Relationship between Osteogenic Characteristics of Bone Marrow Cells and Calcium Phosphate Surface Relief and Solubility

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The capacity of mouse bone marrow cells to adhere to calcium phosphate surfaces and form tissue plates depending on the surface relief and solubility was studied in ectopic bone formation test. Calcium phosphate coating of titanium disks, made by the anodic spark (microarch) oxidation in 10% orthophosphoric acid with hydroxyl apatite particles, differed by the structure (thickness of coating, size of pores, and roughness) and solubility (level of *in vitro* oxidation of 1-week extracts of implants). Chemical (phasic and element) composition of the studied calcium phosphate coatings was virtually the same. The findings indicate that histogenesis is regulated by physicochemical characteristics of the implant surface. It seems that the osteogenic potential of calcium phosphate surfaces is largely determined by their relief, but not by pH of degradation products.

Key Words: *mice; ectopic osteogenesis; implant; structure; pH of extracts*

Osteogenic properties of calcium phosphate (CP) materials were persuasively proven in ectopic bone formation test, when bone structures formed on their surface after their subcutaneous or intramuscular injection [8,10].

Calcium phosphates with different physicochemical properties [6,8] are characterized by different capacity to maintain the process of bone tissue formation. The surface of the implant should possess optimal macro- and microstructure and bioactive characteristics for integration with bone tissue. The key combination of biocompatibility and functional activity of various types of coating remains not found [1,9], including the structure, thickness, porosity, and rate of dissolution of coating. Biological activity of CP ceramic is determined by the chemical composition, structure, and pH of the system. On the other hand, the osteointegration of materials does not directly depend on their solubility [7].

Comparative analysis of the contribution of CP surface relief and solubility to osteogenic potential of the bone marrow seems to be an interesting problem.

MATERIALS AND METHODS

Tested implants (5 per group) were formed from VT-6 titanium discs 12 mm in diameter and 0.5 mm thick, with bilateral CP coating [2]. In order to rule out the effect of technology of the coating formation on its biological properties, both types of bioactive surfaces were obtained by the anodic spark (microarch) oxidation in 10% orthophosphoric acid in the presence of hydroxyl apatite particles (70-100 μ). Phasic and element composition of CP coatings was studied using X-ray diffraction and microrentgen spectral analysis. The thickness of

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coating was measured by VT-1 eddy currents thickness gage. The morphology of CP surfaces was studied by scanning electron microscopy. Roughness of surfaces was evaluated using a Talysurf 5-120 device with 1 nm resolution (surface roughness class was determined according to State Standard 2789-73).

Extracts of implants were obtained (in accordance with ISO 10993-5 requirements, 1992) in 4 ml 0.9% sterile NaCl at 37°C. Every week the solutions were half-replaced with fresh portions (2 ml). The pH values and concentration of ionized (bioactive) calcium were evaluated by amperometric titration [4].

Ectopic bone formation, when an artificial sample is implanted subcutaneously or intramuscularly without using growth factors (*e.g.*, morphogenetic bone protein) [8], is an adequate experimental approach for evaluation of possible osteogenic characteristics of CP materials. The bone marrow is the most frequent source of osteogenic cells in adults.

Preliminary experiments showed the absence of subcutaneous growth of tissue plates from the bone marrow not fixed on carriers. Therefore 15 BALB/cJLac mice (from vivarium of Institute of Pharmacology, Tomsk Research Center) were subcutaneously (under ether narcosis) injected with the implant with a bar of syngeneic femoral bone marrow applied on the implant beforehand under aseptic conditions (1 hybrid implant per mouse). After 1.5 months the implants were removed, the growth of tissue plates on the disc surface and the reaction of the adjacent tissues were evaluated. No tissue plates were detected on titanium discs without CP coating, and hence, this group was excluded from the study.

Histological analysis was carried out by standard light microscopy of fine sections. After fixation in 10% formalin and decalcination of tissue plates grown on the implants, paraffin sections (sliced parallel to the disc surface) were routinely stained with hematoxylin and eosin.

The results were statistically processed using Student's *t* test and nonparametric Wilcoxon—Mann—Whitney *U* test (*Pu*).

RESULTS

Scanning electron microscopy showed qualitative differences in the surface morphology of bioactive CP surfaces. Compact calcium phosphate (CCP) coating had gray surface, rough due to crater-like spherulites, with irregularly shaped pores of different size (mean diameter 5.5±1.0 μ; n=40). The craters were chaotically scattered, separated by cracks at the base, often fusing and overlapping each other. However, comparison of CCP surface with that of loose CP (LCP) coating showed more developed surface structure of the loose coating. For example, the diameters of spherulites was 4.9 μ (n=20) for CCP and 34 μ for LCP coating (Fig. 1). The mean diameter of pores (Table 1) and parameters of surface roughness were also appreciably higher (Table 2).

On the other hand, X-ray diffraction and microrentgenospectral analysis showed close element and phase composition of the studied coatings, in which complex titanium, calcium, and phosphorus compounds predominated (Table 1) with submicrocrystal structure (mean size of grains 460 nm, n=200). Despite similar chemical composition of the coatings, LCP surfaces exhibited higher solubility *in vitro* in a model fluid. The weight and thickness of the coating were lost during 8-week degradation.

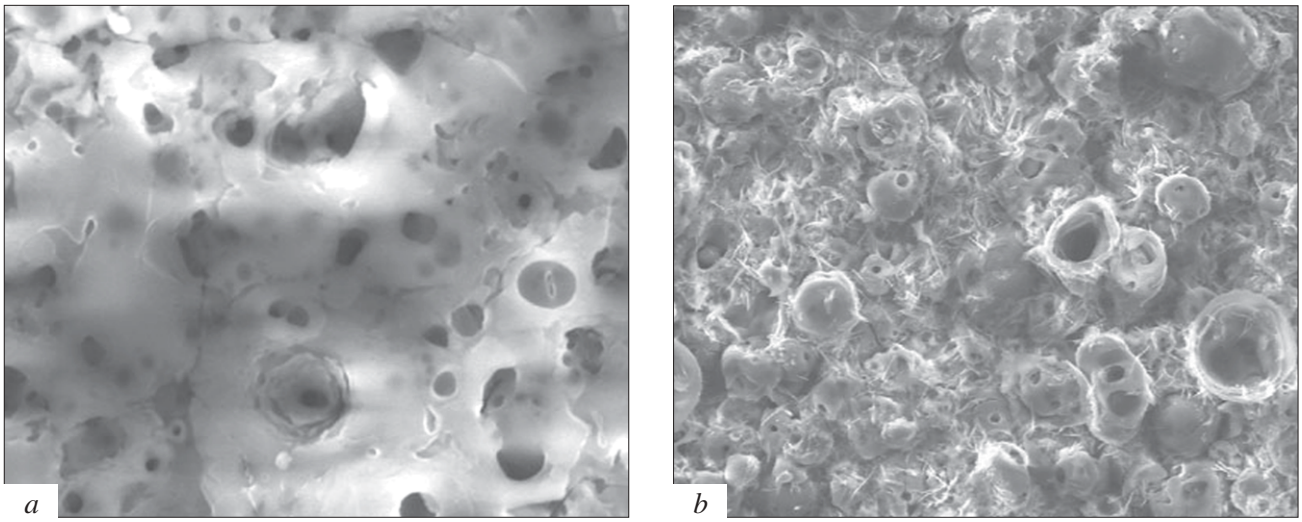

Fig. 1. Surface relief of compact (*a*) and loose calcium phosphate coating (*b*) (scanning electron microscopy; ×2000 and ×250, respectively).

TABLE 1. Surface Parameters of Bioactive Implants Formed on Titanium Discs by the Anodic Spark Oxidation

Coating	Main phase composition of coating	Main element composition of coating, atomic %	Ca/P ratio	Pore diameter, μ
CCP	Titanium hydroxyphosphate $(TiO)_2P_2O_7$, titanium phosphate $Ti_4P_6O_{23}$, calcium titanate $CaTi_4O_9$, calcium titanophosphate $CaTi_4(PO_4)_6$, calcium phosphate CaP_4O_{11}	24.60 titanium, 10.95 phosphorus, 3.45 calcium, 61.0 oxygen	0.315	5.5±1.0 (n=40)
LCP	Calcium titanophosphate $CaTi_4(PO_4)_6$, little amounts of titanium phosphate TiP_2O_7, calcium phosphate $Ca_2P_2O_7$, tricalcium phosphate $Ca_3(PO_4)_2$, titanium oxide	16.8 titanium, 10.95 phosphorus, 5.05 calcium, 67.2 oxygen	0.461	21.0±4.0* (n=40)

Note. *n*: number of measurements. *Significant differences from CCP.

CCP layers were mainly loosened, which was paralleled by decrease in their weight and a certain thickening (Table 3).

About the same loss of ionized calcium was observed after 5-week dissolution of the coatings. However degradation of LCP layers led to a drop of the solution pH (Table 3), which could be due to increase in the concentration of phosphate ions (PO_4^{2-}), specifically, at the expense of high solubility of tricalcium phosphate not detected in CCP coating (Table 1). An additional experiment showed that the mean pH of one-week extracts after 5-week dissolution of synthetic calcium phosphates with Ca/P=1.5 under similar conditions was 4.79±0.11. This value was close to pH of LCP coating solutions.

Hence, differences in bioactivity of the studied CP surfaces can be caused by their relief (structure) and degree of environmental oxidation.

No signs of inflammatory reaction and infection of tissues were detected 1.5 months after subcutaneous implantation in any of the groups. In general, high biocompatibility of implants in both groups induced slight encapsulation, indicating negligible reaction of the connective tissue to their introduction. Biocompatibility of LCP layer in cases with more pronounced dissolution and decrease of environmental pH *in vitro* was at least not lower than that of CCP coating.

Realization of the phenomenon of the implant surface layer bioactivity for orthopedics and traumatology depends on the presence of sufficient amounts of P and Ca ions, constituting the bulk of inorganic bone matrix, in the coating. However, the presence of CP is insufficient for effective ectopic bone formation on the implant surface. Despite similar chemical composition of CP coatings (Table 1), the probability of tissue plate formation from the bone marrow bar was different. The efficiency of CCP layer was 25% (n=4) compared to 80% (n=5, Pu<0.05) tissue formation after implantation of discs with LCP coating. This indicated better adhesion of the bone marrow to LCP surfaces with more developed relief.

Histological study of tissue plates grown from bone marrow bar on bioactive implants in the subcutaneous bone formation test also indicated the relationship between histogenesis and the structure of CP coating. It seems that certain structure of biomaterials can initiate the route of mesenchymal and stem hemopoietic cells development. However, we failed to find reports on the problem. One of mesenchymal stem cell types are cells capable of forming colonies consisting of stromal, osteogenic, chondrogenic, and adipose elements in culture [5].

Calcified, encapsulated sites of the red bone marrow, including blast cells of different hemopoietic stems and numerous megakaryocytes, as well as capillaries filled with erythrocytes were detected on the CCP layer in our experiments. In some samples sites of loose amorphous connective tissue were alternating with longitudinally oriented fibrous cords (Fig. 2). More compact fibrillar elements (collagen fibers) forming the capsule were seen at the periphery. Commonly looking fibrocytes and fibroblasts were detected between the fibers.

The tissue growing on a CCP coating was characterized (on sections) by rarefied sites of the red bone marrow, presented by stromal and parenchymatous cells. Sites of osteoid tissue of different shape and size were detected, forming individual islets (Fig. 3) and cells with bone marrow inside. The main substance of the osteoid tissue was homo-

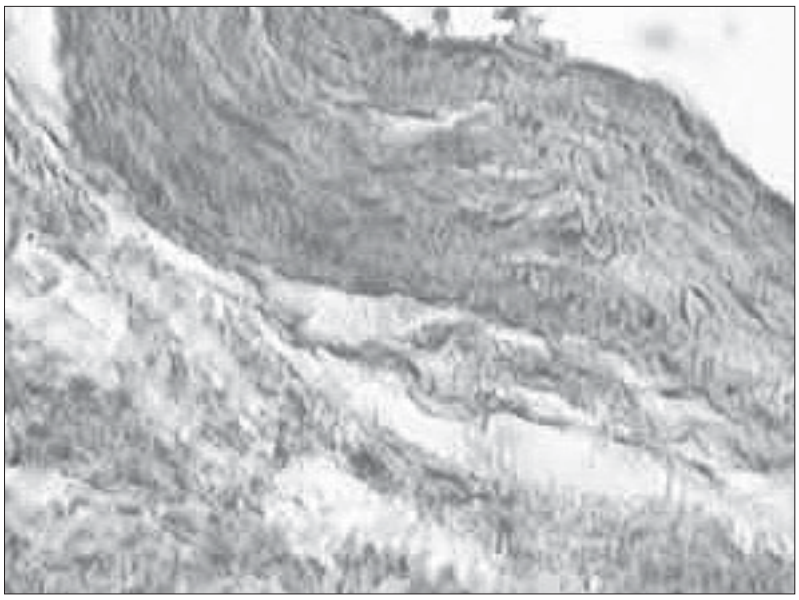

Fig. 2. Areas of loose amorphous and fibrous connective tissues in tissue plates grown on compact calcium phosphate coating. Here and in Fig. 3: hematoxylin and eosin staining, ×400.

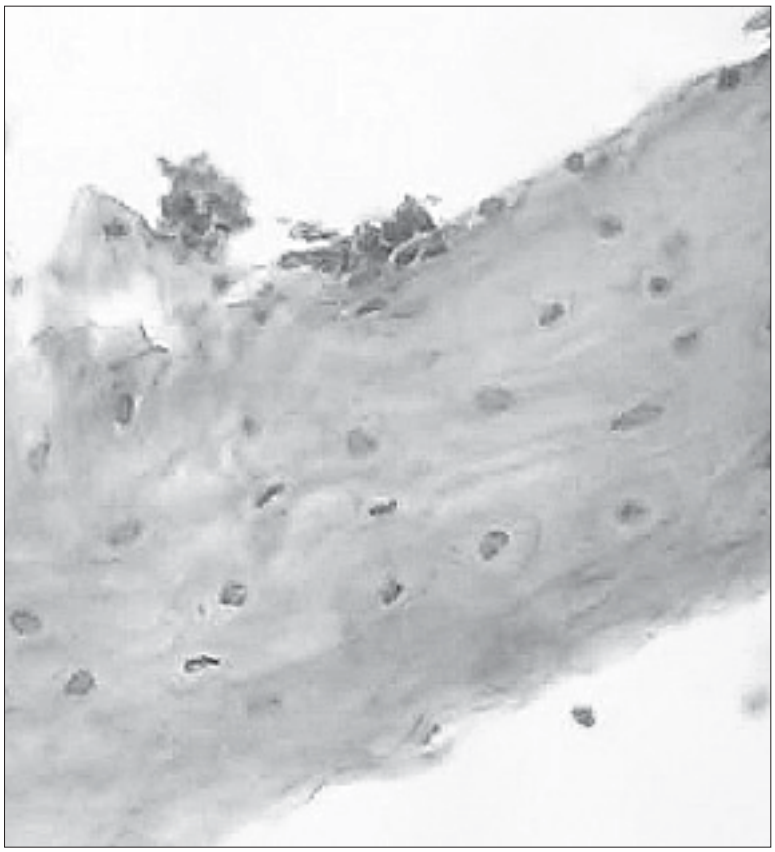

Fig. 3. Areas of osteoid tissue in tissue plates grown on loose calcium phosphate surfaces.

TABLE 2. Relief Parameters of CP Surfaces*

Type of titanium disc coating	Ra, μ	Rz, μ	Roughness class and order
CCP	1.431 (*n*=6)	8.581 (*n*=6)	6b
LCP	6.476^{x} (*n*=6)	33.110^{x} (*n*=6)	4

Note. *State Standard 2789-73. Ra: mean result for several lengths of measured sites; Rz: difference in the means between five highest protrusions and five deepest depressions within appraised length of measured site. *n*: number of measurements. $^{x}Pu<0.001$ compared to CCP.

TABLE 3. Changes in the Parameters of Titanium CP Coating and Their Solutions after Degradation in Isotonic NaCl Solution ($X\pm m$; *n*=5)

Titanium coating	Mean pH of sample solutions	Mean concentration of Ca ions in sample solutions, mg/liter	Alteration of sample weight after 8-week degradation, mg	Thickness of coating before degradation, μ	Alteration of coating thickness after 8-week degradation, μ
CCP	6.12±0.10 (8)	47.03±7.07 (5)	-0.49±0.13	18.00±3.07	+1.60±2.11
LCP	4.10±0.07* (8)	43.00±13.10 (5)	-4.02±0.10*	136.80±8.06*	-11.20±2.30*

Note. *Significant difference ($Pt<0.001$) compared to CCP coating according to Student's *t* test. *n*: number of samples examined. Week of the study is shown in parentheses. "+": increment in coating parameter; "—": decrease of parameter.

geneously stained, mainly oxyphilic, with few basophilic fragments. The main substance of the tissue was clearly "lamellar", determined by properly oriented strictly ordered collagen fibers and cells situated along them. Bone cells were situated regularly, had axons or were round, with oval or round nuclei. Signs of neovasculogenesis and already formed few blood vessels were seen in the osteoid tissue.

Hence, mainly proliferating bone marrow and loose amorphous connective tissue with compact (fibrous) sires were detected on CCP coatings. LCP coating allowed the bone marrow form lamellar bone tissue. The osteogenic potential of myelokaryocytes, presumably parental cells, is realized in the presence of a certain macro- (porosity, thickness of CP layer) and microrelief (roughness), corresponding to the characteristics of LCP coating. This is in line with the data indicating that osteoinductive activity emerges in CP ceramic with irregular size of pores and rough surface [11].

The bone formed on LCP coating under conditions of acid pH of products of its long dissolution in *vitro* (Table 3). Acid phosphatase, participating in bone tissue resorption, is activated at pH 3.5-5.5 [3], but this did not prevent ectopic osteogenesis *in vivo*.

The efficiency of ectopic bone formation test as an osteoinduction model depends on many factors. The key (integral) characteristics of the surface, determining the optimal biocompatibility and functional activity of the implant, are still to be found [1]. Our findings indicate that physicochemical characteristics of the implant surface regulate the differentiation potential of stromal cells. It seems that the osteogenic potential of CP surfaces is largely determined by their relief, but not by the degradation product pH.

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