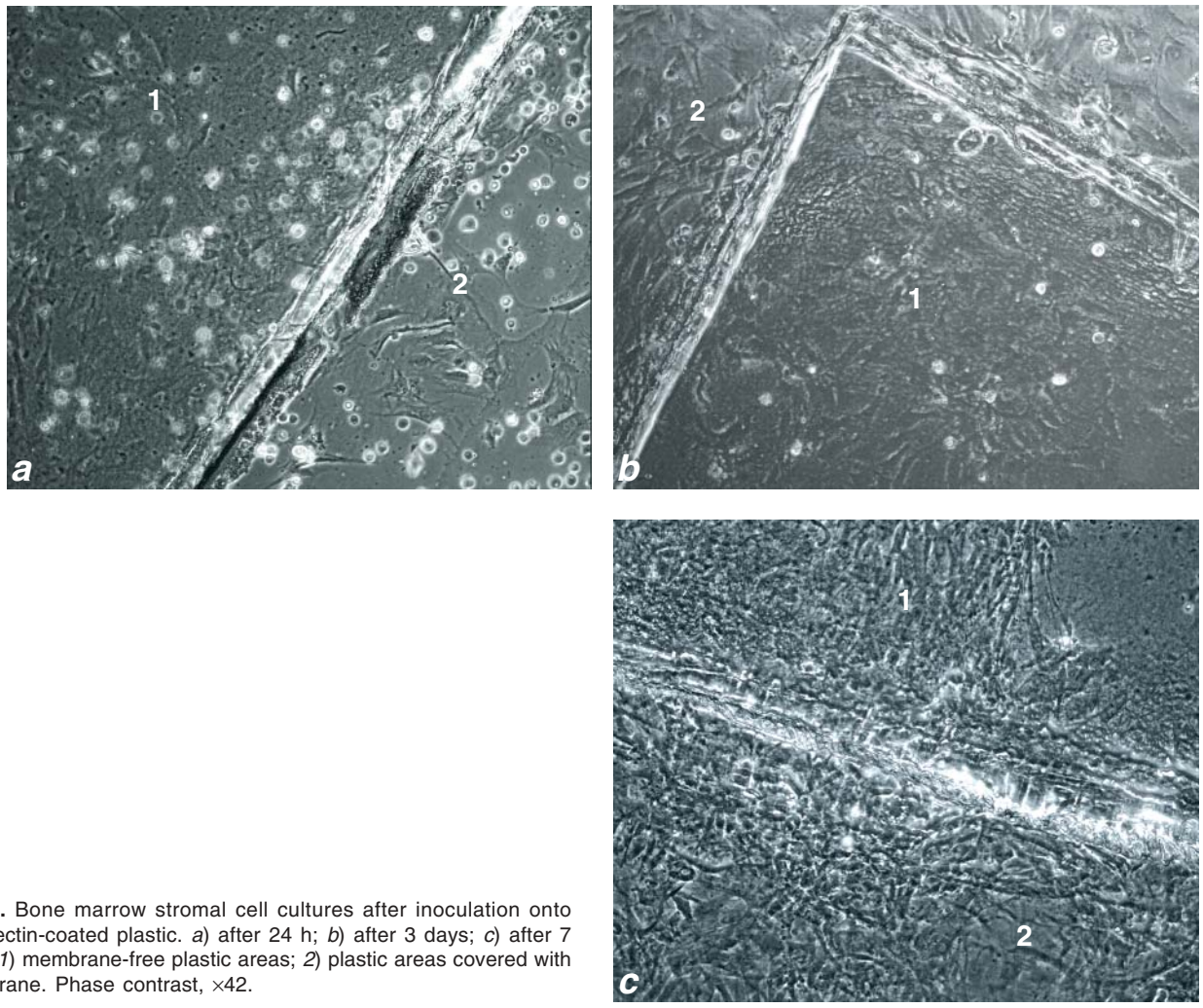

Fig. 1. Bone marrow stromal cell cultures after inoculation onto fibronectin-coated plastic. *a*) after 24 h; *b*) after 3 days; *c*) after 7 days. *1*) membrane-free plastic areas; *2*) plastic areas covered with membrane. Phase contrast, ×42.

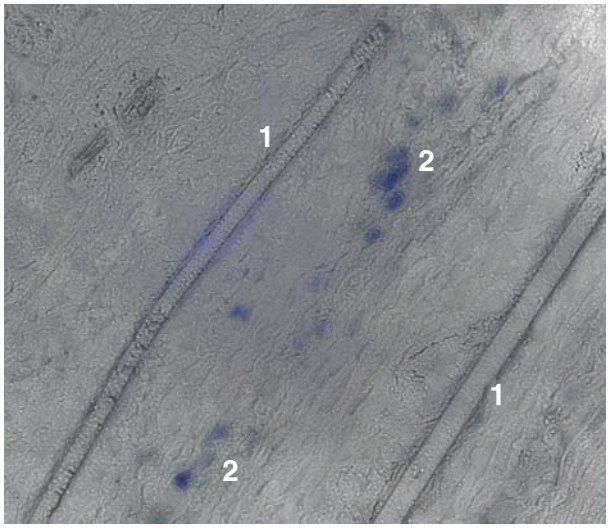

Fig. 2. Fragment of resected muscle wrapped in membrane with immobilized bone marrow stromal cells labeled by LacZ gene 1 month after implantation. *1*) membrane; *2*) cells expressing LacZ gene. Immunohistochemical staining for β-galactosidase activity product, detected after addition of X-Gal substrate, ×120.

plantation they were identified only in tissues adjacent to the membrane (Fig. 2).

Detection of cells in tissues by the product of β-galactosidase activity indicates that transplanted cells are viable and regulate reparative processes in damaged muscles, which confirms that *Elasto*PHB® membranes can be used as biocarriers.

Degradation of hydroxybutyrate homo- and copolymers in biological media is realized by the humoral and cellular pathway with active participation of macrophages and giant multinuclear cells (GMC) [2]. One month after transplantation, loose connective tissue containing numerous connective tissue cells and macrophages formed around the membrane in groups 1 and 2. Granulomas around foreign bodies (partially resorbed membrane) with GMC were sometimes seen (more often in group 2, Fig. 3).

These results are in line with the data on biodegradation of suture threads from PHB and PHB-co-PHV. One month after implantation a connective-tissue capsule formed around sutures, the counts of active macrophages and GMC and activity of acid

phosphatase increased. The capsule consisted of fibroblasts and collagen fibers [2].

Biopsy specimens of muscles from group 3 animals contained, in contrast to those from groups 1 and 2, only solitary GMC, the membrane fragments were found not in the connective tissue, but in the thickness of the muscle tissue. In addition, the cell border surrounding the membrane fragments contained lymphoid cells; inflammatory (predominantly mononuclear) infiltration was sometimes observed (Fig. 4).

Only solitary GMC were detected in muscle preparations from group 4 animals. Tissues adjacent to the membrane contained lymphoid cells (lymphoblasts, minor lymphocytes, plasma cells of different degree of maturity) and polynuclear leukocytes (predominantly neutrophilic). We observed pronounced neoangiogenesis in muscle tissue adjacent to the membrane side with immobilized cells (Fig. 5).

The results in groups 3 and 4 indicate that bone marrow stromal cells transplanted into the focus of

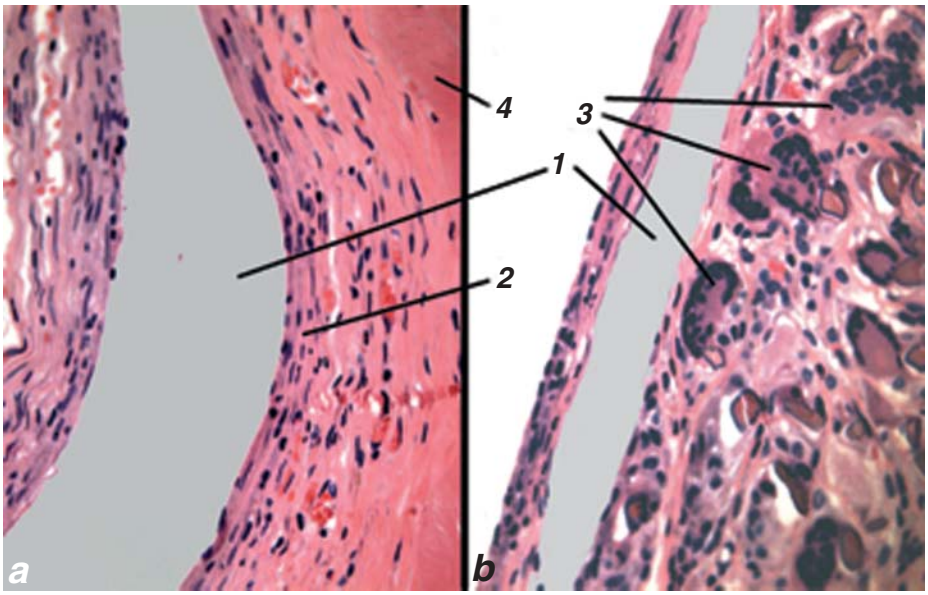

Fig. 3. Fragments of the muscle with implanted membranes. *a*) intact muscle; *b*) damaged muscle. Hematoxylin and eosin staining, ×240. *1*) place of membrane location; *2*) forming fibrous capsule; *3*) giant multinuclear cells; *4*) intact muscle tissue.

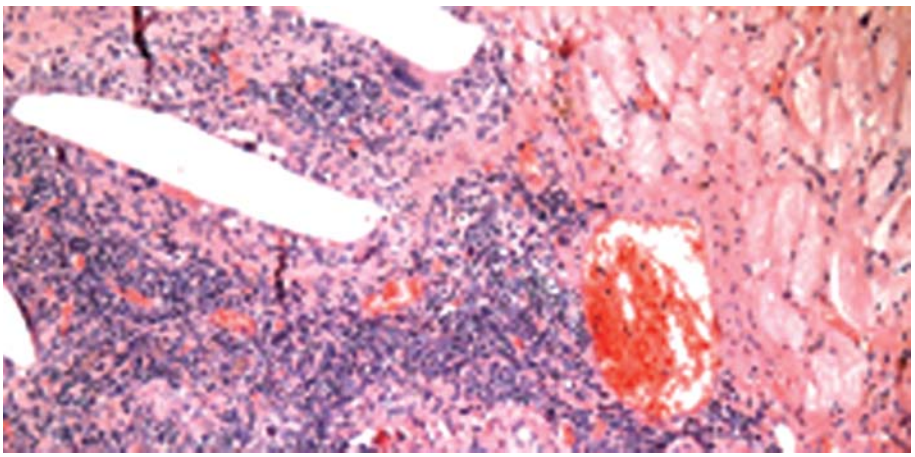

Fig. 4. Muscle fragment with implanted membrane carrying immobilized bone marrow stromal cells. Hematoxylin and eosin staining, ×100.

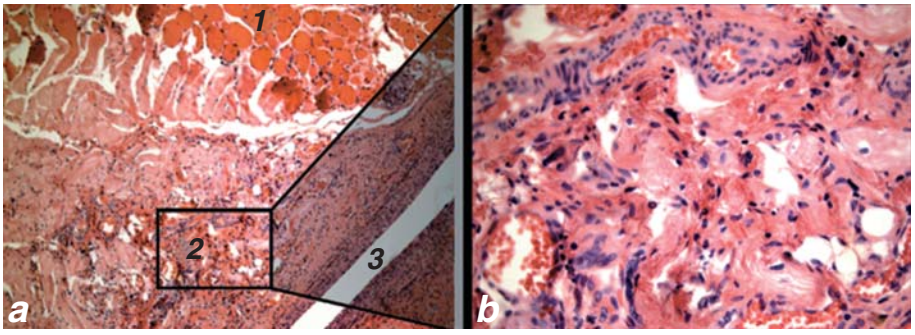

Fig. 5. Muscle fragment with implanted membrane carrying immobilized 5-azacytidine-treated bone marrow stromal cells (*a*); *b*) enlarged fragment. *1*) intact muscle; *2*) area with numerous microvessels; *3*) place of membrane location and cell wall around it. Hematoxylin and eosin staining, ×100 (×400 for *b*).

injury are actively involved in the realization of genetically determined repair program in damaged tissues. This means that substitute (sclerosing) regeneration, whose mechanisms are regulated by the predominant role of mononuclear phagocytes, are inhibited under the effect of bone marrow stem cells, while the processes of reparative (reconstructive) tissue regeneration, the predominant role in which is played by the lymphoid tissue system [1], are activated. In group 2 fibroplastic processes in the muscle tissue were activated: formation of the connective tissue and appearance of numerous macrophages. On the other hand, in group 3 and 4 we observed no intensive development of the connective tissue around the membrane, its formation was far less active than in group 2. Pronounced infiltration of these zones with lymphoid cells with morphoregulatory characteristics was observed [1]. We transplanted autologous cells, and hence, the immunological rejection reactions can be ruled out. FCS was used for cell culturing; presumably, cells were contaminated by xenogenic proteins *in vitro*, which changed their antigenic characteristics [22]. It is hardly possible that cellular infiltration was caused by active proliferation of transplanted stromal stem cells, because immunohistochemical studies documented low number of transplanted cells in tissues under the membrane.

It is noteworthy that there were many young developing cells among lymphoid cells in the focus of injury: minor lymphocytes, lymphoblasts. The appearance of these cells seemed to be due to the release of a complex of bioactive substances (cytokines, peptides, growth factors) by the functioning bone marrow stromal stem cells; these bioactive substances with morphoregulatory and information characteristics acted as regulators of reparative morphogenesis in damaged tissues. Presumably, the formation of new vessels in the focus of injury, especially after treatment by 5-azacytidine, is a manifestation of the lymphocytic morphoorganizing effects. However, this hypothesis requires verification, which was not yet published. It is known that 5-azacytidine promotes myogenic differentiation of stromal stem cells [7,21] and prevents their osteogenic differentiation [3]. It was shown that transplantation of bone marrow mesenchymal stem cells pretreated with 5-azacytidine in culture stimulated neoangiogenesis in damaged myocardium [23].

The concepts on the involvement of cell ensembles into regeneration processes suggest that the basis of these processes in any tissue is the parenchymatous cell—macrophage—fibroblast triad. Macrophage, the central cell in this triad, provides realization of the genetic program of tissue regeneration, as this cell is a component of a universal system of mononuclear phagocytes and regulates the relationship with the central organs of hemopoiesis and immunogenesis in the body responsible for genetic information transfer to the parenchymatous cells.

Many cell transplantation protocols dictate removal of precultured cells from the plastic and their suspension. This procedure reduces viability of transplanted cells. It was shown, for example, that more than 40% cells died 24 h after intramyocardial transplantation of fetal cardiomyocytes [25]. The use of carriers for isolated cells not only increased their functional activity *in vitro* [4,12,15], but promoted the maintenance of this activity after transplantation [9]. Along with variants of injecting the suspension forms, methods for application of cell material immobilized on carriers are developed at Institute of Transplantology. This study confirms the efficiency of this approach: delivery of bone marrow stromal stem cells immobilized on *Elasto*PHB® membranes into the focus of injury optimized functional activity of these cells creating adequate spatial orientation.

Through peptides and cytokines released by the transplanted cells they are involved in the local regulatory system of mononuclear phagocytes, restore the regeneration at the level of cell ensembles: activity of fibroplastic processes decreases, while functional and proliferative activities of parenchymatous cells increase. This recommends the use of bone marrow stromal cells for the treatment of acute myocardial infarction, burns, and traumas in order to reduce the formation of cicatricially-deformed tissues and creation of conditions for reparative regeneration.

Hence, the *Elasto*PHB® membranes can be used for immobilization of bone marrow stromal cells and for implantation of these bioconstructions into the focus of injury. Bone marrow stromal cells immobilized on *Elasto*PHB® membrane proliferate in culture, after transplantation migrate into adjacent tissues, and survive for at least 1 month.

Bone marrow stromal cells modulate the course of inflammatory process: inhibit the formation of fibrous capsule around the membrane and its biodegradation, actively stimulate migration of lymphoid cells into the focus of inflammation. Bone marrow cells treated with 5-azacytidine stimulate angiogenesis in damaged skeletal muscle.

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