Pilot *In Vitro* Study of the Parameters of Artificial Niche for Osteogenic Differentiation of Human Stromal Stem Cell Pool

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The aim of this research is experimental investigation of the topography and evaluation of some parameters of artificial microterritories promoting osteogenic differentiation of stromal stem cells. A technique of short-term culturing of prenatal human lung stromal cells with fibroblastoid morphology on calcium phosphate substrates with known topography was used. Judging from secretory activity of the cell culture (osteocalcin, alkaline phosphatase), stromal stem cells directly interacting with calcium phosphate discs have advantage in manifestation of osteoblast-like functional activity in comparison with cells cultured on plastic. Rough surfaces of calcium phosphate discs stimulate the formation of spatial human fibroblastoid cell culture. The cells with positive reaction to acid phosphatase are located on spheroliths forming the relief of calcium phosphate coatings. The cells with positive reaction to alkaline phosphatase (marker of osteoblasts) populate hollows (niches) of the artificial surface. The niche for induction of osteogenic differentiation of human multipotent mesenchymal stem cells is apparently a structural and functional formation. It can be characterized by an index calculated as the ratio of the total area occupied by alkaline phosphatase-positive cells to the area of artificial surface occupied by one stained cell.

Key Words: *fibroblast-like cells; calcium phosphate surface; osteocalcin; alkaline phoephatase*

For explaining such a fundamental and contradictory phenomenon as self-maintenance of the pool of hemopoietic stem cells (HSC) and subsequent choice of differentiation program into this or that hemopoietic lineage, R. Schofield put forward a hypothesis on a hemopoietic niche (specialized cell microenvironment), *i.e.* a combination of exctracellular matrix molecules and cells producing growth factors and other regulatory molecules essential for the maintenance of stem cell phenotype. Outside the niche HSC differentiate or die (ineffective hemopoiesis) [27]. Committed hemopoietic precursors have their specific microenvironment, so-called hemopoietic islets [17].

R. Schofield paper gave rise to 30-year studies devoted to hypothetic models and various aspects of

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functioning of HSC niches. Detection of close contacts between HSC and osteoblasts located in the endosteum [5] and adventitial cells of blood vessels made a scientific breakthrough in this field and the existence of osteoblastic and vascular niche for HSC was postulated [31].

Osteoblasts, descendants of multipotent mesenchymal stem cell (MMSC), contact with hemopoietic elements, on the one hand, and with the bone containing hydroxyapatite (HAP) and other calcium phosphates, on the other. Numerous conceptions of MMSC proceed from theoretical prerequisites for HSC [25]. In this context, the question on the existence of a niche for MMSC and its descendants, first of all, osteogenic precursors, is quite appropriate.

However, we found only few cautious assumptions on the existence of a hypothetical niche (a structural and functional unit) for MMSC and its descendants [14]. It is emphasized that *in vivo* localization of MMSC is little studied; for instance, they can be located in the perivascular niche [18]. The existence of a specific niche for MMSC probably confined its *in vivo* commitment to 5 differentiation lineages (bone, cartilage, stroma, tendons and ligaments, and fat) [12] in comparison with 11 or more lineages described *in vitro* [14].

Scaffold technology (creation of specific microenvironment for cells and tissues using artificial or natural materials with technologically designated surface properties) is an experimental approach to the search for niches for stromal cells. Researches in the field of medical material technology and tissue bioengineering believe that the main events related to cell vital activity and fundamental biological processes of cell proliferation, commitment, differentiation, and maturation underlying tissue formation take place at the boundary between the artificial material (implant) and cell (tissue) [15,22].

Purposeful design of HAP and/or calcium phosphate scaffold helped to reveal a clear-cut dependence of bone tissue growth *in vivo* on surface relief [10]. At the modern stage, the topography and size of niches for different types of stem cells have to be determined, because the definition of the niche as a morphological (structural and functional) unit (microterritory) providing conditions for vital activity of stem cells implies finite values of its parameters. However, the main factors involved in creation of microterritories for stromal stem cells remain unknown, which hampers the development of cell biology and tissue engineering.

The aim of this research is experimental investigation of the topography and evaluation of some parameters of artificial microterritories promoting osteogenic differentiation of stromal stem cells.

MATERIALS AND METHODS

Characteristics of MMSC populations are similar in different tissues [18]. They were found in the embryonic [14] and lung tissues [23]; hence, the culture of prenatal fibroblast-like cells from human lungs (Bank of Stem Cells) can be a source of MMSC. The preparations represent a population of cells of different shape and size (Fig. 1), which is typical of MMSC pool [14], with limited life-span, stable karyotype upon passaging, and oncogenically safe. The cells are free of viral (HIV, hepatitis and herpes viruses, *etc.*) and bacterial agents (syphilis, mycoplasma, chlamydia, *etc.*). After defrosting, cell viability evaluated in the test with 0.4% trypan blue (according to ISO 10993-5) was 91-93%.

Titanium discs BT1.0 (12 mm diameter, 1 mm thickness) with bilateral calcium phosphate coating (CPC) were used as the carriers of artificial niches for MMSC culturing. The coatings were applied on the titanium carrier by the method of microarc oxidation in 10% phosphoric acid containing a suspension of nanosized synthetic HAP particles. The frequency of pulse current was changed from 1 to 10 Hz and pulse duration from 20 to 200 msec.

Mechanochemical synthesis of nanosized HAP was carried out as described previously [11]. HAP with a stoichiometric composition of $Ca_{10}(PO_4)_6(OH)_2$ was synthesized. Phase composition and crystallinity of the synthesized HAP nano-powder were confirmed by X-ray diffraction analysis and infrared spectroscopy. Electron microscopy (JEM-200CX) showed that the size of HAP particles varied from 10 to 40 nm.

Surface roughness of the coatings was evaluated by the parameters of vertical profile irregularity using

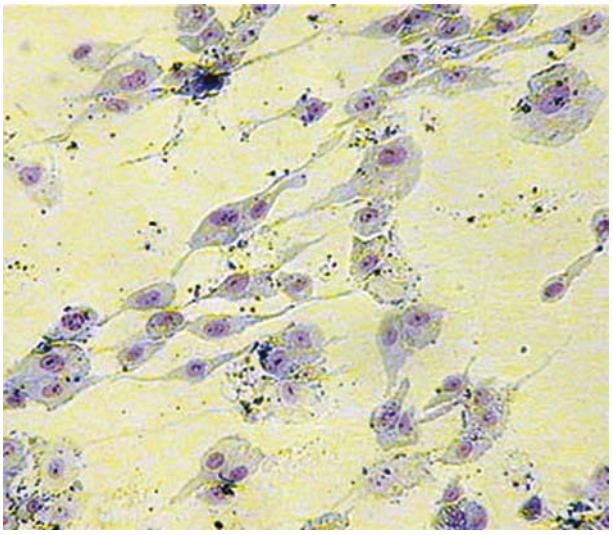

Fig. 1. Culture of prenatal human lung cells on day 5 of culturing in osteogenic medium. Azure II and eosin staining, ×400.

a Talysurf 5-120 measuring system (at 1 nm resolution capacity). *Ra* as a mean roughness value for several measuring sites was determined (GOST 2789-73).

The osteogenic medium contained differentiation inductors (usually, dexamethasone, β-glycerophosphate, and ascorbic acid); the concentrations of inductors somewhat differ in different reports [14, 19,26]. The following medium was used for cell culturing on calcium phosphate substrate: 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10^{-6} M dexamethasone, 280 mg/liter L-glutamine, 50 mg/liter gentamicin sulfate, 10 mM HEPES buffer, 20% FCS, and 80% DMEM/F12 (1:1).

The discs were placed in wells (1.77 cm^2) of 24-well plates (Costar) and cell suspension containing 5×10^4 karyocytes in 1 ml complete nutrient medium was added. Fibroblast-like cells cultured on plastic served as the control.

After 4 days, the implants were removed and dried on air. Fixation of the cells adherent to the surface was performed in two variants: 1) 30 sec in formalin vapors (for cytochemical staining for alkaline phosphatase, AP); 2) 30 min in 2.5% glutaraldehyde on phosphate buffer for scanning electron microscopy (SEM).

The surface morphology of the hybrid implants (base plate+CPC+fibroblast-like cells) was studied in a Collective Usage Center for Material Technology, Tomsk State University. SEM was performed on a Phillips SEM 515 microscope and reflected light microscopy on an Olympus GX-71 metallographic microscope.

For preparing the samples for SEM, the cells adherent to the surface were fixed in glutaraldehyde and 1% osmium tetroxide for 30-40 min and then washed twice with phosphate buffer (pH 7.2-7.4). Then the cells were dehydrated in ascending alcohols (30, 50, 70, 90, and 100°, 15 min each) and 100% acetone.

Stromal cells are heterogeneous by their phenotypes, which can be explained by their multiple functions. None of the known surface antigen markers can be a reliable parameter for evaluation of MMSC culture purity [14,26]. On the one side, even long-term cultures demonstrate certain heterogeneity, and on the other, functionally different cell cultures can have similar immunophenotypical profiles [26].

In light of this, the capacity to differentiate into certain cell lineages and mesenchymal tissues is an important characteristic of stem cells [29]; this can be evaluated by cyto(histo)chemical and biochemical methods. AP and osteocalcin (OC) are often used as molecular markers of osteogenic differentiation of MMSC [14].

Activities of AP and acid phosphatase (AcP) in cells were measured as described previously [9]. The method is based on hydrolysis of naphthol-ASMX-phosphate with the release of α-naphthol in the presence of AP, calcium and magnesium ions. The interaction of α-naphthol with fast garnet dye yields insoluble diazonium precipitate in shades of brown depending on enzyme concentration. Activity of AcP was evaluated by enzymatic hydrolysis of naphthol-AS-BI-phosphate followed by the reaction of the released naphthol with pararosaniline yielding a colored end-product at the site of enzyme localization. In cells, AcP was detected by diffuse rose staining of the cytoplasm.

Supernatants of 5-day cell cultures were collected from the wells into tubes, centrifuged for 10 min at 500*g*, and functional state of fibroblasts was evaluated by activities of AP and AcP and concentration of OC in the supernatant.

The concentration of OC was measured using Osteometer BioTech A/S N-MID Osteocalcin One Step ELISA test system (Nordicbioscience diagnostics). The analysis was performed by the standard scheme for ELISA [3,7]. The results were processed automatically using 4-parametric logistic regression.

Activities of AP and AcP in conditioned media were measured by a standard colorimetrical method recommended by AACC and IFCC [7] using Novofosfal and Fosfacid-novo kits (Vector-Best) followed by photometry at 400-420 nm.

The method of computer morphometry was applied for evaluation of quantitative parameters of cells by measuring their optical characteristics [8]. The area and optical density of objects were calculated using Adobe PhotoShop 7.0 software by gray-scale statistics for non-transparent objects in modification [1]. The area was expressed in μ^2 and optical density in optical density arbitrary units (opt. dens. arb. units).

For analysis of the obtained data, methods of descriptive statistics and verification of statistical hypothesis were used. The hypothesis on equality of the mean sample values was tested using Student's *t* test. For analysis of the correlation between the studied parameters, regression analysis was performed and Spearman rank correlation coefficients (r_S) were calculated. The differences were significant at $p<0.05$.

RESULTS

Differentiation of stem cells can be induced by biological (genetic, cytokine, enzyme, *etc.*) and chemical signals [14] as well as physical [22] and biomechanical factors [2].

The serum added to the culture medium served as a source of bioactive substances and minerals essential for MMSC growth [14]. In the absence of cells in the culture medium, correlation and regression analyses revealed direct relationships between its biochemical (AP, AcP, and OC) and chemical (phosphorus, P)

components essential for the formation and deposition of apatites [4] during their interaction with artificial surface (plastic). For calcium ions these relationships were not shown. Under these conditions, direct (P/OC and P/AcP) and reverse (P/AP) correlations of parameters with high regression coefficients (R=0.996-0.999; p<0.002-0.004; n=4) were observed, which attested to enzymatic control of P concentration in the biological fluid [4].

Addition of fibroblast-like cells adhering to plastic to the culture medium modified the character of the correlation matrix. In contrast to P and AcP, calcium ions actively participated in the molecular processes (r_S=0.85, p<0.008 for OC and r_S=-0.74, p<0.035 for AP; n=8).

Short-term culturing of prenatal cells from human lung on plastic (cell growth control) modified their morphology. Apart from round and oval cells (67% all cells) of varying size typical of cell suspension, elongated (26%) or pyramid fibroblast-like cells (7%) appeared. Round and oval shape is typical of low-differentiated fibroblasts. Changes in fibroblast shape from round to oval indicate cell maturation and differentiation [6].

According to the relative contribution of AP in the total pool of secreted AP, functional activity of stromal stem cells under these conditions was on average 88%. Bone AP is a marker of osteoblasts [4] derived from the stromal precursors during culturing. According to immunophenotyping data, the admixture of cells carrying endothelial, macrophage, and lymphocytic CD markers in the MMSC culture from human bone marrow can reach 10% [14].

Culturing of fibroblast-like cells on disks coated with calcium phosphates completely broke the correlation relationships between biochemical and chemical parameters of the culture medium. Nevertheless, the concentration of AP in conditioned media increased (by 12%, p<0.001) and calcium content decreased (by 15%, p<0.00008), which implies its deposition in the form of amorphous apatites. Under conditions of contact with rough surface, physical (structural) stimulators of cell functional activity probably play the leading role. It should be emphasized that adhesion to the substrate is a crucial condition of MMSC vital activity [14].

Our previous results obtained in *in vivo* experiments showed that microenvironment of stromal cells has certain physical parameters which can be modeled by scaffold technologies. Advances in modern materials science and calcium phosphate materials available prompted a question on quantitative characteristics of artificial microterritories promoting osteogenic differentiation of MMSC. An important characteristic of artificial surfaces affecting adhesion, migration, and osteogenic differentiation of stromal stem cells is their roughness [10].

In vitro reaction of stromal cells to topography of artificial surfaces is now extensively studied [16, 24,30]. However, evaluation of the effects of surface roughness on morphofunctional behavior of osteoblasts yielded ambiguous results.

According to definition of stem cells [12], they can be recognized only during their functioning; at present, the optimal methods for their detection are functional tests, *e.g.* molecular and cytochemical tests.

In our study, regression (Fig. 2) and correlation analyses revealed a direct correlation (r_S=0.75; p<0.019; n=9) between AP and OC secretion into the conditioned medium and the roughness (Ra) of calcium phosphate surfaces. Published data suggest that AP and OC are true molecular markers of osteoblasts [4,14,19]. This suggests that roughness of the surface can be one of key physical factors promoting *in vitro* functional (secretory) osteogenic activity of stromal cells.

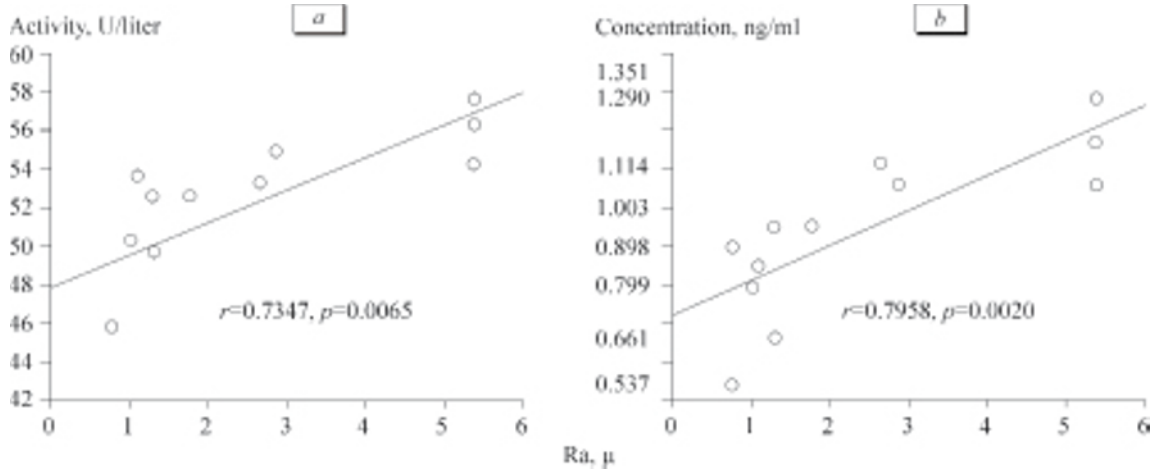

Fig. 2. Correlation between secretion of AP (*a*) and OC (*b*) by fibroblast-like cells and CPC surface roughness. Abscissa: roughness (*Ra*).

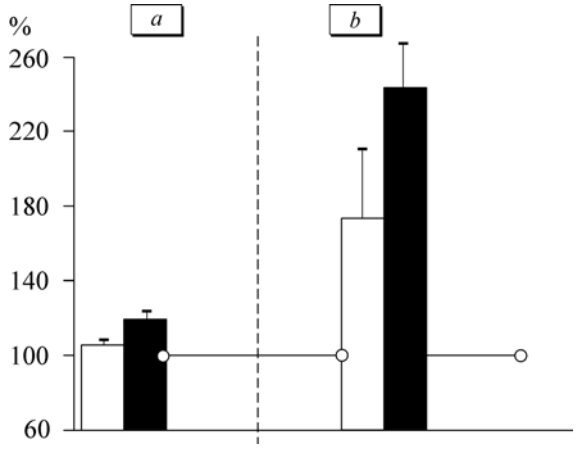

Fig. 3. Markers of secretory activity of fibroblast-like cells on plastic (control, light bars) and calcium phosphate surface (experiment, dark bars). Ordinate: % of corresponding values in cell-free culture medium. *a*) AP; *b*) OC.

The interaction of stromal cells with the calcium phosphate surface considerably stimulated their secretory capacity. Activity of AP and OC content in supernatants of hybrid implants (disk+adherent fibroblast-like cells) significantly increased (Fig. 3) compared to the corresponding parameters in the control cell culture on plastic (by 13 and 70%, respectively).

Thus, stromal cells interacting with calcium phosphate disks more markedly exhibit osteoblast-like functional activity. The implants can modulate vital activity of cells and tissues either directly (surface properties) or indirectly via products of degradation [15]. Experiments allowed us to evaluate the state of fibroblast-like cells adherent to plastic outside the zone of direct contact with calcium phosphate coating of the implant placed in the culture medium. In this case, changes in the state of the cell culture are determined by the combined effect mediated via products of material degradation and secretory activity of cells fixed to the disc surface.

Our findings suggest that the state of fibroblast-like cells adherent to plastic and not directly contacting with the implant does not differ from that of control cells cultured in the absence of the disc (Table 1).

Outside the implant, the cells adherent to plastic demonstrated weak reaction to AP. It is known that positive reaction for AP is typical of stromal cells (fibroblasts, osteoblasts), MMSC derivatives [9,13]. It should be noted that AP activity increases in parallel with cell maturation [6]. Some authors consider staining for AP as a cytochemical characteristic of osteoblasts [4]. Erythroblasts, megakaryocytes, lymphocytes, monocytes, and young granulocytes demonstrate negative reaction for AP [9].

According to computer morphometry data (Table 2), optical density of AP-positive cells fixed on calcium phosphate surface increases by more than 2-3 times ($p<0.00002$; $<10^{-6}$) compared to values presented in Table 1, *i.e.* direct contact of MMSC pool with artificial relief (3D) calcium phosphate surface stimulates their maturation and osteogenic potencies, which manifests, in particular, in enhanced secretion of bone matrix protein (Fig. 3).

HSC and daughter cells, MMSC and its descendants are components of microenvironment for each other [14], therefore, accumulation of stem cells in the endosteum, where they closely contact with the bone, is beyond doubt [4,5]. The physicomechanical properties can substantially modulate differentiation of MMSC into osteoblasts.

In light of this, a question arises about topography of maturating osteoblasts on artificial relief surface. Solution of this question will bring us to the problem of topography and size of niches for osteogenic differentiation of stromal stem cells, because the notion of niche as a morphological (structural and functional) unit (microterritory) providing conditions for vital activity of stem cells implies finite values of its parameters.

Reflected light microscopy revealed the formation of a spatial gradient distribution of the cell culture on relief calcium phosphate surfaces. Cytochemical staining revealed AP in some cells. They are primarily located on spheroliths of the coating (Fig. 4), due to which the degree and area of staining do not depend on the relief of the artificial surface. Correlation and linear regression analyses revealed no significant relationships between cell parameters and *Ra* of the surface.

AcP is a lysosomal enzyme present in various stromal (fibroblasts, osteoblasts) and hemopoietic cells (monocytes/macrophages, granulocytes, lym-

TABLE 1. Characteristics of Prenatal Human Lung Fibroblast-Like Cells Grown on Plastic and Stained for AP ($X\pm m$)

Group	Optical density of cells, opt. dens. arb. units	Area of cells, % of photography area
Control (n=12)	4.36±0.58	1.12±0.11
Experiment (n=11)	5.43±0.35	1.07±0.10

Note. Control: cell culture on plastic in the absence of the implant; experiment: cell culture on plastic around the implant. *n*: number of analyzed cells.

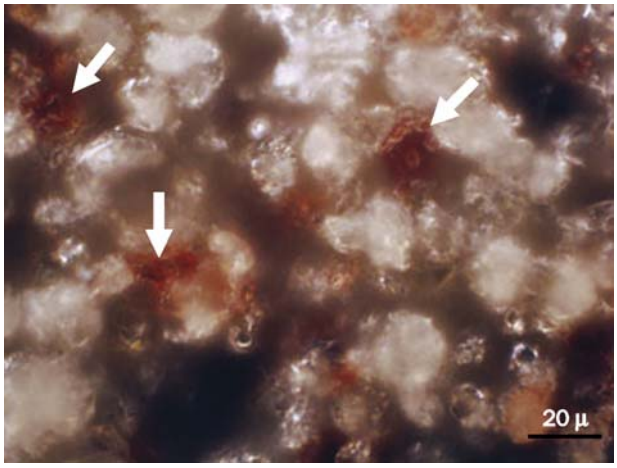

Fig. 4. Location of AcP-positive cells (arrows) on CPC surface (×1000).

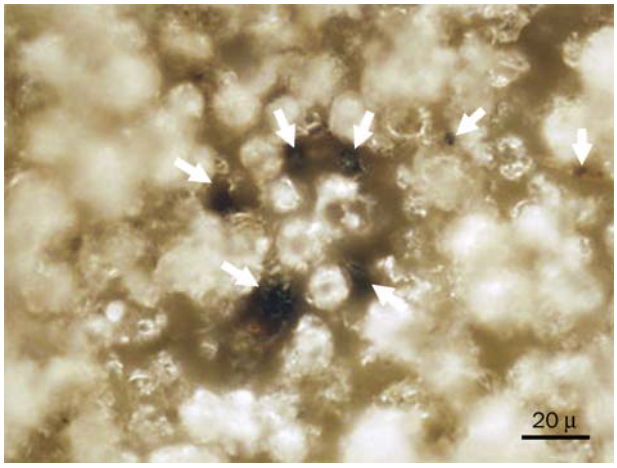

Fig. 5. Location of AP-positive cells (arrows) in hollows of CPC surface according to reflected light microscopy (×1000).

phocytes) [6,9,13]. AcP-positive cells (osteoclasts, monocytes/macrophages) actively participate in bone remodeling [4].

However, in our studies AcP activity in the supernatants of cell cultures virtually did not increase. AcP-positive cells were detected in fibroblast CFU (in particular, in human peripheral blood) representing a heterogeneous population of original mesenchymal cells at different stages of differentiation [21]. In light of this, the detected cells can be stromal precursors from the MMSC pool.

The AP-positive cells occupied only the communicating hollows between the spheroliths. The alternation of hollows and spheroliths determined roughness of the surface, which can be expressed by *Ra* parameter. Big hollows were occupied by several cells of regular shape (Fig. 5) in contrast to fibroblast-like cells with pseudopodia located on the surface, which was confirmed by SEM data (Fig. 6). Stromal stem cells can change their morphology upon interaction with the relief of different geometry (active concept) or peculiarities of the relief promote passive selection of original mesenchymal cells. In any case, epigenetic regulation of gene activity induced by 3D matrix is possible [15], which can determine variations of their differentiation in different microterritories.

During bone tissue remodeling, the stromal precursors colonize dish-shaped depressions with a depth up to 40 μ in the trabecular bone, formed by osteoclasts, and differentiate into osteoblasts actively synthesizing the bone matrix *in vivo* [4]. Our *in vitro* experiments showed that under conditions of uniform distribution of cell culture per well surface area, 63±9% cells from prenatal human lung during short-term interaction with relief CPC populated its hollows similar to those in the bone tissue and demonstrated positive reaction to AP (marker of osteoblasts). About 58% daughter clones obtained *in vitro* from one colony of human bone marrow MMSC can form the bone after subcutaneous implantation to immunodeficient mice [14].

The density of osteoblast distribution is important for normal remodeling of the bone; this parameter decreases in osteoporosis [4] and can be an important characteristic of implant suitability for bone tissue bioengineering. At least, osteogenic activity of discs tested in our *in vitro* experiments correlated with their capacity to stimulate bone formation *in vivo* established in our previous study [10].

According to light microscopy data (Table 2), the area of depression in the artificial coating preferentially occupied by each AP-positive cell was about 302 μ^2, 86% stained cells were seen in hollows with areas of 100-625 μ^2. These hollows can be considered as microterritories where stromal stem and original cells differentiate and maturate into osteoblasts. The mean area of AP in the cell, according to immunochemical staining, was 23.73±1.95% (SD=12.79%) from the microterritory area. SEM showed that the cell occupied on average 217 μ^2 (118-316 μ^2 with consideration for SD) or 42% its microterritory.

Heterogeneity of human MMSC cultures by the size, morphology, and differentiation potential was previously demonstrated [14]. The scatter of the experimental data can reflect selection of different classes of stem and committed cells in appropriate microterritories.

Thus, rough calcium phosphate surfaces stimulate the formation of spatial culture of human fibroblastoid cells. The cells with positive reaction to AcP are located on spheroliths forming the relief of calcium phosphate coatings. The cells with positive reaction to AP populate hollows of the artificial surface.

Functional activity of stromal cells increases with increasing the size of hollows on the surface. CPC with roughness *Ra*=0.5-5.5 μ stimulated secretory ac-

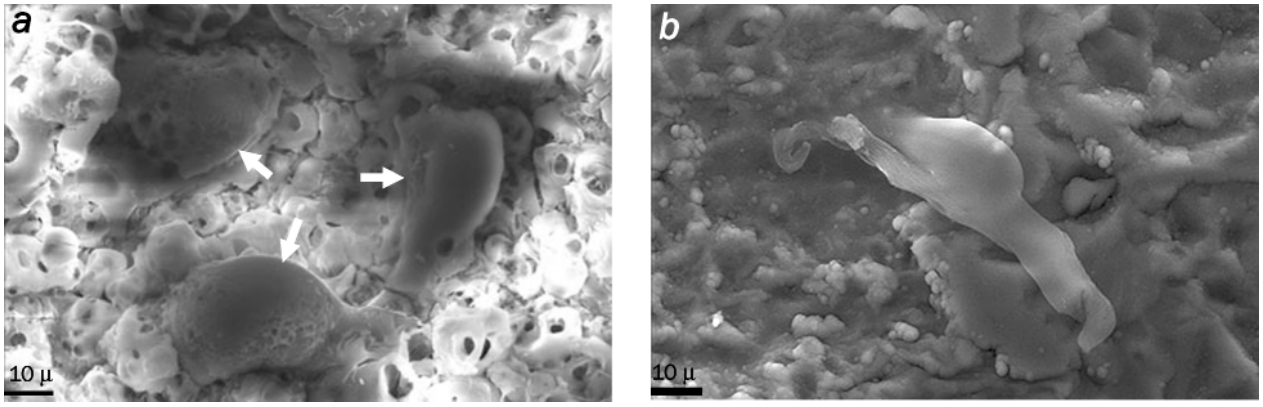

Fig. 6. SEM of fibroblast-like cells (×1250). *a*) hollows on calcium phosphate surface. Arrows: location of cells; *b*) calcium phosphate surface.

tivity of osteoblasts (Fig. 2). We revealed a 71% correlation dependence between the area of AP-positive staining and the area of hollow occupied by each cell. In turn, the staining area by 34% correlated with optical density of stained zones (Table 2).

Of particular importance is the fact that the niche for induction of osteogenic differentiation of human multipotent mesenchymal stem cells is apparently a structural and functional formation. It can be characterized by an index (in %) calculated as the ratio of the total area of AP-positive cell staining to the area of artificial surface occupied by one stained cell (microterritory): S_{AP}/S_{MT}. This index (in our case ~24%) apparently characterized the distribution of active enzyme (marker of osteoblasts) depending on the area of the hollow (microterritory, niche) occupied by the cell. The proposed parameter can be used in further studies aimed at the search for optimal structural and functional parameters of osteogenic niche on surfaces with different relief.

The obtained results are of fundamental (theoretical and experimental) and applied importance for critical technologies of science development in the Russian Federation (including cell technologies and nanotechnologies, creation of new materials and implants, tissue bioengineering). Of particular importance are new impulse to the search of regulatory mechanisms of MMSC differentiation to various lineages and scientifically substantiated principles of improving the efficiency of bioengineering of supporting tissues and development of biotechnological bases of medical materials technologies for creation of implants for the bone tissue. In view of well-known trans- and dedifferentiation capacity of osteoblast culture [19,20], the results can be useful for the study of niches for other MMSC differentiation lineages.

Moreover, understanding of close interaction between the bone and hemopoietic tissues in the formation of the hemopoietic niche [28] brings us (on the basis of the presented methodology) to the problem of quantitative evaluation of microterritories providing vital activity of HSC.

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TABLE 2. Results of Computer Morphometry of Fibroblast-Like Cells Stained for AP and APA Parameters (X±m, *P*)

Parameter	Mean value (*n*=43)	Coefficient of correlation between the parameters, r_S		
		1	2	3
Area of AP-positive staining, μ²	65±9	–	0.34* <0.026	0.71* $<10^{-6}$
Optical density (D) of stained fragments of cells, opt. dens. arb. units	14.42+0.96	0.34* <0.026	–	0.19 >0.2
Area of hollow on the surface occupied by stained cell, μ²	302±34	0.71* $<10^{-}$	0.19 >0.2	–

Note. *n*: number of staining cells; *significant differences (*t* test).

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