Tissue Engineering Construct on the Basis of Multipotent Stromal Adipose Tissue Cells and Osteomatrix for Regeneration of the Bone Tissue

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We developed a new method of creation of tissue engineering constructs for regeneration of the bone tissue based on the principle of free distribution of cells in a fibrin clot within a scaffold. The tissue engineering construct includes multipotent stromal adipose tissue cells committed in osteogenic lineage, platelet-rich plasma, and resorbed material on the basis of xenogeneic bone collagen. The culture of bone progenitor cells was characterized by the main markers of osteoblastic differon. The material meets all requirements for materials intended for tissue engineering. An innovative high-technological tissue engineering product for clinical application is prepared.

Key Words*: tissue engineering construct; bone tissue regeneration; multipotent stromal adipose tissue cells; Osteomatrix*

Creation of bone tissue equivalents is one of the major trends in tissue engineering and regenerative medicine. Rapid development in this field is dictated by high interest of research clinical centers and medical preventive institutions to the use of tissue engineering products for reparative osteogenesis in various bone diseases, such as non-uniting fractures, vast defects of the bone tissue, intervertebral disk degeneration, *etc*. Commercial osteoplastic materials for directed tissue regeneration on the basis of allogeneic and xenogeneic collagens are widely used in oral surgery, traumatology, and orthopedics [1,3]. However, they are not always efficient.

The main requirement of regenerative medicine to tissue engineering constructs (TEC) for neoosteogenesis is promotion of organotypic regeneration at the site of transplantation due to the presence of osteoprogenitor cells, the source of neohistogenesis. The carrier materials should promote osteoconduction, maintain the shape, and retain mechanical durability for at least 6 months; they should be also a substrate for vascular growth at the site of transplantation [8,9,13].

One of the main problems during the development of TEC for bone tissue regeneration is the choice of the source of osteoprogenitor cell culture. Multipotent stromal cells (MSC) isolated from the bone marrow are most widely used. The adipose tissue (AT) can be an alternative source of MSC. The cell culture derived from the stromal vascular AT fraction, similarly as BM MSC, are characterized by clonogenicity, high proliferative activity, and the capacity of directed differentiation towards osteogenic, chondrogenic, adipogenic,

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and myogenic lineage cells [15]. Moreover, AT MSC culture is identical by its major cytodifferentiation markers to BM MSC culture [10,12].

For directed regeneration of the bone tissue, preliminary differentiation of MSC from any source towards osteogenic lineage is required; to this end, the cells are cultured in an inductive medium containing sodium β-glycerophosphate, ascorbic acid, and dexamethasone or vitamin D_3. Addition of dexamethasone to differentiation medium is not always justified because of stimulation of adipogenic differentiation on AT MSC culture [2], while the role of 1,25-dihydroxyvitamin D_3 as differentiation inductor and regulator of mineralization is well documented [11,14].

The use of the carrier for TEC is determined by some principles known as golden standard of the material: the absence of cytotoxicity, promotion of cell adhesion and proliferation, controlled resorption, unchanged necessary mechanical characteristics, and porous 3D structure. Various commercial products on the basis of natural or synthetic materials are used as the matrix for TEC [8,9]. We believe that Osteomatrix is the most suitable domestic material exhibiting satisfactory cytological and biomechanical characteristics for the development of viable equivalents of the bone tissue.

Here we developed a new-generation TEC for reparative osteogenesis according to an original protocol on the basis of AT MSC and Osteomatrix with the use of autologous platelet-rich plasma (PRP) and fibrin gel as the immobilizing component.

MATERIALS AND METHODS

For creation of TEC we used a culture of MSC from the stromal vascular fraction of AT (SVF AT).

SVF AT was isolated from lipoaspirate obtained from adult donors. Lipoaspiration was performed on the anterior abdominal wall according to a standard method under local anesthesia. The blood in required volumes was taken for preparing of PRP and autologous serum.

The lipoaspirate was washed with Versen solution and disaggregated in Versen solution with 0.25% trypsin at 37°C. The cell suspension was centrifuged at 1100 rpm for 10 min, the pellet was dissolved with DMEM/F12 (1:1) supplemented with 10% autologous serum and amikacin (final concentration 500 mg/liter), transferred into Petri dishes and incubated under standard conditions (37°C, 5% CO_2). Seeding density was 1500-2000 cells/cm^2. The growth medium was replaced every 3 days.

Expression of specific surface markers in the studied cultures was performed on a FACS Calibur flow cytofluorometer (BD Biosciences). Mouse monoclonal antibodies (PharMingen, Chemicon) were used. Nonspecific mouse (rabbit) IgG from the same manufacturers were used as the negative control. The data were processed statistically using MDI 2.8 software.

For directed osteogenic differentiation, the cells were seeded on Petri dishes and after attaining 80% confluence the growth medium was replaced with a differentiation medium (DMEM with 10% autologous serum, 100 μg amikacin, 50 mg/liter L-ascorbic acid, 10 mmol/liter sodium β-glycerophosphate, and 10 nM 1,25-dihydroxyvitamin D_3). Differentiation was performed for 14 days; the medium was replaced every 3 days. For evaluation of the efficiency of osteogenic differentiation, mineralization foci in the cell culture were detected and expression of markers of osteogenic differentiation was analyzed.

For detection of mineralization foci, the cells were twice washed with BPS (pH 7.2-7.4, PanEko), fixed with 4% formalin, and stained with 40 mM alizarin red S aqueous solution (pH 4.1; PanEko) for 5 min. Mineralization foci were stained orange-red.

Gene expression was analyzed by real-time PCR using Sybr Green intercalating dye (Sintol). Osteopontin and osteocalcin were used as markers of osteogenic differentiation (Table 1). Total RNA was isolated (RNeasy Mini Kit, QIAGEN) and reverse transcription reaction was performed according to Fermentas protocol using oligo-dT-primers. The level of mRNA of the analyzed genes was standardized by averaged results of amplification of house keeping genes GAPDH and β-actin. The relative content of mRNA was calculated by the ΔC(T) method.

Osteomatrix samples (Konektbiofarm, Federal Service over Surveillance in the Field of Public Health and Social Development, certificate No.

TABLE 1. Primers for PCR

Gene	5' primer	3' primer	Annealing	Amplicon, b.p.
Osteopontin	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT	60.2	230
Osteocalcin	CACTCCTCGCCCTATTGGC	GCCTGGGTCTCTTCACTACCT	62.5	138
β-Actin	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC	60	144
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	60	87

FS01033997/ 2696-05) were used as the matrix for preparing TEC. The material represents a highly purified bone matrix with preserved collagen and mineral components and natural architectonics. Osteomatrix is manufactured as crumbs, small blocks ~5×5×5 mm, and larger blocks with linear dimensions of more than 1 cm. Crumbs and small blocks are ready to use, while large blocks are used for preparing matrix of individual shape fitting the defect geometry determined by 3D model.

The toxic effect of the carrier on the cell culture and proliferation of MSC on carrier matrix were studied using MTT test.

For evaluation of the toxic effect of Osteomatrix, MSC were seeded to 96-well plates at a density of 10^4 cells per well. After 24 h, the samples were added and the cells were incubated for 24 h. Then, MTT (PanEko) was added in a concentration of 0.5 mg/ml and incubation was performed for 2 h at 37°C. Formazan crystals were eluted with DMSO (PanEko) for 20 min (on a shaker at 200 rpm). Adsorption of formazan was evaluated by measuring optical density of the eluate at λ=570 nm and subtracting the background value measured at λ=620 nm. Wells without cells served as the control. Measurements were performed on Anthos Reader 2020 plate reader (Anthos Labtec).

For evaluation of MSC proliferation on the surface of Osteomatrix carriers, 5×5×5 mm^3 blocks were populated with cells. To this end, 50 µl cell suspension (2×10^6 cells per 1 ml growth medium) was applied onto each block. Due to high capillary properties of the carrier determined by its porous structure, the suspension rapidly got into the sample. After 1 h (*i.e.* after initiation of cell adhesion to the walls of pores), the growth medium was added. The samples were incubated under standard conditions; the medium was replaced every 3 days. The cells were cultured on the carriers for 1, 3, and 7 days and then the number of viable cells on the carriers was determined (using the above-described method).

Cell adhesion to the carrier surface and their distribution within TEC was evaluated by SEM. The samples were washed with PBS, fixed with 2.5% glutaraldehyde (Panreac) for 24 h, dehydrated, and dried with hexamethyldisilazane. The samples were coated with gold (10 nm) in an IB 3 apparatus (Eiko). The preparations were analyzed under SEM S-570 (Hitachi) at accelerating voltage of 15 kV.

For PRP preparation, the blood was centrifuged 10 min at 1100 rpm. The supernatant containing blood plasma with platelets was transferred into a new tube. The platelets were pelleted by centrifugation at 3600 rpm for 15 min and the pellet was resuspended in $^1/_2$ supernatant volume.

For TEC assembly, the sterile shape-generating material Osteomatrix in the form of blocks or crumbs was washed with physiological saline and dried. MSC committed towards the osteogenic lineage were harvested from plastic with Versen containing 0.25% trypsin and centrifuged 10 min at 1100 rpm. The pellet was resuspended in PRP: 14×10^6 cells per 1 ml PRP. The cell suspension in PRP was applied drop by drop on the carrier matrix (0.5 ml per 1 cm^3). For fibrin formation from fibrinogen present in PRP, thrombin dissolved in 10% $CaCl_2$ was added drop by drop to the system. Polymerization took 3-5 min.

Statistical processing of the results was performed using SigmaStat 9.0 software. Mann–Whitney *U* test was used for comparison of small groups and the data not conforming the normal distribution. The differences were significant at $p<0.05$.

RESULTS

The cell culture isolated from the lipoaspirate represents a population containing primarily small spindle-shaped or polygonal mononuclear cells forming rapidly growing colonies and admixture of large, often polynuclear cells (the number of the latter decreases during passaging) (Fig. 1).

Immunophenotyping of the obtained AT MSC cultures showed that 60-90% cells expressed markers of stromal cells (CD13, CD29, CD44, CD73, CD90, CD105, and CD166) and were negative by markers of hemopoietic (CD34, CD45) and endothelial (CD31) cells.

For TEC preparation, the cells were committed to osteogenic differentiation by incubation in osteogenic medium for 2 weeks. The efficiency of differentiation was confirmed by the presence of mineralization foci stained with alizarin red (Fig. 2) and intensive transcription of osteopontin and osteocalcin genes, markers of osteogenic differentiation and the main

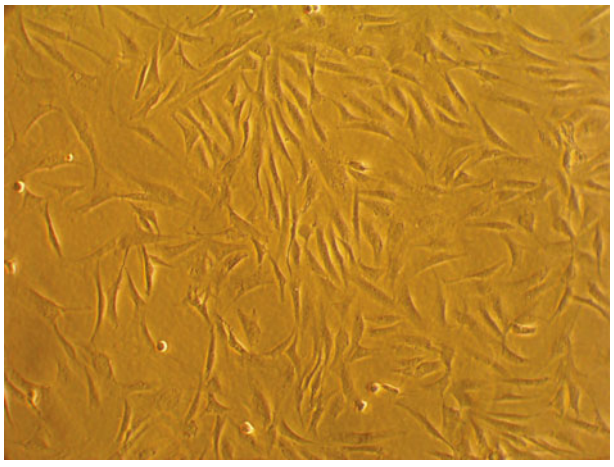

Fig. 1. AT MSC culture (×100).

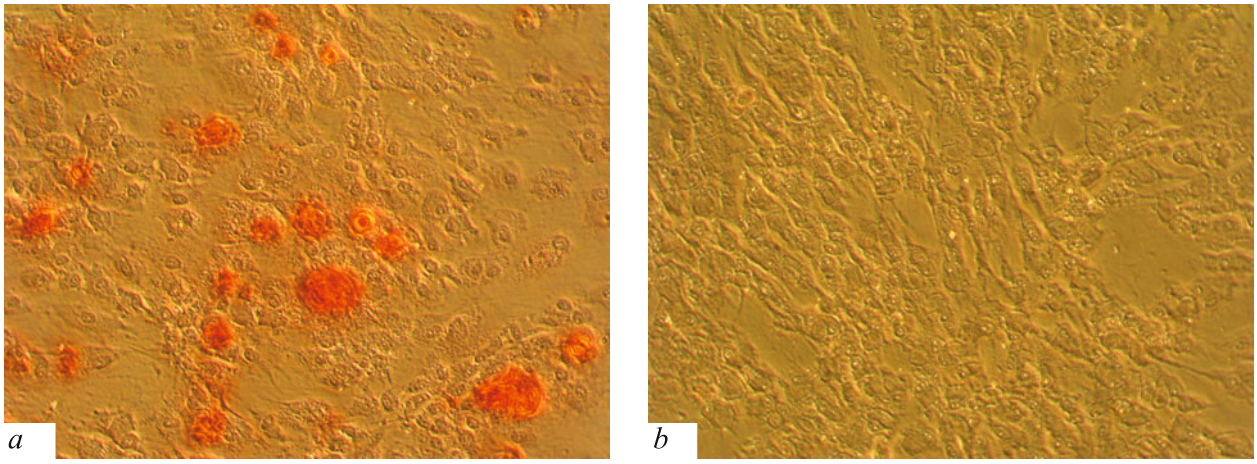

Fig. 2. Detection of mineralization foci in AT MSC culture, ×200. Alizarin red staining. *a*) experiment; *b*) control.

components of non-collagen bone matrix (real-time PCR data, Fig. 3).

MSC isolated from AT were similar to osteogenic cultures obtained from BM stroma by cell morphology, immunophenotype, and osteogenic differentiation capacity *in vitro*. Thus, MSC culture from the stromal vascular fraction of AT can be an alternative source of preosteoblasts and can be used for creation of TEC for reparative osteogenesis. Isolation of the material by lipoaspiration can be a relatively low invasive method in comparison with BM aspiration, which makes AT MSC the most promising culture for clinical use.

Osteomatrix, a highly purified bone matrix retaining architectonics of native bone, was chosen as the scaffold for TEC. According to MTT test, Osteomatrix produced no toxic effects on cells.

After application of MSC suspension on the Osteomatrix carrier, pronounced cell adhesion was observed as soon as on the next day (Fig. 4). Most cells

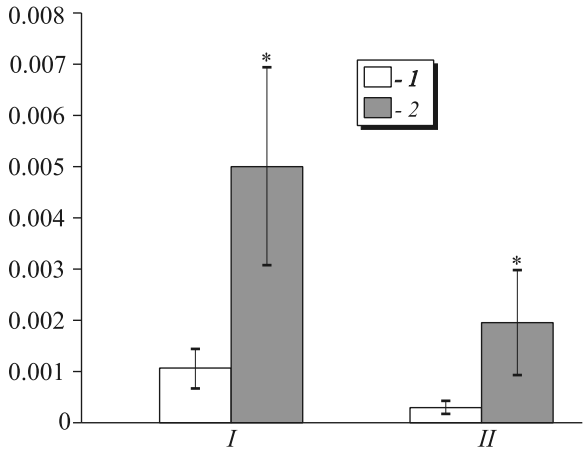

Fig. 3. Expression of osteopontin (*I*) and osteocalcin (*II*) gene: real-time PCR. Ordinate: relative mRNA content. *1*) control medium; *2*) differentiation medium. *$p<0.05$ in comparison with control medium.

were flattened on the surface (Fig. 4, *b*). Proliferative activity of MSC during culturing on the matrix carriers was studied by MTT test. The choice of this test is dictated by difficulties in harvesting the cells from carriers with complex spatial organization. The experiments demonstrated the increase in the number of adherent cells on the carrier surface during 7-day culturing (Fig. 5).

It was found that the material meets all requirements for carriers intended to tissue engineering: it is not cytotoxic, promotes adhesion and proliferation of AT MSC, *i.e.* is characterized by high cytocompatibility. Moreover, the material possesses required biomechanical characteristics and showed good results in clinical practice [3,4,7]. Experiments on rabbits revealed no signs of bone tissue formation [5] after Osteomatrix implantation into critical defects of the parietal bones and under the skin, which suggests that this material exhibits no osteoinductive activity. At the same time, Osteomatrix possesses sufficient osteoconductive functions retaining its shape for at least 6 months, which in combination with its high cytocompatibility allows using it for preparation of TEC.

For cell immobilization on the carrier, platelet gel was used as the third element of the construct. The use of PRP is believed to improve the regenerative potential of TEC [6]. First, degranulation of platelet present in PRP leads to the release of angiogenic factors, such as platelet-derived growth factor-β, vascular endothelial growth factor, and transforming growth factor-β, which accelerates the formation of the vascular network in the TEC and improving trophism of transplanted cells. Second, PRP with cells occupies the entire volume of the carrier, thus forming the substrate for morphogenesis. Third, the use of platelet gel makes it possible to rapidly assemble the construct without preliminary cell culturing on the carrier. Moreover, very high cell concentration can be thus created 10-

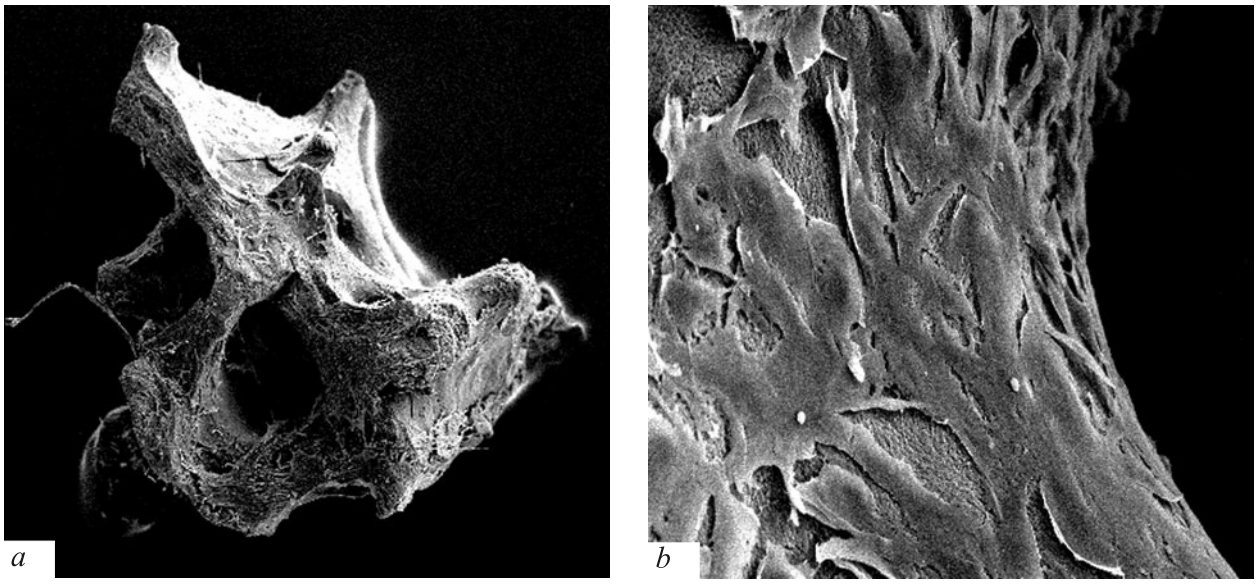

Fig. 4. Adhesion of MSC on the carrier surface. SEM. ×50 (*a*), ×300 (*b*).

15-fold surpassing the cell concentration in the two-component system (carrier populated with cells).

The developed construct consists of the matrix (67%), PRP (33%), and cells (1%); 1 cm^3 TEC contained 5×10^6 cells. On microphotographs (SEM) made immediately after TEC assembly, cell clusters of spherical shape are seen. The cells are surrounded by fibrin threads forming dense network adjacent to the carrier surface (Fig. 6, *a*, *b*). During preparation of specimens for electron microscopy, the material was dehydrated, which led to compaction of the fibrin network near the carrier surface. The cells inside the network were also pressed to the carrier surface. One day after assembly, many cells were flattened on the carrier surface (Fig. 6, *c, d*).

Thus, we created a TEC for organotypic regeneration presented by a three-component system on the basis of AT MSC, biodegradable material Osteomatrix, and PRP. The use of osteoprogenitor cells determines osteogenic properties of the construct due to participation of the transplanted cells in the formation of the bone tissue and inductive influences of released growth and differentiation factors on resident cells. Osteomatrix is responsible for osteoconduction, while PRP determines angiogenic properties, improves cell survival after transplantation, and serves as a substrate for morphogenesis. The developed three-component construct is principally new high-technological tissue engineering product for regeneration of the bone tissue intended for clinical use in traumatology, orthopedics, pediatric surgery, craniomaxillofacial surgery, *etc*.

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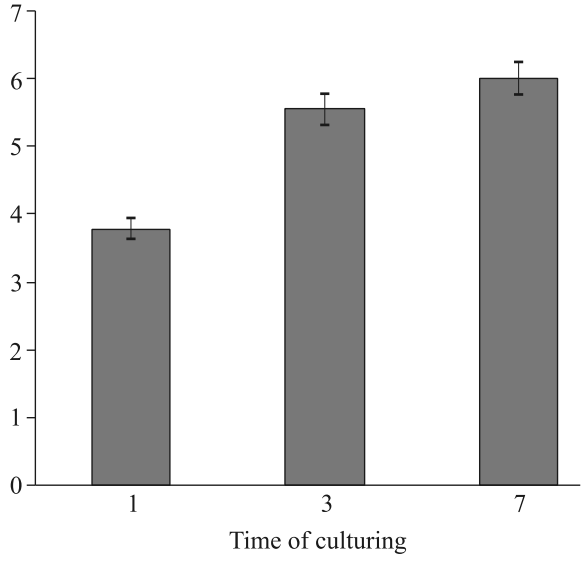

Fig. 5. Proliferation of AT MSC on Osteomatrix carrier surface. MTT-test. Ordinate: cell number (10^3) per 1 μg sample.

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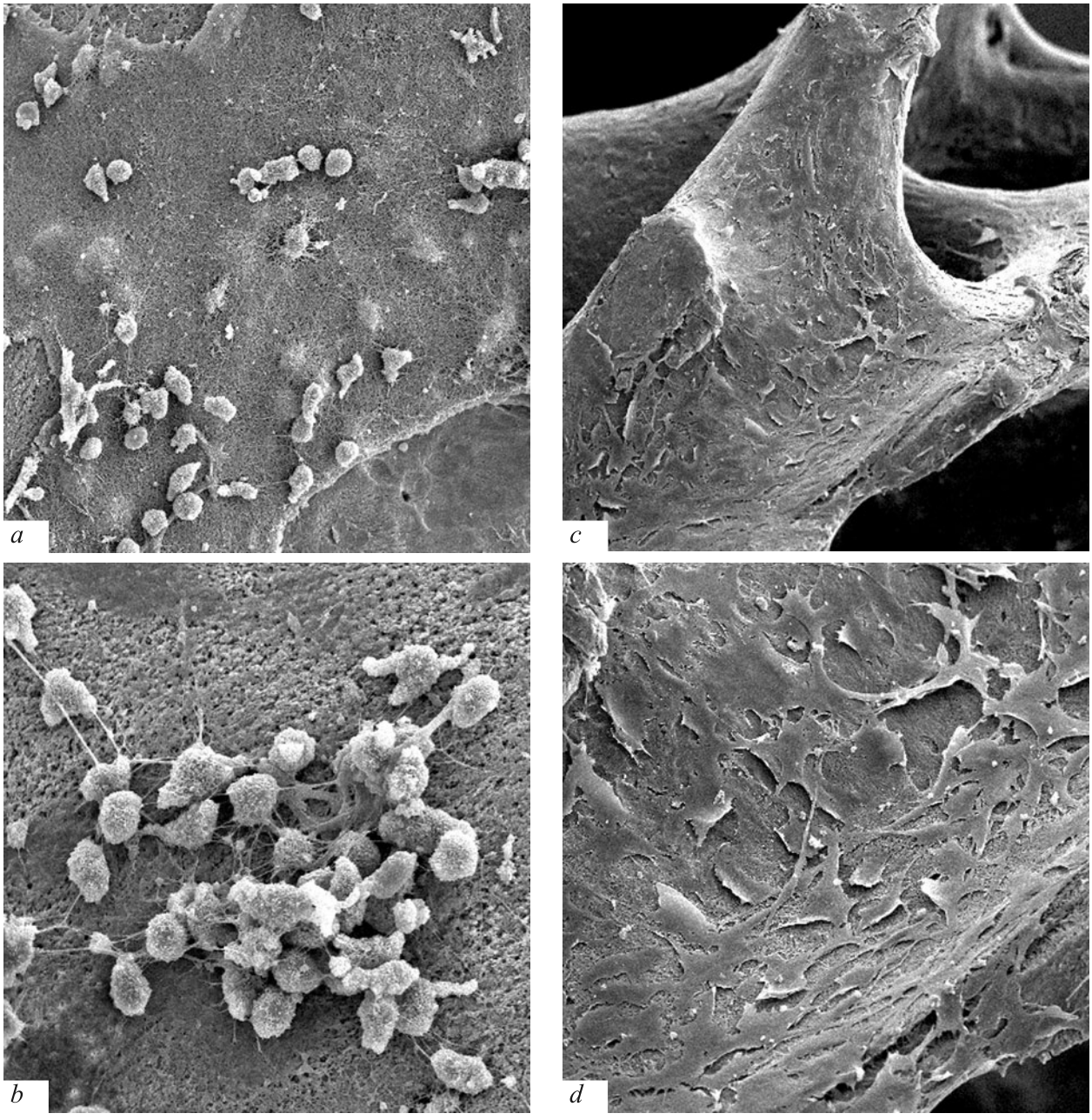

Fig 6. Cells in TEC. SEM. *a*, *b*: immediately after TEC assembly cell clusters had spherical shape. The cells were surrounded by fibrin fibers; *c*, *d*: one day after assembly, cells were flattened on the carrier surface. ×500 (*a*), ×1000 (*b*), ×100 (*c*), ×250 (*d*).

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