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Microscale mechanical and mineral heterogeneity of human cortical bone governs osteoclast activity☆

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ABSTRACT

Human cortical bone permanently remodels itself resulting in a haversian microstructure with heterogeneous mechanical and mineral properties. Remodeling is carried out by a subtle equilibrium between bone formation by osteoblasts and bone degradation by osteoclasts. The mechanisms regulating osteoclast activity were studied using easy access supports whose homogeneous microstructures differ from human bone microstructure. In the current study, we show that human osteoclasts resorb human cortical bone non-randomly with respect to this specific human bone microstructural heterogeneity. The characterization of this new resorption profile demonstrates that osteoclasts preferentially resorb particular osteons that have weak mechanical properties and mineral contents and that contain small hydroxyapatite crystals with a high carbonate content. Therefore, the influence of human bone microstructure heterogeneity on osteoclast activity could be a key parameter for osteoclast behaviour, for both *in vitro* and clinical studies.

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1. Introduction

Bone remodeling occurs throughout life and provides the bone turnover required to adapt both structure and architecture of bone tissue to its mechanical environment. Harmonious remodeling is vital to maintain the biological and mechanical characteristics of healthy bone [1–3]. Osteoclasts - cells of a hematopoietic origin - are one of the three main bone cell types that play a crucial role in the bone remodeling cycle [4]. Mature bone-resorbing osteoclasts adhere tightly to the bone surface at the sealing zone and dissolve both the inorganic and organic components of the bone matrix by secreting protons and enzymes [5]. This resorption phase initializes bone remodeling and thus determines the bone areas that will be substituted. Impaired osteoclast activity is involved in many bone diseases, such as osteoporosis [6] and osteogenesis imperfecta [7]. Thus, osteoclasts are prime targets in the development of new therapeutic treatments [8]. Moreover, resorption is an essential parameter in the formulation of better performing biomaterials whose bioresorbability has to be controlled [9]. Recent studies on osteoclasts have allowed for a better understanding of the mechanisms involved in the differentiation of these cells and the intracellular mechanisms that are responsible for the resorption, the regulation of the resorption process remains unclear. Particularly, the behaviour of osteoclasts and their capacity to function in a cyclic manner - alternating between resorption and migration phases - has not been fully elucidated. In this study, we postulated that the *in vitro* observations of these alternating resorption and migration phases are regulated by the microstructure of the support used. The resorption profile was observed, for the first time, on human cortical bone in which the heterogeneous microstructure differs from the supports that are routinely used to investigate osteoclasts *in vitro*.

Previous *in vitro* studies on resorption were carried out using substrates that were either synthetic or natural - such as bovine bone or dentin. However, the osteoclast behaviour (adhesion, activity) varies according to the nature of the substrates [10,11]. For synthetic substrates, the osteoclastic activity varies according to the composition, roughness, and size of the hydroxyapatite crystals [11]. Regarding natural substrates, each has its own composition and microstructure; although their composition is similar, dentin has a tubular structure [12], whereas bovine bone is plexiform. Under the same culture conditions, the surface area of dentin resorbed by osteoclasts is 11 times larger with 7 times more pits per square centimetre than bovine bone [10]. Thus, the characteristics and organization of the material microstructures predispose them to be more or less resorbed. This observation suggests that the intracellular function of osteoclasts is modulated by the support (adhesion capacity – enzyme efficiency).

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Human cortical bone has a specific heterogeneous haversian microstructure composed of osteons [13] that are formed from adjacent concentric lamellae of type 1 collagen sprinkled with hydroxyapatite crystals and are delimited at their periphery by cement lines [14,15]. The microstructural heterogeneity is observed for mechanical [16] and mineral properties between osteons and interstitial bone. Although the microstructure of human cortical bone is different from other species [17] or other substrates that are usually used to study osteoclast behaviour, the only previous study investigating the resorption of human bone is from 1986. Using techniques available at the time and a mixture of chick osteoclasts, it was reported that resorption by such osteoclasts was confined at 60% to a single mineral density phase [18]. In the present study, direct correlations between human cortical bone mechanical and mineral properties and resorption localization by human osteoclasts were analysed. Resorbable and non-resorbable areas were characterized in terms of their mechanical and mineral properties. The mechanical properties with the Young's modulus and hardness were determined on the microscale by nanoindentation. The quantity and quality of minerals on the microscale were measured by RAMAN spectroscopy.

2. Material and methods

2.1. Bone sample preparation

Human cortical bones were collected from fresh cadavers by JC Auregan at Ecole de chirurgie du fer à Moulin, Lariboisière hospital (three male donors with a mean age of 72 years old), and immediately frozen at −20 °C. The age of the three bone male donors was respectively: donor 1: 69 years old; donor 2: 77 years old; donor 3: 71 years old. Every anatomical subject was free of systemic disease and did not take any medication before its inclusion in the study. However, given that no information about the BMD was available before the completion of the study, we collected the hip, distal radius and vertebras of each subject to ensure that none of these bones displayed any sign of osteoporosis. Nine plane-parallel samples (4 mm-long, 4-mm wide and 3-mm high) were cut in the diaphysis with a diamond saw (Secotom-15, Struers A/S, Ballerup, Denmark) transversally from the osteon direction (see Fig. 1a). The upper surface perpendicular to the osteon's direction was polished using a 1-μm diamond powder. Samples were then cleaned by ultrasonication in distilled water.

2.2. Human osteoclasts

Monocytes were purified from blood of healthy adult volunteer donors (Etablissement Français du Sang, Lyon Gerland, France) as previously described [19]. Mononuclear cells were isolated by density gradient centrifugation using Ficoll (Eurobio®), then centrifuged through a 50% Percoll gradient (GE Healthcare®). The light density fraction from the pellet was recovered and incubated for 10 min at room temperature in 3% human serum-PBS. Monocytes were purified from the light density fraction by immunomagnetic depletion (Dynal, Invitrogen®) using monoclonal antibodies (Immunotech, Beckman Coulter®) directed against CD19 (J3-119), CD3 (UCHT1), CD56 (C218) and CD235a (11E4B-7-6). Then, monocytes were cultured at 37 °C in 5% CO_2 using α-minimum essential medium (α-MEM, Life technologies®) supplemented with 2 mM L-glutamine (Gibco®), 100 U/mL penicillin (Gibco®), 100 μg/mL streptomycin (Gibco®), and 10% foetal bovine serum (FBS, Pan biotech Dutscher®). Monocytes were seeded in the presence of 50 ng/mL human M-CSF (PeproTech®) and 30 ng/mL human RANKL (PeproTech®). The medium and cytokines were changed after 3 days; M-CSF at 25 ng/mL and RANKL at 100 ng/mL. Osteoclasts were then detached from the plastic plates using Accutase (Sigma-Aldrich®) as previously described [20] and were seeded onto bone samples (50,000 cells/sample) with 25 ng/mL M-CSF and 100 ng/mL RANKL for 72 h (Fig. 1b). In the present study, osteoclasts from two blood donors were seeded on nine bone samples (see Table 1).

2.3. Nanoindentation

Prior to osteoclast seeding, nanoindentation tests were performed on the nine bone samples surrounded by a physiological saline solution at ambient temperature using a commercial nanoindenter (Agilent Nanoindenter G200, ScienTec, Les Ulis, France) (Fig. 1a). Fused silica was used to calibrate the Berkovich diamond tip contact surface. A grid of 81 measurement points per sample with a spacing of 150 μm

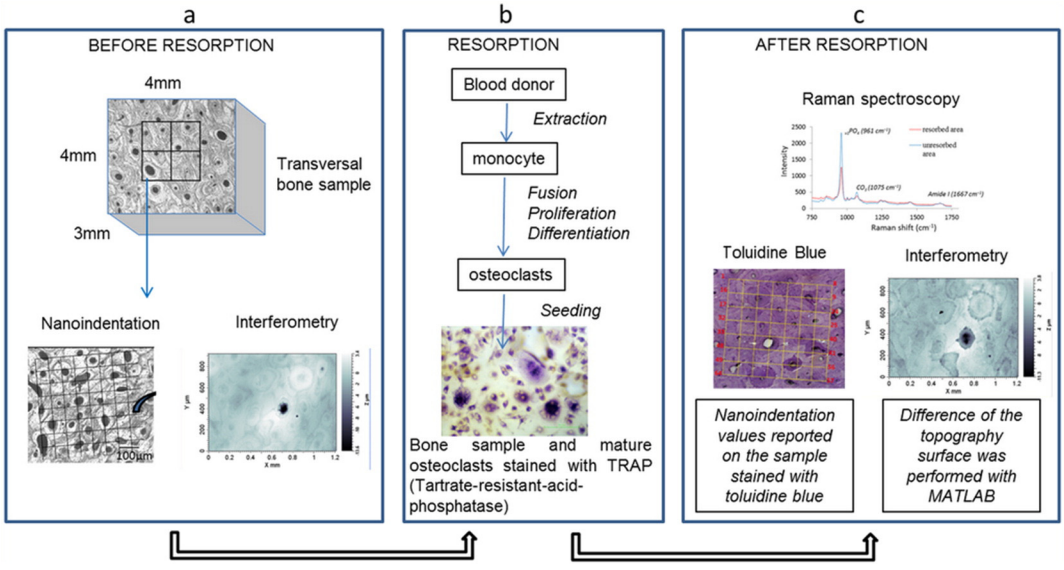

Fig. 1. Experimental design. The surface of each bone sample was characterized before seeding mature osteoclasts. First, nanoindentation and interferometer, respectively, provided local mechanical properties values and surface topography before resorption, respectively (panel a). In parallel, monocytes from human donors were extracted from peripheral blood and differentiated into osteoclasts. Then, osteoclasts were seeded on each sample in an optimal medium for osteoclast activity. After 72 h, osteoclasts were stained with TRAP, counted and removed from the bone surface (panel b). Next, the resorbed bone sample was stained by toluidine blue to determine the resorbed areas (intense violet). Analysis of the surface topography after resorption was carried out by interferometry on the same areas that were characterized before osteoclasts seeding. The variation of z (μm) at each point was measured and the mineral properties were characterized by Raman spectroscopy (panel c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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