Acta Biomaterialia 8 (2012) 1137–1145

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Hydroxyapatite surface roughness: Complex modulation of the osteoclastogenesis of human precursor cells

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ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form 27 November 2011
Accepted 29 November 2011
Available online 6 December 2011

Keywords:
Hydroxyapatite surface roughness
Osteoclast differentiation
Peripheral blood mononuclear cells
Complex modulation

ABSTRACT

It is recognized that the surface roughness affects osteoblastic differentiation, but little information is available regarding its effect on osteoclastogenesis. With this work, the osteoclastogenic behaviour of human peripheral blood mononuclear cells (PBMCs), cultured isolated (1.5×10^6 cells cm^{-2}) or co-cultured with human bone marrow cells (hBMCs; 10^3 cells cm^{-2}), was assessed on surface-abraded hydroxyapatite disks with three different surface roughnesses (R_a 0.0437–0.582 μm). Monocultures and co-cultures were performed for 21 days in the absence or presence of recombinant M-CSF and RANKL. Results showed that PBMCs supplemented with M-CSF and RANKL or co-cultured with hBMCs displayed typical osteoclastic features, i.e. multinucleated cells with actin rings, vitronectin and calcitonin receptors, gene expression of TRAP, cathepsin K, carbonic anhydrase 2, c-myc and c-src, TRAP activity and resorbing activity. The osteoclastogenic response increased with surface roughness in PBMCs cultured with M-CSF and RANKL but decreased in PBMCs co-cultured with hBMCs. However, co-cultures supplemented with the osteoclastogenic inducers displayed high and similar levels of osteoclast differentiation in the three tested surfaces. In conclusion, modulation of osteoclast differentiation by surface roughness seemed to be dependent on the mechanisms subjacent to the osteoclastogenic stimulus, i.e. the presence of soluble factors or direct cell-to-cell contacts between osteoblastic and osteoclastic cells.

1. Introduction

Bone tissue has the ability to regenerate itself when subjected to partial damage [1–3]. Nevertheless, in the presence of a large loss of bone mass, caused by trauma or disease, the self-regenerative ability might be insufficient to promote a proper bone healing [1,2]. In such cases, bone regeneration can be achieved with a bioactive biomaterial, which modulates cellular activities in order to stimulate the self-regeneration ability of the tissue, allowing a progressive replacement of the biomaterial by new bone mass. Hydroxyapatite (HA), an inorganic calcium phosphate material, is one of the most widely used bone regenerative biomaterials, as its composition mimics the inorganic extracellular matrix of bone tissue [4,5].

The events taking place at the bone–material interface are determinant for the success of a bone regeneration process. A high, but coordinated, rate of bone remodelling is usually required, in order to replace the old bone and the biomaterial, repair fatigue damaged foci and maintain the structural integrity at the interface [6,7]. Therefore, as happens on a healthy bone, a successful biomaterial therapy requires a correct equilibrium between osteoblastic and osteoclastic activities [2]. These cells are not only responsible for bone metabolic activities, but are also key players on the regulation of the differentiation and activation of each other, through the production of either membrane-bound or soluble molecules [8]. The role of osteoblasts on osteoclastogenesis is well known [8] but the inverse relationship is less elucidated. In a recent study that detailed some reciprocal interactions occurring on a co-culture of osteoblastic and osteoclastic cells, we observed that osteoclastic cells have an important function on the regulation of osteogenesis [9]. In this context, both the osteoblastic and osteoclast activities and the reciprocal interactions between the two cell types are relevant at the bone–material interface.

The behaviour of osteoblastic and osteoclastic cells at the bone-implant surface is affected by the physical and chemical properties of the biomaterial surface [2,10,11]. The proper surface topography depends on the proposed application of the biomaterial [2]. The influence of the biomaterial surface on the osteoblastic behaviour has been reported in a variety of studies, and the increase in the surface roughness appears to favour osteoblast activity and material integration [11–16]. However, as referred above, osteoclast activity is also a key event at the bone–material interface [6,17]. Several studies were conducted with pure or chemically modified

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doi:10.1016/j.actbio.2011.11.032

HA, and, globally, the majority revealed a good osteoclastic biocompatibility of the material [18–21], although with some contradictory results regarding the influence of chemical modifications. However, the effects of surface roughness of biomaterials on osteoclast development and functional activity are poorly elucidated. The few published reports in this issue were performed mainly on titanium implants (an inert material) or bone slices [7,10,17], being only one study conducted with nanoceramic materials [22]. The aim of this work was to compare the differentiation of human osteoclast precursors from peripheral blood mononuclear cells (PBMCs) cultured over hydroxyapatite disks with three surface roughnesses. Osteoclast precursors were cultured isolated in the presence of the osteoclastogenic inducers M-CSF and RANKL [23,24] and co-cultured with osteoblastic cells, due to the role of osteoblasts in osteoclast differentiation [3,8,25,26]. Colonized HA samples were assessed for osteoclast-associated markers, including the resorption activity.

2. Materials and methods

2.1. Preparation of hydroxyapatite disks with different surface roughness

Commercial hydroxyapatite (HA) (Plasma Biotal Ltd) was used to prepare disks 16 mm in diameter (~1 g). The disks were obtained using metal moulds and uniaxial pressure (90 MPa), followed by sinterization at 1300 °C for 1 h in a sintering furnace (Termolab/Eurotherm 2408). After, the disks were polished with a Dual Disc Polishing Machine (Stuers Rotopol-1). In order to obtain different roughness surfaces, three grinding processes were performed using the following SiC paper grit sequence: roughness 1: # 320; # 500 # 1000 # 2500 # 4000; roughness 2: #320, #500 and #1000; roughness 3: #320.

2.2. Surface topography analysis

A profilometry (Hommelwerke T8000) was used for surface roughness and three-dimensional (3-D) topography measurements. The measurement conditions used were randomly orientated (no less than 12), and the acquisition conditions were according to the recommendations of the standards DIN EN ISO 4288 and DIN EN ISO 3274 (cut-off wavelength, l_c = 0.8 mm; measuring length: 4.8 mm). The measured parameters were: arithmetic mean deviation of the surface (R_a), root-mean-square deviation of the surface (R_q), maximum height of summits (R_p), maximum depth of valleys (R_v), and total height of the surface (R_t).

2.3. Cell cultures

2.3.1. PBMCs

PBMCs were isolated from blood of healthy male donors (25–35 years old), as described previously [27]. Briefly, blood was diluted with phosphate buffered saline (PBS) (1:1) and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). Samples were centrifuged at 400*g* for 30 min. After centrifugation, PBMCs were collected at the interface between Ficoll-Paque and PBS and washed twice with PBS. On average, for each 100 ml of processed blood, $\sim 70 \times 10^6$ PBMCs were obtained. PBMCs were cultured at 1.5×10^6 cells cm^{-2} over HA disks placed in 24-well plates, for 21 days, in α-MEM supplemented with 20% human serum (from the same donor from which PBMCs were collected), 100 IU ml^{-1} penicillin, 2.5 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B and 2 mM L-glutamine. Cells were maintained in the absence (base medium) or presence of 25 ng ml^{-1} recombinant M-CSF (R&D Systems) and 40 ng ml^{-1} RANKL (Insight Biotechnology) [26]. The seeding concentration used was chosen based on previous experiments [28], in order to maximize the osteoclastogenic response. All the cells were seeded at the same time, and culture medium (1 ml) was changed in the following day. In the first medium change, cells were washed with PBS in order to remove non-adherent cells. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37 °C. Culture medium was replaced once a week, as determined by preliminary experiments [28]. Colonized HA disks were characterized at day 21.

2.3.2. Co-culture of human bone marrow cells with PBMCs

Bone marrow was obtained from patients (25–35 years old) undergoing orthopedic surgery procedures, after informed consent. Human bone marrow cells (hBMCs) were cultured in α-MEM supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin, 2.5 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B and 50 μg ml^{-1} ascorbic acid until reaching ~70–80% confluence. Then, cells were enzymatically detached with 0.05% trypsin and 0.5 mM EDTA. The cell suspension was seeded at 10^3 cells cm^{-2} over hydroxyapatite disks and cultured in the same experimental conditions. After 24 h, PBMCs were added at 1.5×10^6 cells cm^{-2} and co-cultures were maintained for 21 days in the experimental conditions described for PBMC cultures. The seeding densities were chosen according to the results obtained in previous experiments [28]. HA disks colonized with the co-cultures were characterized at day 21.

2.4. Characterization of the cell cultures

2.4.1. Protein quantification

Cellular protein was determined by Bradford's method [29], using bovine serum albumin as a standard. For that, after being washed twice with PBS, cell layers were solubilized with 0.1 M NaOH. Cellular extracts were incubated for 2 min at room temperature with Coomassie® Protein Assay Reagent (Fluka). The absorbance of the samples was determined at 595 nm in an ELISA plate reader (Synergy HT, Biotek).

2.4.2. Tartarate-resistant acid phosphatase (TRAP) activity

TRAP activity was quantified by the *para*-nitrophenylphosphate (*p*NPP) hydrolysis assay as described before [9]. Cells were washed twice with PBS and solubilized with 0.1% (v/v) Triton X-100. Then, the samples were incubated for 1 h at 37 °C with 12.5 mM *p*NPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8). The reaction was stopped with 5 M NaOH, and the absorbance was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). Results were normalized with total protein content and expressed as nmol (min $mg_{protein}$)$^{-1}$.

2.4.3. RT-PCR analysis

RNA from PBMC cultures and co-cultures of PBMCs and hBMCs was extracted with an Rneasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. Following quantification by UV spectrophotometry at 260 nm, RNA was analysed by RT-PCR for the expression of the housekeeping gene glycerol-3-phosphate dehydrogenase (GAPDH), the osteoclast functional genes TRAP, cathepsin K (CATK) and carbonic anhydrase 2 (CA2), and the osteoclast-associated differentiation and activation factors c-myc and c-src, respectively [24]. When indicated, co-cultures were also assessed for the expression of the osteoclastogenic modulators osteoprotegerin (OPG) and RANKL. For that, 0.5 μg of RNA was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55 °C. The primers used are listed on Table 1. After separation on a 1% (w/v) agarose gel, RT-PCR bands were analysed by densitometry