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Osteoclast differentiation from human blood precursors on biomimetic calcium-phosphate substrates

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ABSTRACT

The design of synthetic bone grafts to foster bone formation is a challenge in regenerative medicine. Understanding the interaction of bone substitutes with osteoclasts is essential, since osteoclasts not only drive a timely resorption of the biomaterial, but also trigger osteoblast activity.

In this study, the adhesion and differentiation of human blood-derived osteoclast precursors (OCP) on two different micro-nanostructured biomimetic hydroxyapatite materials consisting in coarse (HA-C) and fine HA (HA-F) crystals, in comparison with sintered stoichiometric HA (sin-HA, reference material), were investigated. Osteoclasts were induced to differentiate by RANKL-containing supernatant using cell/substrate direct and indirect contact systems, and calcium (Ca^{++}) and phosphorus (P^{5+}) in culture medium were measured.

We observed that OCP adhered to the experimental surfaces, and that osteoclast-like cells formed at a rate influenced by the micro- and nano-structure of HA, which also modulate extracellular Ca^{++}.

Qualitative differences were found between OCP on biomimetic HA-C and HA-F and their counterparts on plastic and sin-HA. On HA-C and HA-F cells shared typical features of mature osteoclasts, i.e. podosomes, multinuclearity, tartrate acid phosphatase (TRAP)-positive staining, and TRAP5b-enzyme release. However, cells were less in number compared to those on plastic or on sin-HA, and they did not express some specific osteoclast markers. In conclusion, blood-derived OCP are able to attach to biomimetic and sintered HA substrates, but their subsequent fusion and resorptive activity are hampered by surface micro-nano-structure. Indirect cultures suggest that fusion of OCP is sensitive to topography and to extracellular calcium.

Statement of Significance

The novelty of the paper is the differentiation of human blood-derived osteoclast precursors, instead of mouse-derived macrophages as used in most studies, directly on biomimetic micro-nano structured HA-based surfaces, as triggered by osteoblast-produced factors (RANKL/OPG), and influenced by chemistry and topography of the substrate(s). Biomimetic HA-surfaces, like those obtained in calcium phosphate cements, are very different from the conventional calcium phosphate ceramics, both in terms of topography and ion exchange. The role of these factors in modulating precursors' differentiation and activity is analysed. The system is closely reproducing the physiological process of attachment of host cells and further maturation to osteoclasts toward resorption of the substrate, which occurs in vivo after filling bone defects with the calcium phosphate grafts.

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1. Introduction

During implant integration toward a successful clinical outcome, surfaces of orthopaedic materials interact with host bone cells, triggering a chain of biological events where osteoclasts and osteoblasts are involved in the replacement of damaged bone by new bone [1].

Synthetic materials for bone replacement are widely tested for their osteoconduction and/or osteoinduction, with substrates hosting osteoblasts or osteoblastic cell lines scored as promising materials. But the replacement of bone substitutes with newly formed bone implies that the material is gradually removed to leave space for new bone tissue, and recent research has shed light on the close osteoclast/osteoblast cooperation for bone formation and remodeling. Osteoclasts have been shown to secrete products to promote osteoblast precursor recruitment and differentiation and thereby promote bone formation [2]. Therefore, understanding the nature of the interactions of the different biomaterials used as synthetic bone grafts and osteoclasts is of paramount importance.

Osteoclasts, both from humans and mice, have been tested using *in vitro* protocols and shown to be able to resorb hydroxyapatite (HA) by imprinting pits on the HA surface [3,4]. Murine RAW.246.7 or mouse marrow-derived cells are largely employed in studies of osteoclasts on ceramics [5–7], with only a few publications addressing osteoclast differentiation of human monocytes on ceramic substitutes [8,9]. These studies have shown that osteoclasts are sensitive to different material properties, such as chemical composition and topography. However, the most crucial parameters of this interaction have not been fully identified.

In this work we investigated the effect of the composition and surface topography of HA substrates on osteoclast differentiation of human blood precursors. Biomimetic HA substrates can be obtained by low-temperature setting reactions, where a calcium deficient hydroxyapatite (Ca/P ratio = 1.5) is formed through the hydrolysis of α-tricalcium phosphate (α-TCP) [10], which is very similar to the mineral phase of bone, in terms of chemical composition, crystallinity and specific surface area. We have previously shown that the microstructure of these materials can be reliably controlled by tuning the particle size of the starting powders [11,12], obtaining materials with the same chemistry and different surface topographies [13].

In addition, sintered stoichiometric HA (Ca/P ratio = 1.67) with surface topography quite different from that obtained in biomimetic HA was included in the study. This type of stoichiometric HA is also commonly used as bone filler in clinics and is typically fabricated by high temperature treatments. In order to better understand the effect on the cells of the ionic exchange between the material and the cell culture medium, osteoclast precursors were also exposed to material extracts.

To mimic the bone tissue microenvironment, peripheral blood mononuclear cells (PBMC) were isolated and induced to differentiate to osteoclasts on biomimetic and sintered stoichiometric HA substrates by RANKL-containing supernatants from osteoblast cultures. This method closely mimics the bidirectional osteoclast/osteoblast interaction occurring *in vivo* at the sites of bone remodeling [14].

2. Materials and methods

2.1. Synthesis and characterisation of biomimetic HA substrates with different topographies

Biomimetic HA substrates with identical composition, but different micro/nanostructural features were obtained by low-temperature setting of a α-TCP cement, using starting powders with different particle sizes, as previously reported [11].

Briefly, α-TCP was obtained by heating in a furnace (Hobersal CNR-58), in air, an appropriate mixture of calcium hydrogen phosphate ($CaHPO_4$, Sigma–Aldrich C7263) and calcium carbonate ($CaCO_3$, Sigma–Aldrich C4830) at 1400 °C for 2 h followed by quenching in air. Two different sizes of α-TCP powder were prepared following two different milling protocols. The powder with larger size (coarse, C) was milled in an agate ball mill (Pulverisette 6, Fritsch GmbB) with 10 balls (d = 30 mm) for 15 min at 450 rpm. The powder with smaller particle size (fine, F) was first milled with 10 balls (d = 30 mm) for 60 min at 450 rpm followed by a second milling for 70 min at 500 rpm with 100 balls (d = 10 mm). Precipitated hydroxyapatite (2 wt%; Alco 1.02143) was added as a seed in the powder. The cement's liquid phase consisted of an aqueous solution of 2.5 wt% disodium hydrogen phosphate (Na_2HPO_4, Panreac 131679.1210). A liquid to powder (L/P) ratio of 0.35 ml/g was used to prepare disks of 14 mm diameter and 0.25 mm high in Teflon moulds. The cements were allowed to set in Ringer's solution (0.9 wt% NaCl) for 7 days at 37 °C to obtain the calcium deficient HA. In addition, stoichiometric HA disks were obtained by sintering in air at 1100 °C for 20 h a pre-compacted mixture of $CaHPO_4$ and $CaCO_3$ at 50 MPa, with a calcium to phosphorus ratio of 1.67. Biomimetic HA samples were named HA-C and HA-F in accordance with the powder size used to prepare them, and the stoichiometric HA, used as a reference material for cell cultures, was named sin-HA.

The microstructure on the surface of the samples was observed by scanning electron microscopy (SEM, Zeiss Neon40, 5 kV). A thin gold/palladium layer was deposited on the sample surface through vapour deposition before analysis to enhance conductivity. The surface roughness was characterized by optical interferometry (Veeco Wyko NT1100), using a 50× magnification and a scanned area of 125 × 95 mm^2. Images were processed using Vision32 software. The specific surface area (SSA) of the samples was determined by nitrogen adsorption (ASAP 2020 Micromeritics) using the Brunauer- Emmett-Teller (BET) method.

2.2. Osteoclast cultures

Peripheral blood mononuclear cells (PBMC), containing the precursors of human osteoclasts, were isolated from buffy coats of healthy voluntary blood donors to the National Blood Transfusion Service. Donation was anonymous, and institutional review board (IRB) approval was not required.

The mononuclear cells were isolated by density centrifugation using Ficoll-Histopaque gradient (Sigma–Aldrich), washed with phosphate-buffered saline (PBS), re-suspended in Dulbecco's Modified Eagle's Medium – High Glucose (DMEM, Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Euroclone) (complete DMEM), and seeded for the direct and indirect assays.

To induce the differentiation of osteoclast precursors (OCP) the RANKL-containing human osteoblast (HOB) supernatant was used [15,16]. HOB were seeded (1.5×10^4 cells/cm^2) in 75-cm^2 tissue-culture flasks in complete DMEM medium. After reaching 90% confluence, the cell supernatant was collected, centrifuged at 2000*g* at 4 °C for 10 min, and stored at −80 °C until use. In both the culture systems the OCP were re-stimulated with 25% HOB supernatant every 3 days with a medium change along the 21-day culture period.

2.2.1. Direct contact assay

The biomimetic HA (HA-C and HA-F) and sintered HA (sin-HA) disks were placed at the bottom of 24 well-polystyrene culture plates (Nalge-Nunc International, Rochester, New York, USA), and were pre-conditioned with complete DMEM, in humidified 5% CO_2 atmosphere at 37 °C for 4 days. Then the medium was removed and 6×10^6 cells per cm^2 were seeded on the preconditioned disks,