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MULTILEVEL CALCIUM PHOSPHATE COATING: A MODEL OF BONE-LIKE TOPOGRAPHY FOR OSTEOIMMUNOLOGY

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Close interactions between blood immune cells and resident cells, mainly human adipose-derived multipotent mesenchymal stromal cells (hAMMSCs), form the basis of successful cell migration, vascularization, osteogenesis, wound and fracture healing, culminating in the emerging term "osteoimmunology" (1). There is a current trend towards modeling cell behavior under 3D culture conditions. Biocompatible micro-arc CaP coatings with a roughness index R_a=2-4 μm promote MMSC osteogenic differentiation both *in vitro* (2) and *in vivo* (3). In this regard, *in vitro* interaction of hAMMSCs and blood mononuclear leukocytes (hBMLCs) modulated by micro-arc CaP coating was investigated with the help of Cell-IQ and RTCA advanced tools for continuous monitoring.

The method used was described closely by (2). Commercially available pure titanium VT1-0 plates (99.58 Ti, 0.12 O, 0.18 Fe, 0.07 C, 0.04 N, and 0.01 H, wt.%; $10 \times 10 \times 1$ mm^3) were used as substrates to deposit the bilateral CaP coatings by the micro-arc oxidation (MAO) method using the Micro-Arc 3.0 system (ISPMS SB RAS, Tomsk, Russia). DC pulsed power was supplied in the anodic regime. The electrolyte suspension contained 20 wt.% aqueous solution of phosphoric acid, 6 wt.% dissolved hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) powder, and 9 wt.% dissolved calcium carbonate ($CaCO_3$). Before biological testing, the samples were dry-heat sterilized.

One of each substrates with CaP coating was placed in a well vertically at one edge of sterile 12-well plastic culture flat-bottom plates and attached to the wall of the well with a clip. In this position, the samples were not shifted when the plates were moved on the sample stage of the instrument and did not damage the forming cell layer. An average velocity of cell division (AVCD) of hAMMSCs in *in vitro* contact with allogenic hBMLCs was studied by means of Cell-IQ v2 MLF integrated phase-contrast microscopic platform for real-time surveillance imaging of living cells. Both 50 μL suspensions (5×10^4 viable cells) of the $CD73^+CD90^+CD105^+$ hAMMSCs and hBMLCs were applied into the center of the well of 12-well plastic plates for 7 days at 100% humidity in a 5% CO_2 atmosphere at 37°C until the formation of a monolayer. A nutrient medium was once replaced. To determine cell invasion (chemotactic motility) through 8 μm pores the real-time cell analyzer (RTCA; xCELLigence RTCA DP system) with the 16-well CIM-plate was used as described previously (4). Statistical processing of results was carried out using the STATISTICA 13.3 software package for Windows. The mean (X) and standard deviation (SD) or median (Me) and 25% (Q1) and 75% (Q3) quartiles were calculated. Because of non-normal distribution, non-parametric tests were used to evaluate the significant differences between the samples.

SEM showed the microreliefs of the CaP surface with R_a = 2-4 μm with similar irregularities. The peaks of the CaP topography consisted of spherulites of up to 10–20 μm in diameter. The optical microscopy demonstrated interconnected valleys as vast dark fields between ranges of bright spherulites. Single or open interconnected pores (1–10 μm in diameter) were observed by SEM in both spherulites and valleys independent of the roughness index.

An average velocity of cell division (AVCD) of fibroblast-like adherent hAMMSCs was 0.4 divisions per 1 hour. No micro-arc CaP coating or hBMLCs influenced this process statistically (Table 1) but a 10-fold increase in the total area of calcification around the CaP-coated samples was detected in the 3D mixed culture.

Table 1. Behavior of hAMMSCs contacted in vitro with hBMLCs in 2D- or 3D-cultures for 7 days, Me (Q1-Q3), X±SD, n=3.

Parameters of bilateral CaP coating			Average velocity of cell division (AVCD), number of divisions per hour before monolayer formation
Ra, μm	Thickness, μm	Mass, mg	
1) hAMMSC culture on plastic surface			
-	-	-	0.40 ± 0.29
2) hAMMSC culture in contact with the CaP-coated titanium substrates			
3.5 (2.4-4.3)	52.0 (30.5-56.5)	14.0 (9.0-17.1)	0.46 ± 0.30
3) hAMMSC + hBMNC coculture on plastic surface			
-	-	-	0.44 ± 0.31
4) hAMMSC + hBMNC coculture on plastic surface in contact with the CaP-coated titanium substrates (3D mixed culture)			
3.3 (2.3-4.3)	51.0 (31.5-52.5)	13.7 (9.3-14.7)	0.39 ± 0.29

Note: n, number of wells in each plate for each group; six visualization fields were located in each well.

RTCA system showed an invasion of hAMMSCs towards hBMLCs and not vice versa. Therefore, chemokines secreted by hBMLCs can promote hAMMSC recruitment into sites of inflammation and/or regeneration.

Thus, multilevel micro-arc CaP coating forms bone-like inorganic structure to modulate interaction of hAMMSCs and hBMLCs. Cell-IQ and RTCA are useful tools for *in vitro* real-time simulation and imaging of morphofunctional interplay between inflammatory and regeneratory cells. Our data emphasize the significance of the hBMLCs for CaP implantation, which is not always successful at inducing vascularization and bone formation without osteogenic cells or bone growth factors (5, 6).

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