Biochimica et Biophysica Acta 1860 (2016) 825–835

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Osteoclastogenic differentiation of human precursor cells over micro- and nanostructured hydroxyapatite topography

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ARTICLE INFO

Article history:
Received 31 August 2015
Received in revised form 27 December 2015
Accepted 15 January 2016
Available online 19 January 2016

Keywords:
Osteoclastogenesis
Hydroxyapatite
Microtopography
Nanotopography

ABSTRACT

Background: Surface topography is a key parameter in bone cells–biomaterials interactions. This study analyzed the behavior of human osteoclast precursor cells cultured over three hydroxyapatite (HA) surfaces ranging from a micro- to nanoscale topography.
Methods: HA surfaces were prepared with microsized HA particles, at 1300 °C (HA1), and with nanosized HA particles at 1000 °C (HA2) and 830 °C (HA3). Human osteoclast precursors were cultured in the absence or presence of M-SCF and RANKL.
Results: HA surfaces had similar chemical composition, however, HA1 and HA3 presented typical micro- and nanostructured topographies, respectively, and HA2 profile was between those of HA1 and HA3. The decrease on the average grain diameter to the nanoscale range (HA3) was accompanied by an increase in surface area, porosity and hydrophilicity and a decrease in roughness. Compared to HA1 surface, HA3 allowed a lower osteoclastic adhesion, differentiation and function. Differences in the cell response appeared to be associated with the modulation of relevant intracellular signaling pathways.
Conclusions: The decrease in HA grain size to a biomimetic nanoscale range, appears less attractive to osteoclastic differentiation and function, compared to the HA microsized topography.
General significance: This observation emphasizes the role of surface topography in designing advanced biomaterials for tailored bone cells response in regenerative strategies.

1. Introduction

Tissue engineering aims at the reconstitution of lost or damaged organs and tissues, circumventing the problems associated with traditional transplants [1]. Nowadays, this strategy is widely used in many clinical applications such as in the orthopedic context. Indeed, the clinical demand for synthetic bone substituting materials is high [2]. The success of both orthopedic implants and tissue engineered constructs is dependent on the properties of the selected biomaterial. Since the interactions of the biomolecules and cells with the biomaterial surface arecrucial elements in the evaluation of its applicability, biomaterial research is continuously searching for pertinent host–cell interactions in order to design materials that allow and potentiate favorable interactions and enhance tissue regeneration [3]. In native tissues, nanoscale protein interactions are crucial to control cell functions such as proliferation, migration, and extracellular matrix production [4]. Therefore the development of bioceramics for applications in bone tissue engineering may include chemical and physical modifications, such as the use of nanostructured and nano-crystalline materials [5], in order to modulate those interactions. This is a very important issue, since osteoblastic and osteoclastic cells at the bone-implant surface are known to be affected by the surface properties of the biomaterial [6–9].

Bone tissue has an extracellular mineralized matrix, composed essentially by calcium phosphate salts in the form of hydroxyapatite (HA) [10,11]. HA is a suitable biomaterial for bone regeneration applications because it displays bioactive and osteoconductive properties [12] mainly due to its chemical and structural similarity with natural mineral bone matrix [13]. With the advent of nanoscale technology, nano-structured HA (nanoHA) is being increasingly studied and applied, revealing a high potential for bone regeneration applications [14,15]. Nanoscaled materials show improved performances due to their large surface to volume ratio and especially to their surface reactivity (unusual chemical/electronic synergistic effects) [12,13]. In particular, the characteristics of nanophased as compared to microphased hydroxyapatite, such as

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http://dx.doi.org/10.1016/j.bbagen.2016.01.014
0304-4165/

surface grain size, pore size and wettability may modulate protein properties (like adsorption, conformation and bioactivity) and thus affect cellular responses [16,17].

During the bone regenerative events, the biomaterial is a dynamic player in the bone microenvironment establishing a close interaction with the osteoclasts and osteoblasts, during the ongoing bone formation and remodeling, [10,18]. According to several reports, nanoHA substrates appear to stimulate the behavior of osteoblastic cells [19–24]. However, and although osteoclasts are essential for skeletal remodeling, regeneration and repair [26–28], the behavior of osteoclastic cells on bioactive materials with different surface topographies, especially nanoHA substrates, is scarcely documented. Previously, it was observed that microsized HA substrates, with different surface roughness, have a significant effect on human osteoclastogenesis, which were dependent on the osteoclastogenic subjacent stimuli, that means, the presence of soluble factors or direct cell-to-cell contacts between osteoblastic and osteoclastic cells [9]. Detsch R et al. observed that nanostructured calcium phosphate substrates have the ability to modulate cell viability, and resorbing ability of RAW 264.7 rat cell cultures [2]. Recently, Hoffmann and his co-workers have shown that disks of β-tricalcium phosphate (β-TCP) (another calcium phosphate bioceramic) with different degrees of microporosity elicited a differential behavior of rabbit osteoclasts [29].

In this context, the aim of the present study was to analyze the osteoclast differentiation over HA substrates displaying similar chemical composition, but presenting surface features ranging from a micro- to a nanoscale topography. For that, osteoclast precursors, from human peripheral blood mononuclear cells (PBMC) were cultured over the HA substrates in the absence and in the presence of osteoclastogenic promotors, in order to evaluate the influence of the surface topography in undifferentiated precursors and, also, on precursors actively engaged in the differentiation process. Osteoclastic response was assessed for several differentiation markers and, in addition, for the resorption activity, as the ultimate osteoclastic functional parameter. Furthermore, the involvement of several signaling pathways on the cellular response was also investigated.

2. Materials and methods

2.1. Preparation of HA disks

Microsized hydroxyapatite particles (Plasma Biotal Limited) and nanosized hydroxyapatite particles nanoXIM.Hap202®, Fluidinova S.A., Portugal) were used to prepare the material samples. Cylindrical disks of HA were obtained using 75 mg of dry power under uniaxial compression stress of 20 bar (Mestra snow P3). For the disks prepared with microsized hydroxyapatite, the sintering temperature used was 1300 °C (HA1), with a 1 h plateau and applying a heating rate of 4 °C/min. For the disks prepared with nanosized hydroxyapatite, two different sintering temperatures were used, namely 1000 °C (HA2), with a 1 h plateau and applying a heating rate of 4 °C/min, and 830 °C (HA3), with a 15 min plateau and applying a heating rate 20 °C/min. Samples were cooled inside the furnace, until reaching room temperature. The mass of the HA disks was assessed with an analytical balance (Melter AG285). The diameter and thickness of the disks were measured with a digital caliper (Duratool).

2.2. Physicochemical characterization of HA disks

2.2.1. X-ray diffraction (XRD) and Fourier Transformed Infrared Spectroscopy (FTIR)

For XRD studies, the three types of HA disks were ground and the resulting fine powders were analyzed by an X-ray diffractometer Rigaku Dmax-III-VC, using a Cu-Kα radiation (Kα = 1.54056 Å). Data were acquired for 2Θ values between 4 and 80° at intervals of 0.02°/s. The chemical characterization of the disks was determined using the Fourier Transformed Infrared Spectroscopy (FT-IR) technique with a FT-IR spectrometer Perkin-Elmer 2000. To this end, HA disks were analyzed as KBr pellets with a spectral resolution of 2 cm^{-1} in transmission mode. One hundred scans were performed per sample.

2.2.2. Contact angle

Surface wettability was assessed by means of contact angles measurements, using a System Data physics Instruments, model OCA 15, equipped with a charge-coupled video camera (CCD), an electronic unit with a syringe (Hamilton) and SCA 20 software. Ultrapure water with resistivity equal to 18.2 MΩ·cm was used. The contact angles were obtained using the sessile drop method at 25 °C in a chamber saturated with liquid from the sample. Digital images were collected every 40 ms for 30 s with a CCD camera. Due to the nature of absorption of HA disks, the contact angle was calculated at the time that the drop of water (4 μL of ultrapure water) contacted the surface through the application of the tangent function. The results presented are a mean ± standard deviation (SD) of at least 10 measurements (one per sample).

2.2.3. Scanning electron microscopy (SEM)

Analyses were performed using a SEM microscope FEI Quanta 400FEG/EDAX Genesis X4M under high vacuum conditions. HA disks (HA1, HA2 and HA3) were coated before analysis with a thin gold film using a sputter coater (SPI-Module) in an atmosphere of argon. The diameters of some randomly selected grains were measured from the SEM images of the different HA substrates.

2.2.4. Atomic force microscopy (AFM)

The studies using AFM were performed with a scanning probe microscope with a controller Picoscan 2500 (both from Molecular Imaging). Each sample was scanned with a piezoelectric scanner over an area of 150 × 150 μm^2. Image and roughness analyses were performed at room temperature under Tapping mode® using a cantilever with a spring constant of 25–75 N/m (tip radius <10 nm). Three samples of each type were analyzed at randomly chosen locations. To characterize the roughness, the root mean square roughness (Sq) was obtained from scanned areas of 20 × 20 μm. This area was chosen taking into account that the material is small enough to accurately assess the nanoroughness, but is also large enough to cover several crystals in each measurement. The results for the roughness of each surface and diameter measurements are represented as the mean values ± SD.

2.2.5. Mercury porosimetry

Apparent density, total surface area, and pores volume percentage on HA substrates were assessed by mercury porosimetry method (Quantachrome Poremaster model No. 60). Detection of open porosity was conducted in the range of 0.0035–10.6379 μm. Helium picnometry was also used to determine the real density. Around 0.7 g of each sample was used for the measurements and data were obtained using Quantachrome Poremaster for Windows, version 3.0.

2.3. Osteoclastic cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors with 25–35 years old, after informed consent, as described previously [9]. The blood was diluted at a ratio of 1:2 with PBS and was transferred to tubes containing Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400 g for 30 min, PBMC were collected and transferred to a tube where they were washed twice with PBS, by centrifugation at 400 g for 10 min. Cells were counted in a cytometer (Celltac MEK-5103). Typically, for each 100 ml of processed blood about 70 × 10^6 mononuclear cells were obtained.

PBMC were seeded over HA disks placed in 48-well plates at a density of 1.5 × 10^6 cells/cm^2. Cell cultures were performed in α-Minimal Essential Medium (α-MEM) supplemented with 30% (v/v) human