Effect of Hydroxyapatite as a Component of Biostable Composites on Population and Proliferation of Mesenchymal Stem Cells

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Three groups of biostable composite materials were studied. The initial binder polymers (polymethylmethacrylate, polyamide-12, superhigh-molecular-weight polyethylene) and hydroxyapatite-containing composites on the basis of these polymers were tested. Biostable polymers, including those containing hydroxyapatite, were nontoxic for fibroblasts and mesenchymal stem cells: the adhesion parameters for these cells were maximum for polyamide-12 and superhigh-molecular-weight polyethylene and did not depend on the presence of hydroxyapatite. Cell adhesion to "pure" polymethylmethacrylate was significantly lower than to other composites, but increased after integration of hydroxyapatite. The efficiency of proliferation of fibroblast and mesenchymal stem cell on the surface of polyamide-12 and superhigh-molecular-weight polyethylene was maximum and did not depend on the presence of hydroxyapatite. The efficiency of cell proliferation on the surface of "pure" polymethylmethacrylate was low, but increased significantly if it was combined with hydroxyapatite, particularly in areas of mineral particles accumulation. It seems that the presence of high amounts of hydroxyapatite in polymethylmethacrylate samples promotes cell adhesion and proliferation.

Key Words: *mesenchymal stem cells; polyamide; polymethylmethacrylate; superhigh-molecular-weight polyethylene; hydroxyapatite*

Biocompatible composite materials close to bone tissue by their physical and mechanical characteristics are now used for repair of bone defects. The materials of biological origin are effectively substituted by such biostable composites as polymethylmethacrylate (PMMA), superhigh-molecular-weight polyethylene (SHMPE), and polyamide-12 [1,3,4,6,10]. They are not hydrolyzed in biological media and are characterized by mechanical strength, low immunogenicity, and resistance to infection. Synthetic hydroxyapatite (HAP) is added to composites for improving their mechanical and osteointegrative characteristics [1,3]. Stimulation of the formation of full-value bone tissue in patients with metabolic disorders and for repair of large bone defects requires appreciable increase of the osteogenic potential of implantation materials, which can be attained by various methods, including modern cell technologies [7-9]. The toxicity of biostable osteoplastic materials and their effects on proliferative activity of cells covering them have never been evaluated, nor the role of HAP as a component of these materials was studied.

We developed a method for population of biostable HAP containing osteoplastic material with mesenchymal stem cells (MSC).

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MATERIALS AND METHODS

Three composite biostable materials were compared: polyamide-12, PMMA, and SHMPE (Table 1).

The initial binding polymers (copolymer PMMA, polyamide-12, SHMPE) and composites on their basis containing HAP, were studied. Wells from culture plastic without composite materials served as the control. Polyamide-12 with carbon fiber and HAP is a composite material developed at Laboratory of Filled Polymeric Systems, A. N. Nesmeyanov Institute of Elementorganic Compounds, Russian Academy of Sciences [1]. PMMA was obtained from polymer/monomer composition polymerized at 100°C. The GUR 4120 SHMPE with mol. weight of 4×10^6 Da [2] was used. Plates (20×10 mm, 2 mm thick) were prepared from composites. Synthetic HAP was introduced into composites (50% weight) by a previously developed technology during their preparation [3]. HAP (Polistom Company) with the main fraction particles of 5-10 μ was used. The plate surfaces were polished using dental technological paste. Chemical composition of the outer layers of composites' was evaluated by X-ray photoelectron spectroscopy.

Cytotoxicity of the test materials and their effects on the efficiency of cell adhesion and proliferation were evaluated using cultured human diploid postnatal fibroblasts and bone marrow stem cells. The cells were cultured in DMEM with 10% FCS (PanEco) in disposable plastic flasks and in 24- or 96-well plates (Nunk) at 37°C and 5% CO_2 under conditions of saturating humidity. The number of cells on the surface of composites was evaluated by photometry and direct counting in a Goryaev chamber after cell removal with trypsin solution. Screening MTT test was used. Formazan was eluted with OM 80 for 30 min at 37°C and optical density of the eluate was measured on an EFOS 9305 (EFOS) microplate reader at λ=570 nm. Wells with cells without samples served as the control.

The efficiency of cell adhesion to the sample surface was evaluated by the number of adherent cells, their morphology, viability, and distribution on the sample surface. The optimal time of cell adhesion to the substrate (plastic for cell culturing) was preliminary evaluated. After 120 min the cells completely adhered to the substrate and started flattening. The composite samples were then placed into 24-well plates and 100 μl cell suspension (50,000 cells per sample) was applied onto their surfaces. After 120 min, the samples were washed with culture medium, cleansed from nonadherent cells, and transferred to new plates with fresh medium. After 24 h, the number of cells on samples was evaluated using the MTT test and routine counting in a Goryaev chamber. Cell viability *in situ* was evaluated by fluorescein diacetate and ethidium bromide staining. The images were obtained using color CCD Sanyo 6975 TV camera. Adherent cells were stained with acridine orange for cell visualization and evaluation of cell morphology and density of population in the samples. The images were obtained with the Kodak MDS-290 system.

The efficiency of cell proliferation on the test samples was evaluated by the number of cells, their morphology, and pattern of their distribution on the sample surface. In order to obtain cell suspension, the samples were treated with Versene and trypsin solutions at 37°C. Organization of the cell layer on the surface of the test materials was evaluated by scanning electron microscopy (SEM, Phillips SEM-515 microscope) after 14 days in culture.

TABLE 1. Specification of Studied Groups of Samples

Group	Sample	Sample composition
Control		Culture plastic (Nunk)
1	1	Polyamide-12
	2	Polyamide-12+carbon fiber+30% HAP
2	3	PMMA copolymer
	4	PMMA+30% HAP
3	5	SHMPE
	6	SHMPE+30% HAP

RESULTS

Specimens of biostable composite materials containing and not containing HAP exhibited no cytotoxic effect towards fibroblasts or MSC contacting with them. The results of screening MTT test were at the level of control values (Fig. 1).

Fibroblasts and MSC more intensely adhered to the surface of polyamide-12 and SHMPE. Introduction of HAP into these composites did not promote cell adhesion to their surface (Table 2). For PMMA samples these values were significantly lower (no more than 60% of control). HAP as a component of PMMA significantly improved the efficiency of cell adhesion to the composite. Cell number on the samples was evaluated by the MTT test (Fig. 2). The results of evaluation by the two methods were similar. The reactions of fibroblast and MSC to polymers were similar.

Evaluation of cell viability *in situ* by fluorescein diacetate and ethidium bromide staining showed solitary dead cells on the surfaces of all studied samples. The cells were located evenly on polyamide-12 and SHMPE samples and formed cords on the surface of PMMA samples.

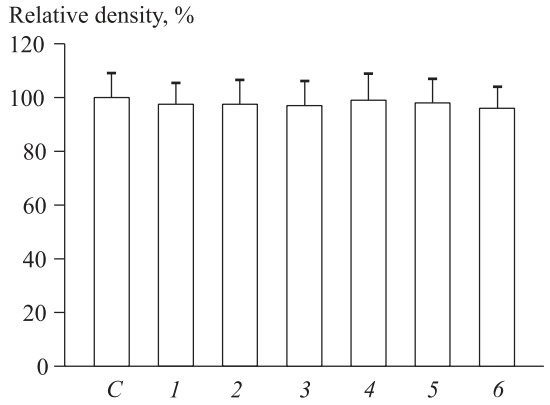

Fig. 1. Optical density of eluate for each sample (*1-6*) populated by MSC. Here and in Figs. 2, 3: *C:* control.

Fibroblast and MSC proliferation was most effective on samples 1, 2, 5, and 6. Evaluation of fibroblast proliferation by the MTT test showed the same trends in changes of the proliferation parameters as in direct cell counting: the values were minimum on samples 3 and 4.

The efficiency of MSC proliferation on days 7 and 14 evaluated by direct counting in a Goryaev chamber and by the MTT test (Fig. 3) was maximum on polyamide-12 and SHMPE samples and minimum on PMMA samples, which was in line with the data on fibroblast proliferation. Fibroblast and MSC growth rates varied: the number of fibroblasts surpassed that of MSC on day 7, while by day 14 of culturing the cell counts were similar. This result can be due to contact inhibition during the formation of cell monolayer. It was found that the presence of HAP in polyamide-12 and SHMPE composites was inessential for the efficiency of cell proliferation, while as an ingredient of PMMA, HAP significantly increased this parameter. This effect can be explained by peculiar cell distribution pattern on the surface of these samples.

Scanning electron microscopy (Fig. 4) of polyamide-12 and SHMPE samples after 7 days of culturing showed that MSC formed single layer, were sharply flattened and had a polygonal shape (Fig. 4, *a*). The back side of these cells was smooth, though often with short microvilli (Fig. 4, *b*). Dividing cells were sometimes seen (Fig. 4, *c*). They had more round shape and their back surface had microvilli. Cell bodies and processes were usually interwoven and formed an almost continuous layer. No HAP areas open for cells were detected on the surface of these composites, which seems to explain negligible increment in cell number during their proliferation in comparison with the control. On uneven surface of PMMA samples, the cell layer was looser with gaps, in which globular structures of the composite could be discerned, the cell processes penetrating into the spaces between the granules. Crystal accumulations on PMMA samples containing HAP were easily accessible for cells, which formed a substantial layer on HAP-containing areas of the composite. MSC bodies were located at some distance from each other.

HAP particles on polyamide-12 and SHMPE were not abundant, they were separated from the surface with a thin layer of the polymer and therefore were inessential for cell proliferation. X-Ray photoelectron spectroscopy for the depth of 3-4 nm showed that by the ratio of Ca and P elements in different layers of the composites the PMMA samples contained 4-5-fold more HAP than SHMPE samples.

After 14 days in culture, the main regularities of cell organization on the surface of samples were preserved, but the cells were more compactly packed and often formed several layers; mitoses were rarer than after 7-day culturing.

Biostable polymers, including the HAP-containing ones, are not toxic for fibroblasts and MSC. The parameters of these cells adhesion were maxi-

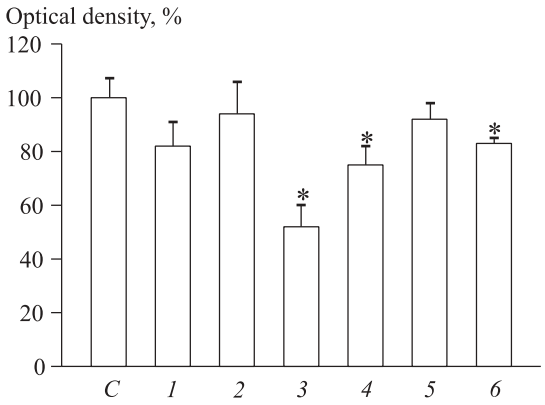

Fig. 2. MTT test: MSC adhesion to surface of samples (*1-6*). Here and in Fig. 3: **p*<0.05 compared to the control.

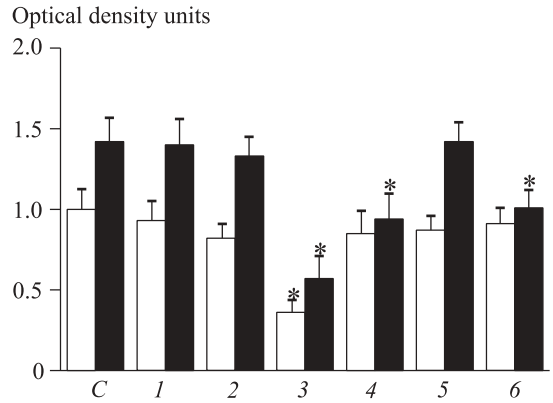

Fig. 3. MTT test: efficiency of MSC proliferation. Light bars: day 7 of culturing; dark bars: day 14.

TABLE 2. Number of Cells ($\times 10^3$) Removed from Each Composite Sample: Counting in a Goryaev Chamber

Parameter	Sample					
	1	2	3	4	5	6
Fibroblasts	44±5	43±4	29±4	42±5	41±3	39±4
MSC	47±5	46±4	28±3	38±3	42±3	36±4

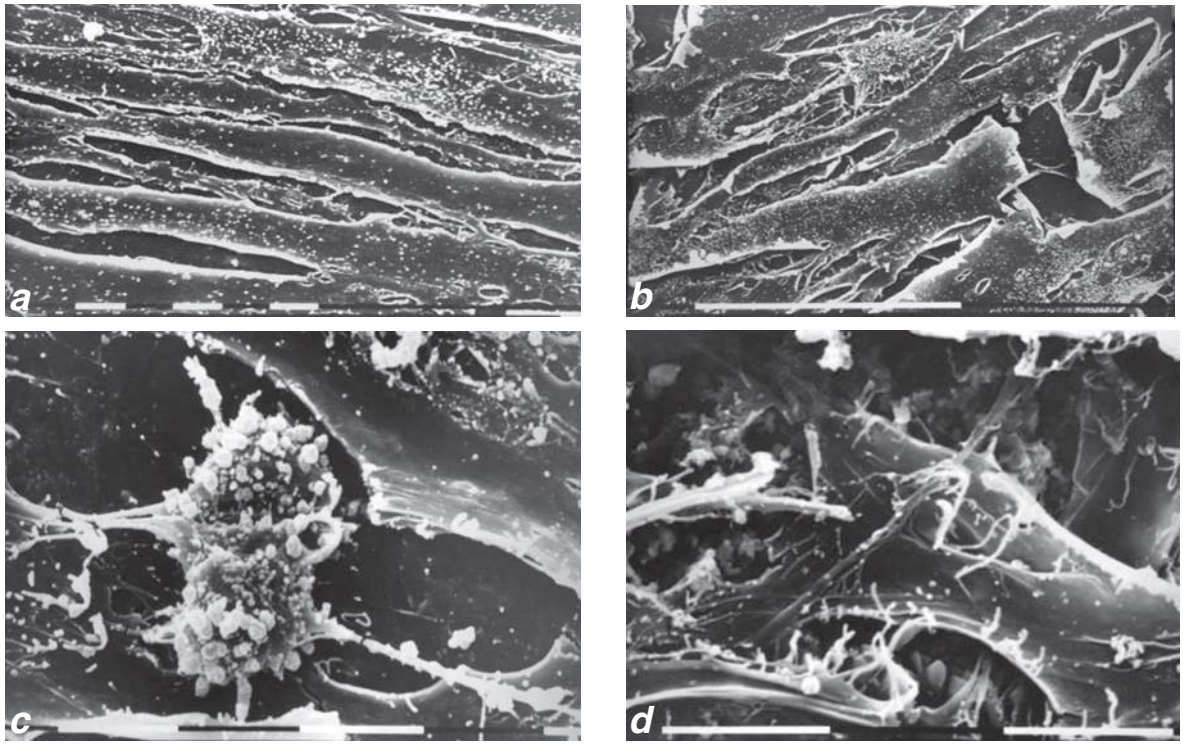

Fig. 4. Scanning electron microscopy of cells on the surface of composite samples on day 7 of culturing. *a*) MSC on the surface of polyamide-12 sample (scale 100 μ); *b*) short microvilli on the back surface of MSC on a sample from polyamide, carbon fiber, and HAP (scale 10 μ); *c*) mitotic cell on the surface of sample from polyamide-12, carbon fiber, and HAP (scale 10 μ); *d*) HAP-containing areas under MSC on the surface of a sample from non-modified PMMA and HAP (scale 10 μ).

mum for polyamide-12 and SHMPE and did not depend on the presence of HAP. Cells less intensely adhered to "pure" PMMA, but introduction of HAP into this composite increased this parameter. The maximum efficiency of fibroblast and MSC proliferation was detected on the surface of polyamide-12 and SHMPE samples, irrespective of HAP presence. The efficiency of cell proliferation on the surface of "pure" PMMA was low, but increased significantly on the surface of HAP-containing PMMA, particularly in foci of mineral particles accumulation. The detected difference in the intensity of fibroblasts and MSC growth on the test composites on days 7 and 14 of culturing can be due to contact inhibition during monolayer formation. Inefficiency of HAP, judging from cell adhesion and proliferation on polyamide-12 and SHMPE, can be explained by shielding of mineral particles by the polymeric binders of these composites, which prevents cell adhesion to them, in contrast to PMMA composite, the surface of which contains numerous GAP minerals.

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