Tissue Engineering Construction from 3D Porous Ceramic Carriers and Multipotent Stromal Cells for the Repair of Bone Tissue Defects

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The tissue engineering construction was developed from human bone marrow multipotent stromal cells and 3D porous foamed—ceramic carriers of a zirconium oxide--aluminum oxide system. The carriers had no cytotoxic activity and were potent in maintaining the cell adhesion and proliferation. We developed the method for inoculation and cultivation of bone marrow multipotent stromal cells on these carriers. The optimal time of incubation to obtain a tissue engineering construction was estimated. Bone marrow multipotent stromal cells could be cultured at a depth of 9 mm from the edge of the matrix. The tissue engineering construction holds promise for the repair of extensive defects in bone tissue.

Key Words: *multipotent stromal cells; 3D porous carriers; bone regeneration; tissue engineering construction*

Osteoplastic materials are use for the repair of extensive defects in bone tissue. The main requirements for these materials are biocompatibility, osteoconductivity, absence of immunogenicity, and mechanical strength. The introduction of an additional component (stem/progenitor cells) allows the tissue engineering construction (TIC) to gain osteoinductive properties. Bone marrow multipotent stromal cells (MSC) are of considerable interest for tissue engineering. These cells can differentiate in the osteogenic, chondrogenic, adipogenic, and other directions [9]. Much attention is paid to the development of TIC from MSC for stomatology, oral and maxillofacial surgery [7], traumatology, and orthopedics [2]. Administration of cells is followed by the induction of tissue regeneration due to the release of factors for growth and differentiation, stimulation of intercellular substance synthesis and, probably, replacement of lost cells in the bone tissue. These processes contribute to the recovery of tissue injury [10]. Immobilization of cultured cells on a carrier matrix provides the maximal concentration of cells in the zone of injury, contributes to histotypic differentiation, and increases cell survival after transplantation.

The carriers from hydroxyapatite and xenogeneic collagen of type 1 (Collagraft [13], Gapkol, Kolapol [1], *etc*.) are widely used at the present time. They have high osteoconductive activity and some osteoinductive properties. The weakness of these materials is the induction of immune and allergic reactions [8]. Synthetic biodegradable carriers from polylactic and polyglycolic acids or their copolymers have no immunogenicity. However, lysis of these polymers is followed by a local decrease

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in medium pH and damage to the surrounding tissue [5]. Moreover, the rate of matrix resorption significantly exceeds that of tissue formation. These properties reduce the effectiveness of carriers.

Non-resorbed carriers (*e.g.*, from bioceramics and metal alloys) can be used in clinical practice to maintain high mechanical strength of the transplant for a long period of time. Predifferentiated bone marrow MSC were used for bone tissue reparation. These cells are put on the porous framework of a titanium nickelide alloy precoated with collagen solution [3].

A three-dimensional porous structure is the optimal carrier for bone tissue replacement with TIC. This structure has high specific area for cell adhesion and provides perfusion of the culture medium in a whole volume of the carrier. These properties contribute to high-efficacy adhesion, proliferation, and differentiation of cells *in vitro* [11]. This spatial configuration (*in vivo*) of the carrier corresponds to the spongy bone tissue, which provides invasion of blood vessels, migration of proper cells, and optimal microenvironment for regeneration and osteointegration.

In our experiments, the carrier was prepared from non-resorbed oxide foamed—ceramics of a zirconium oxide--aluminum oxide system. Composite matrices from these compounds, hydroxyapatite, and other components are characterized by high biocompatibility [12], absence of antigenic activity, and mechanical strength [6]. Osteoinductive properties are provided by administration of human bone marrow MSC to the transplant.

Foamed—ceramic carriers are manufactured by duplication of a polyurethane foam base. This approach allows us to obtain the samples with an extremely high value of porosity (82-90%) and well-developed labyrinth-and-arch structure, which consists of interrelated open pores. It provides the optimal conditions for cell cultivation and bone tissue formation *in vivo*. A microporous surface of the matrix is favorable for cell adhesion and proliferation. Moreover, this method of matrix synthesis allows us to obtain the samples of desired size and shape, which depends on the geometry of bone defect.

This work was designed to develop TIC for bone tissue regeneration. We evaluated the optimal conditions for inoculation and cultivation of human bone marrow MSC on three-dimensional foamed—ceramic carriers of a zirconium oxide—aluminum oxide system. The following medical-and-biological properties of carriers were studied: cytotoxicity, effectiveness of cell adhesion, and dynamics of proliferative activity.

MATERIALS AND METHODS

Preparation of the composite ceramic from a zirconium oxide--aluminum oxide system. Carriers were prepared from the highly-porous ceramic material of a zirconium oxide-aluminum oxide system with cellular structure. The material was obtained by duplication of a polyurethane foam base. Porosity of the ceramic material (GOST 24468-80) and size of pores were estimated (STP 231-5.02-98 "Size of pores in highly-porous cellular materials"). The topography and microstructure of sample surface were evaluated under a CamScan scanning electron microscope (Opton Instruments).

Foamed—ceramic carriers were sterilized by γ-irradiation with 2.5 Mrad. This method of sterilization does not cause a change in the physicochemical and medical-and-biological properties. The carriers were washed with physiological saline (PanEco) and dried.

Experiments were performed on the samples, which appeared as cubic blocks (5×5×5 mm) with a pore size of 50-100 m (PPI=60; PPI, number of pores per linear inch) or 100-200 m (PPI=45). The samples of 2×2×2 mm were used in the MTT test. Inoculation of large carriers was performed with spherical samples (18 mm in diameter).

Preparing the culture of MSC. MSC were isolated from the bone marrow suspension. It was extracted from the posterior border of the ilium (crest) in adult healthy donors. The bone marrow aspirate was layered to a separating Ficoll gradient (density 1.077 g/ml, PanEco) and centrifuged at 1300 rpm and 4°C for 20 min. The interphase ring with nucleated cells was collected and centrifuged at 1100 rpm and 4°C for 10 min. The supernatant was removed. The pellet of cells of suspended in DMEM/F12 culture medium (1:1) with 2 mM L-glutamine (PAA Laboratories), 10% fetal bovine serum (HyClone-Perbio), and 0.5 mg/ml amikacin (Sintez). The cell suspension (10^4 cells/ml) was inoculated on plastic petri dishes (diameter 90 mm, Nunlon). Incubation was performed under standard conditions (37°C, saturation moisture content, 5% CO_2). Nonadherent cells were removed after 24 h. Adherent cells were washed with the culture medium and incubated to achieve a 75-85% confluence. The medium was replaced at 2-3-day intervals, which depended on culture growth. We analyzed the growth and cell morphology in newly formed colonies. The small-cell and rapidly growing colonies were selected for further proliferation. The selected cells were reinoculated to produce a partial monolayer (not more than 50%, inoculum density not more than 50 cells/cm^2).

Immunophenotyping of MSC on a FACS Caliber flow cytofluorometer (BD Biosciences) showed that 60-90% cells express stromal markers CD90 and CD105, but not CD34.

Functional activity of MSC was evaluated from the ability for directed differentiation into mesodermal lines (myogenesis, chondrogenesis, osteogenesis, and adipogenesis) in induction media.

Cytotoxicity assay. Cytotoxicity of samples was studied in the screening MTT test. This method is based on the ability of mitochondrial succinate dehydrogenase from living cells to reduce light-yellow MTT into insoluble dark-colored formazan. Formazan crystals were extracted from the cell using organic solvents (dimethylsulfoxide, DMSO). Optical density of the eluate was measured at 540 nm. The measurements were performed with the cell culture of human MSC.

The cells were placed in 96-well plates (5-10 thousands cells per well). One day after cell adhesion, the samples were maintained in wells for various periods. MTT (PanEco) at a concentration of 0.5 mg/ml was added. Incubation was performed at 37°C for 2 h. Staining was visualized. The reaction was stopped by removing the MTT-containing medium. Formazan was eluted with DMSO (PanEco) for 20 min under conditions of agitation on a shaker (200 rpm).

Formazan absorption was estimated. The initial optical density at 620 nm was subtracted from optical density of the eluate at 540 nm. The samples were not added to control wells with cells. The measurements were performed on a Fluoroscan Ascent plate photometer (Labsystems).

Method for inoculation of MSC samples. The cell suspension was prepared. The culture of passage 2-3 MSC was removed from petri dishes with trypsin-Versen solution (PanEco) and centrifuged at 1100 rpm and 18°C for 10 min. The pellet was resuspended in the culture medium. The suspension (1-2 million cells per ml) was put on samples (100 ml suspension per 100 mg sample). Due to high capillarity of the carrier, this suspension was rapidly incorporated into the inner space. Cell adhesion to the wall of pores was initiated by incubation for 60 min under standard conditions. The culture medium was added. The samples of cells were put in new plates after 1 day.

MNC cultivation on carriers. Bone marrow MSC on carriers were cultured in 12-well or 24-well plates (Nunclon) under standard conditions. The medium was replaced at 2-day intervals.

The medium was perfused using a Heidolph magnet stirrer. The rotation rate of a magnet armature was 350-400 rpm. A culture flask with carriers was placed in a CO_2 incubator. MSC were cultured under standard conditions. The culture medium was replaced at 2-day intervals.

Study of cell morphology and inoculum density in the carrier with MSC. Adherent cells were stained with eosin and methylene blue (May—Grunwald method) to visualize the cells and to evaluate the inoculum density of samples. A detailed study of cell morphology and distribution inside the sample was performed by means of scanning electron microscopy (SEM).

Staining with eosin and methylene blue was performed by the May—Grunwald method (Mini-Med). The samples were washed with phosphate buffered saline (PBS, PanEco), treated with the concentrated dye for 3-5 min, and repeatedly washed with distilled water. The samples were examined under an Olympus CK 40 inverted light microscope equipped with an Olympus Camedia C-5050 hardware-software system (Olympus).

For SEM, the samples were washed with PBS and fixed in 2.5% glutaraldehyde (Panreac) for 24 h. The samples were dehydrated, dried with hexamethyldisilazane, and covered by a gold layer (10 nm) in an EIKO IB 3 device. The samples were examined under an S-570 scanning electron microscope (Hitachi, accelerating potential 15 kV).

Studying the dynamics of cell proliferation on carriers. The dynamics of cell growth was studied on a foamed—ceramic carrier. Adherent cells were homogenized. Cell number was estimated from total protein concentration in the homogenate. An indirect method was used due to the loss of a considerable number of cells during elution from three-dimensional samples with trypsin-Versen solution.

The homogenate was prepared. The cell-containing sample was washed with PBS (PanEco). The cells were disintegrated by threefold freezing in liquid nitrogen and defrosting. The sample was taken from a tube. The homogenate was centrifuged at 10,000 rpm for 3 min to remove debris. Protein content in homogenates was measured by the method of Bradford. The calibration curve was constructed using a standard solution of BSA (1-125 mg/ml, PanEco). The measurements were performed on a Fluoroscan Ascent plate spectrophotometer (Labsystems) equipped with a 595-nm absorption filter.

The increase in cell count at fixed time intervals was evaluated by means of light microscopy and SEM.

Evaluation of viable cells. Cell viability in TIC was estimated by staining with trypan blue. The cells adherent to a carrier were removed by trypsin-Versen solution (PanEco) and stained with 0.4%

trypan blue solution (Dia M) for 5 min. The ratio of viable cells was evaluated from dye exclusion by means of direct counting in a Goryaev chamber (MiniMed).

The results were analyzed by Student's *t* test (SigmaStat software). The differences were significant at $p<0.05$.

RESULTS

Characteristics of foamed—ceramic carriers. The porous framework was obtained from powders by duplication of a polyurethane foam base. This structure was characterized by a homogeneous distribution of pores (by size) and had the rounded bridges (Fig. 1). The breaking strength of a foamed—ceramic framework (porosity 82-84%) was observed at 50-MPa compression (similarly to bone tissue). The microstructure of carrier surface was formed by 700-900-nm particles of oxide compounds.

Cytotoxicity of samples. In the MTT test, the cells were incubated with samples from 1, 3, and 7 days. The relative optical density (determinant for the number of living cells) on days 1, 3, and 7 was 100.1±7.8, 107.7±10.2, and 97.7±7.6%, respectively (100% in the control). No significant differ-

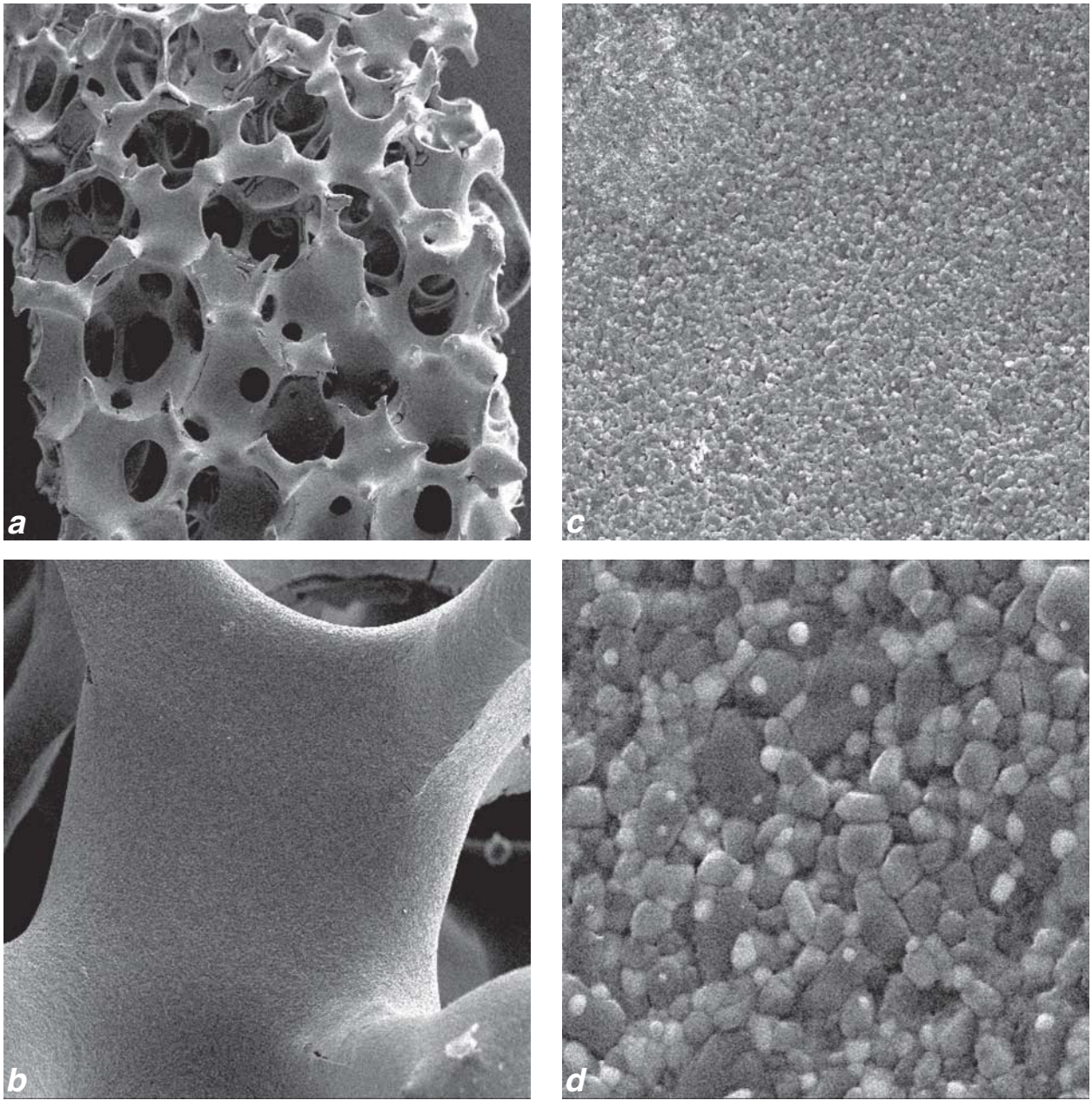

Fig. 1. Structure of the foamed—ceramic carrier with a pore size of 50-100 m. Magnification: 35 (*a*), 250 (*b*), 1300 (*c*), 6000 (*d*).

ences were found between the treatment and control groups. Hence, test samples did not produce the toxic effect on bone marrow MSC.

Cell adhesion to the carrier surface. A SEM study showed that at various stages of cultivation, the cells were spread and closely adjacent to the matrix surface (Fig. 2). Strong adhesion of cells is associated with the microstructured relief of carrier surface, which contributes to the accumulation of biologically active substances from the culture medium. One of these substances is fibronectin, which interacts with glycosaminoglycans and proteins of the cytoskeleton and stimulates cells adhesion and spreading [4]. A strong capillary effect of carriers is related to high specific area of the surface. In the minimum volume of suspension, the cells may be applied to samples at high density.

Morphological characteristics of MSC cultures on carriers. One day after inoculation of samples with cells, the wall of carrier pores included only individual cells of elongated or polygonal shape. These cells were spread on the matrix surface. Some cells were located on the concave surface of wells and had elongated processes. Due to the presence of processes, these cells were attached to adjacent surfaces (Fig. 3, *a*, *b*). On day 4 of cultivation, we revealed the cluster of cells that were close to each other. In some regions, these cells covered each other. Some cells were arranged in rows and formed the bridges between adjacent walls of wells (Fig. 3, *c*, *d*). After 7-day cultivation, a major portion of the surface in carrier pores was covered by several rows of cells. Some wells were completely closed by multilayers of cells (Fig. 3, *e*, *f*). The density of cell distribution on day 10 did not differ from that in the previous stage (Fig. 3, *g*, *h*). An examination of SEM micrographs revealed the presence of cell layers that lied separately from the carrier surface. It results from drying of test samples. The cells were located on wide transverse regions and narrow longitudinal bridges of the construction.

Dynamics of MSC growth on carriers. The proliferation rate of MSC on carriers was analyzed quantitatively for 10 days. Total protein concentration in the cell homogenate on days 1, 3, and 7 was 0.5900±0.0771, 1.240±0.155, and 2.380±0.346 mg/mg sample, respectively. These data illustrate cell growth on the carrier surface over the first 7 days. Proliferative activity of cells decreased by the 10th day. Total protein concentration in this period was 2.010±0.314 mg. It was probably associated with contact inhibition due to high inoculum density. Moreover, the impairment of culture conditions in test sample could be related to inadequate supply of nutrient substances and oxygen and accumulation of metabolic products. A correlation was found between quantitative characteristics of MSC growth in test sample and results of light microscopy and electron microscopy. These tests allowed us to evaluate the optimal time of cell cultivation on the carrier surface, which corresponds to an active growth phase of cells. During this period, the cells occupy a considerable area of the carrier. In

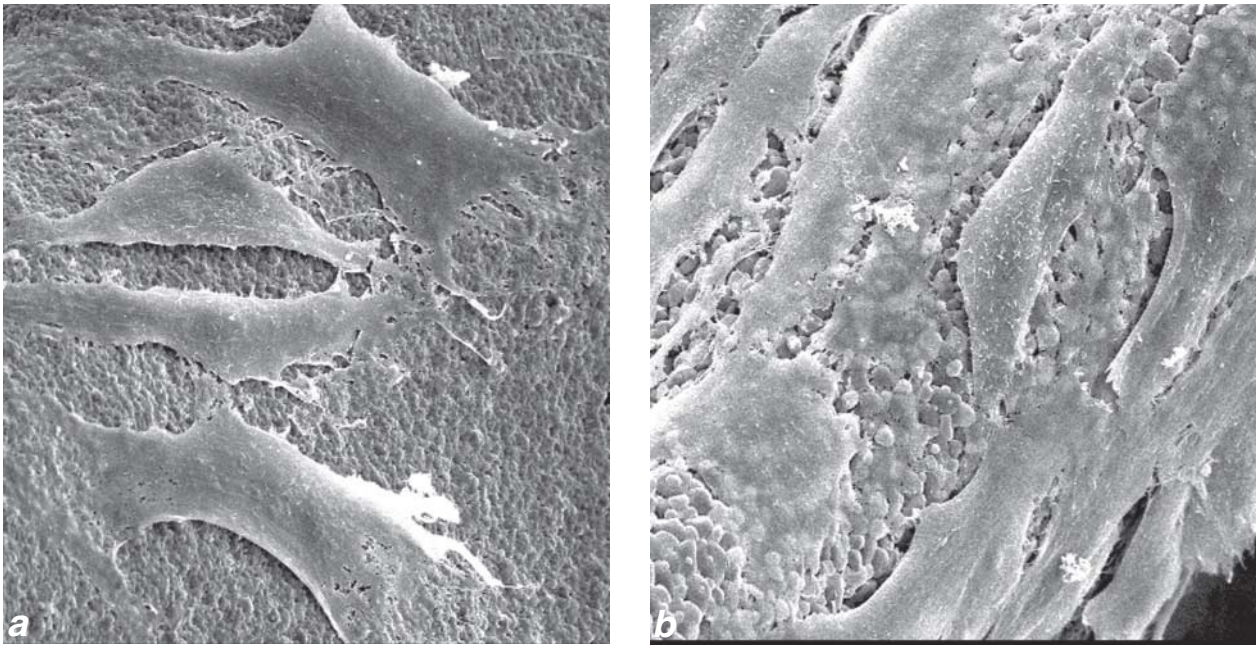

Fig. 2. Cell adhesion to the carrier surface: SEM (×100). Day 4 of incubation (*a*); day 7 of incubation (*b*).

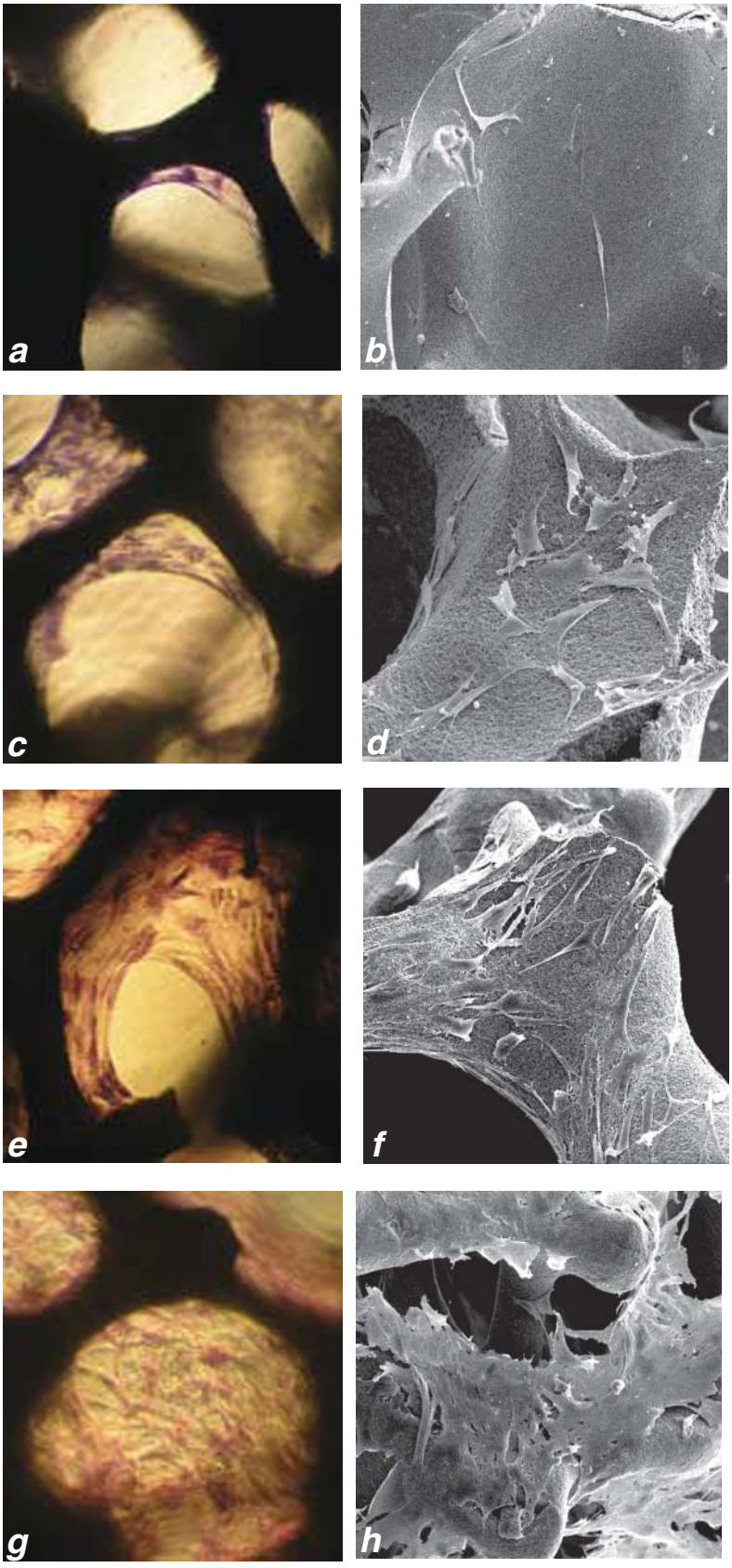

Fig. 3. Cultivation of MSC on the carrier surface. May-Grunwald staining (*a*, *c*, *e*, *g*); SEM (*b*, *d*, *f*, *h*). Day 1 (*a*, *b*); day 4 (*c*, *d*); day 7 (*e*, *f*); day 10 (*g*, *h*). Magnification: 200 (*a-c*, *e*, *g*); 250 (*d*, *f*, *h*).

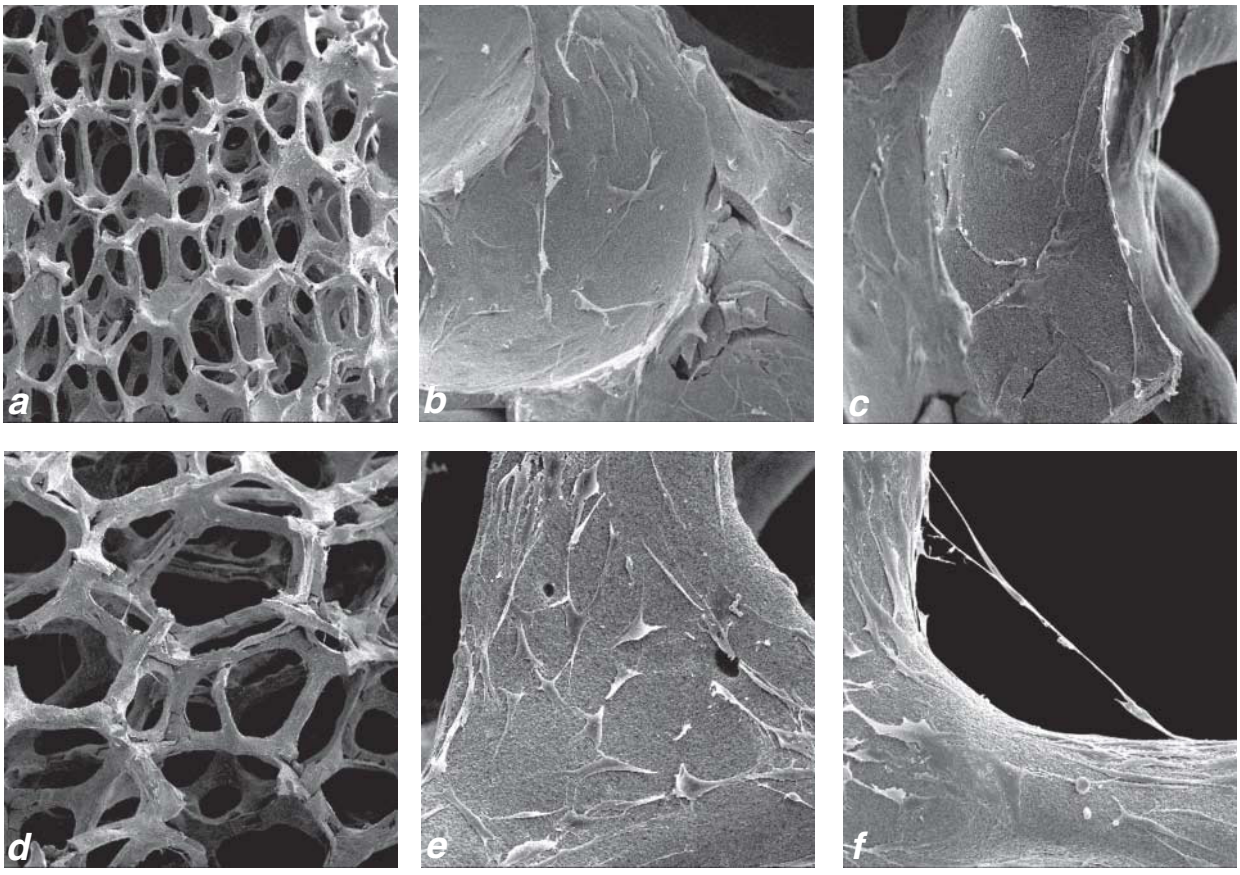

Fig. 4. Cell distribution inside and on the surface of carriers (5×5×5 mm) with different pore size on day 7 of cultivation: SEM. Structure of carriers at low magnification (×35; *a*, *d*)); cell distribution in the central zone of sample (*b*, *e*); cell distribution in the peripheral zone of sample (*c*, *f*). Samples with a pore size of 50-100 m (*a*, *b*, *c*); samples with a pore size of 100-200 m (*d*, *e*, *f*). Magnification: 200 (*b*, *c*); 250 (*e*, *f*).

our experiments with the cell culture (specified method of inoculation under standard culture conditions), this period was 7 days. Survival of cultured cells in samples was not less than 95%.

Distribution of MNC inside the carrier. We studied the effectiveness of cell distribution over the volume of test samples. A SEM study was performed to evaluate the type of cell distribution in test sample (from the periphery to the center). The conditions of cell growth in central regions are less favorable due to inadequate supply of nutrient substances and oxygen. MSC were cultured on carriers (5×5×5 mm) with different pore size for 7 days. Cell number was evaluated in the peripheral (1.5 mm from the edge) and central zone of the fracture plane (1.5-2.5 mm from the edge) and expressed per 1 mm^2 sample surface. The samples with a pore size of 50-100 m had the same populations of cells in the central and peripheral zone. No significant between-group differences were revealed. In samples with a pore size of 100-200 m, cell number in the central zone was 35% greater than in the peripheral zone. Therefore, cell density in the central zone was comparable with that in the peripheral

TABLE 1. Cell Distribution in the Samples (5×5×5 mm) with a Pore Size of 50-100 and 100-200 m under Various Conditions of Cultivation (Cell Number per 1 mm^2 Carrier Surface)

Pore size, μ	Steady-state conditions		Perfusion of medium	
	center*	periphery**	center*	periphery**
50-100	337.0±18.5	345.0±17.8	307.0±13.2	345.0±11.6
100-200	389.0±11.3	252.0±8.0	212.0±5.2	165.0±8.0

Note. *Depth 1.5-2.5 mm from the edge; **depth up to 1.5 mm from the edge.

zone. These data indicate that cell number in the central zone was higher than in the peripheral zone of carriers with a pore size of 100-200 m. A local increase in the concentration of growth factors probably improves the microenvironment for cell growth and development.

No morphological differences were revealed between adherent cells in the central and peripheral zone of a carrier. They were spread and had a similar elongated or polygonal shape (Fig. 4). Our results show that MSC colonize the whole volume of carriers. Cell viability was high in the central zone. The formation of a new tissue may occur on the surface and deep layers of the carrier.

Selection of culture conditions. One of the major problems of cultivation on three-dimensional carriers is the reduction of cell survival at a distance of more than 100-200 m from the edge of the matrix. It is related to inadequate supply of oxygen and nutrient substances. This problem is usually solved by agitation of the medium in bioreactors. In our experiments, bone marrow MSC were cul-

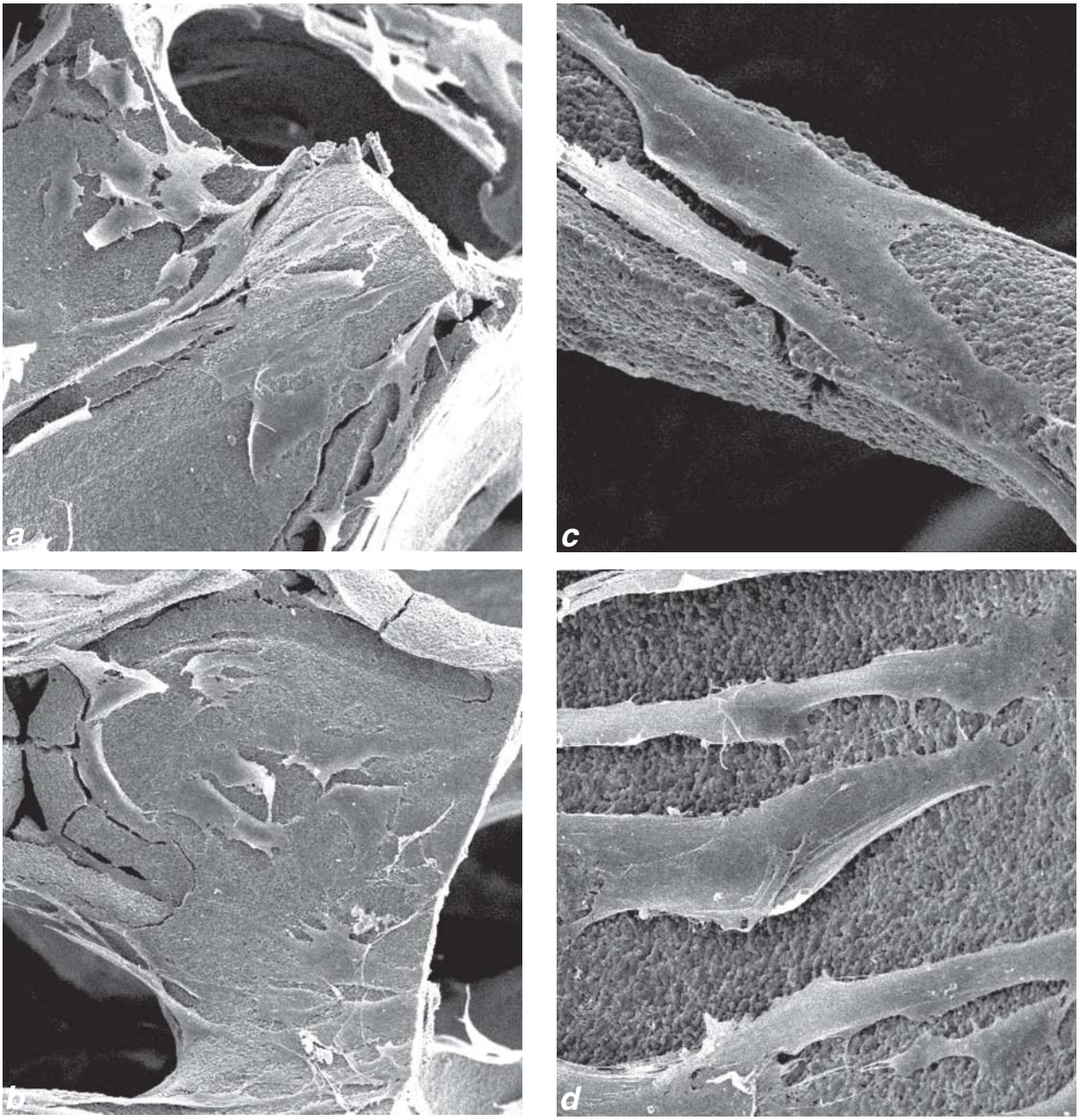

Fig. 5. Distribution and adhesion of cells in samples (diameter 18 mm) on day 7 of cultivation: SEM. Cells in the central zone of sample (*a*, *c*); cells in the peripheral zone of sample (*b*, *d*). Magnification: 250 (*a*, *b*); 1000 (*c*, *d*).

tured on carriers (5×5×5 mm, pore size 50-100 and 100-200 m) under conditions of medium perfusion for 7 days. A SEM study was performed to evaluate cell distribution in the sample (at the fracture plane). The results of previous experiments served as the control (cultivation of cells on the carrier with no agitation of the medium, Table 1). Cell number in the peripheral zone of samples with a pore size of 50-100 m did not differ in the treatment and control groups. No differences were revealed in the central zone of test samples under agitation of the medium and steady-state conditions. Cell number in the central zone of samples with a pore size of 100-200 m was lower during perfusion of the culture medium (by 45% compared to the control). Cell number in the peripheral zone of these samples was lower during agitation of the medium (by 35% compared to steady-state conditions). These results illustrate the presence of a considerable number of cells in the central zone of the carrier with a pore size of 100-200 m. Agitation of the medium probably prevents an increase in the concentration of growth factors at the site of cellular localization, which suppresses cell proliferation. Mitotic cells could be mechanically removed from the wall of the carrier with a medium flow, which results from the decrease in adhesive properties. We conclude that this structure of the carrier with a pore size of 50-200 m provides gas exchange and cell supply with nutrient substances at a depth of up to 2.5 mm. The specific conditions of cultivation are not required.

Effectiveness of cell colonization on large carriers. Test matrices hold promise for the repair of extensive defects in bone tissue. It is necessary to develop new carriers of different size and shape. Our experiments were performed with spherical carriers (diameter 18 mm, pore size 50-100 m). Cell survival on these carriers was 90%. A SEM study of the fracture plane was performed on day 7 of cultivation under standard conditions. Spread cells were revealed in the peripheral and central zone of the carrier at a depth of 6-9 mm from the edge (Fig. 5). Cell number in the central and peripheral zone was 184±7 and 231.0±5.4, respectively ($p<0.05$). The cells were morphologically similar to those cultured on compact carriers. The shape of cells was typical of MSC. They were located close to each other. These cells were found on longitudinal beams and transverse bridges. Cell density on such carriers was one-third lower than that on the carriers with a size of 5×5×5 mm. This fact is not surprising and results from the impairment of trophic function. These changes are probably accompanied by the decrease in proliferative activity, but not in cell viability. Our results indicate that MSC may be cultured on foamed—ceramic matrices at a distance of up to 9 mm from the edge of the carrier under standard conditions.

The examined foamed—ceramic matrices of a zirconium oxide--aluminum oxide system have no cytotoxic activity. Due to a specific structure, the carriers are characterized by high capillarity and adhesiveness. These features provide the effectiveness of cell inoculation. High proliferative activity of MSC on the carrier surface allows to obtain TIC over a short period of time (7 days). This construction is densely populated by viable and functionally active cells. All parameters remain unchanged in the cells that are cultured at a depth of 9 mm from the carrier surface with no additional perfusion of the medium. The carriers are characterized by high mechanical strength. Moreover, any desired shape can be given to carriers. Hence, these carriers hold much promise for the repair of bone defects.

REFERENCES

1. L. A. Grigor'yants, V. A. Badalyan, S. G. Kurdyumov, and K. S. Desyatnichenko, *Paradontologiya*, **3**, No. 24, 71-72 (2002).
2. R. V. Deev, A. A. Isaev, A. Yu. Kochish, and R. M. Tikhilov, *Kletochnaya Transplantatsiya Tkanevaya Inzheneriya*, **4**, No. 46, 18-30 (2007).
3. V. V. Stupak, V. V. Ostanin, M. Yu. Sizikov, and E. R. Chernykh, *Method for Autotransplantation of Immobilized Stromal Stem Cells from the Bone Marrow* [in Russian], Inventor's Certificate No. 2269961 of 20.02.2006.
4. L. Bacakova, E. Filova, F. Rypacek, *et al.*, *Physiol. Res.*, **53**, Suppl. 1, S35-S45 (2004).
5. O. Bostman, U. Paivarinta, E. Partio, *et al.*, *J. Bone Joint. Surg. Am.*, **74**, No. 4, 1021-1031 (1992).
6. H. Kim, S. Shin, H. Kim, *et al.*, *J. Biomater.*, **22**, No. 6, 485-504 (2007).
7. N. W. Marion and J. J. Mao, *Methods Enzimol.*, **420**, 339-361 (2006).
8. G. F. Muschler, S. Negami, A. Hyodo, *et al.*, *Clin. Orthop. Relat. Res.*, **328**, 250-260 (1996).
9. M. F. Pittenger, J. D. Mosca, and K. R. McIntosh, *Curr. Top. Microbiol. Immunol.*, **251**, 3-11 (2000).
10. A. J. Salgado, J. T. Oliveira, A. J. Pedro, and R. L. Reis, *Curr. Stem Cell. Res. Ther.*, **1**, No. 3, 345-364 (2006).
11. S. Srouji, T. Kizhner, and E. Livne, *Regen Med.*, **1**, No. 4, 519-528 (2006).
12. R. Quan, D. Yang, X. Wu, *et al.*, *J. Mater. Sci. Mater. Med.*, **19**, No. 1, 183-187 (2008).
13. W. R. Walsh, J. Harrison, A. Loefler, *et al.*, *Clin. Orthop. Relat. Res.*, **375**, 258-266 (2000).