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Energy metabolism in osteoclast formation and activity

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ABSTRACT

Osteoclastogenesis and osteolysis are energy-consuming processes supported by high metabolic activities. In human osteoclasts derived from the fusion of monocytic precursors, we found a substantial increase in the number of mitochondria with differentiation. In mature osteoclasts, mitochondria were also increased in size, rich of cristae and arranged in a complex tubular network. When compared with immature cells, fully differentiated osteoclasts showed higher levels of enzymes of the electron transport chain, a higher mitochondrial oxygen consumption rate and a lower glycolytic efficiency, as evaluated by extracellular flux analysis and by the quantification of metabolites in the culture supernatant. Thus, oxidative phosphorylation appeared the main bioenergetic source for osteoclast formation. Conversely, we found that bone resorption mainly relied on glycolysis. In fact, osteoclast fuelling with galactose, forcing cells to depend on Oxidative Phosphorylation by reducing the rate of glycolysis, significantly impaired Type I collagen degradation, whereas non-cytotoxic doses of rotenone, an inhibitor of the mitochondrial complex I, enhanced osteoclast activity. Furthermore, we found that the enzymes associated to the glycolytic pathway are localised close to the actin ring of polarised osteoclasts, where energy-demanding activities associated with bone degradation take place. In conclusion, we demonstrate that the energy required for osteoclast differentiation mainly derives from mitochondrial oxidative metabolism, whereas the peripheral cellular activities associated with bone matrix degradation are supported by glycolysis. A better understanding of human osteoclast energy metabolism holds the potential for future therapeutic interventions aimed to target osteoclast activity in different pathological conditions of bone.

1. Introduction

The skeleton is a metabolically dynamic tissue that undergoes continuous remodelling during life. Bone homeostasis is maintained by a delicate balance between deposition and resorption that is mediated by osteoblasts and osteoclasts that form and degrade bone, respectively (Teitelbaum, 2000). Pathological increases in osteoclast activity induce trabecular bone erosion which can result in various diseases, such as rheumatoid arthritis (Sweeney and Firestein, 2004), Paget's disease of bone (Erzurumlu et al., 2013), primary (Teitelbaum, 2000) or secondary laminopathies-associated osteoporosis (Zini et al., 2008), and osteolysis associated with bone cancer (Avnet et al., 2008).

Osteoclasts are giant, multinucleated, not-proliferative bone resorbing polykaryons that are formed by the differentiation and fusion of hematopoietic precursors of the monocytic and macrophagic lineage (Massey and Flanagan, 1999). Induction towards the osteoclast lineage is achieved through two essential cytokines, macrophage colony-stimulating factor (M-CSF) (Boyle et al., 2003) and receptor activator of nuclear factor-kappa B (NFkB) ligand (RANKL) (Blair et al., 2007; Novack, 2011). These and other pro-osteoclastogenic factors are locally produced by osteoblasts

Abbrevations: ATP, adenosine triphosphate; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-kappa B ligand; OxPhos, oxidative phosphorylation; TCA Cycle, tricarboxylic acid cycle; NF-kB, nuclear factor-kappa B; PGC-1β, peroxisome proliferator-activated receptor-Γ coactivator 1β; V-Atpases, vacuolar H(+)-ATPases; PBMC, peripheral blood mononuclear cells; TRAP, tartrate-resistant acid phosphatase; HSP60, heat shock protein 60 KDa; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PKM2, pyruvate kinase isozymes M2; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide 4-trifluoromethoxy phenylhydrazone; mtOCR, mitochondrial OCR; ROI, region of interest; mtDNA, mitochondrial DNA; PI, propidium iodide.

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and other cells of bone microenvironment (Cenni et al., 2006; Granchi et al., 2004; Granchi et al., 2005). Interestingly, beside proteic factors, a reduced oxygen tension is also able to strongly induce osteoclast formation and function in different osteolytic conditions associated with hypoxia, such as osteoporosis, giant cell tumor of bone, and rheumatoid arthritis (Knowles and Athanasou, 2009; Knowles et al., 2010; Morten et al., 2013; Utting et al., 2010). Oxygen availability dictates the metabolic profile of living cells that, in turn, possibly modulates osteoclast formation and activation. In bone tissue, a correlation between metabolism regulation and cell differentiation has already been described. As an example, in osteoblasts, changes in metabolic pathways have been observed during their differentiation with glycolysis and respiration continuously shifting one to the other in a delicate balance to support the different energetic demands during sequential differentiation stages (Komarova et al., 2000). In osteoclasts, similarly, high metabolic activity might be required for the energy-demanding steps of the differentiation process, including migration, fusion, actin reorganisation, acid degradation and enzymatic activities for bone resorption. A few previous reports on osteoclasts derived from rats (Baron et al., 1986), from mice (Miyazaki et al., 2012) or from a murine myeloid cell line (Czupalla et al., 2005) showed that osteoclasts are mitochondria-rich cells with high expression of tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OxPhos) enzymes. Moreover, intact and active mitochondria are required for osteoclast differentiation (Indo et al., 2013; Jin et al., 2014; Morrison et al., 1998). However, the basal metabolism of mouse and tumor cells is quite different from that of normal human cells. Indeed, metabolic enzymatic activities might significantly differ between mice and humans since murine cells have a lower metabolic stability (Demetrius, 2006). Regarding the osteoclast model that derives from murine myeloid cells, tumor cells are also notoriously more glycolytic than normal cells as a consequence of the Warburg effect (Pavlova and Thompson, 2016) and, therefore, are not the optimum model to study the metabolism of normal cells.

In this study, by using an in vitro models from peripheral blood precursors, for the first time we performed a comprehensive analysis of bioenergetics in human osteoclasts during their differentiation and function.

2. Methods

2.1. Human osteoclast cultures

Primary cultures of osteoclasts were obtained from monocytic precursors isolated from fresh buffy coats obtained from 15 different healthy volunteers (AVIS, Bologna, Italy; Saint-Luc University Hospital, Brussels, Belgium), as previously described (Avnet et al., 2011). Buffy coats obtained from AVIS in Italy were collected after their expiration date (1days after harvesting), whereas for buffy coats obtained from Saint-Luc University Hospital, Belgium, cells were isolated immediately after harvesting and after the ethical committee approval was obtained. Briefly, peripheral blood mononuclear cells (PBMC) were layered over Hystopaque (GE Healthcare) and seeded on tissue-culture glass or plasticware (3,000,000 cells/cm^2) in DMEM (Sigma) supplemented with 25 mM glucose (Merck), 10% heat-inactivated characterized FBS (Celbio), plus 20 U/mL penicillin, 100 mg/mL streptomycin (Euroclone), and incubated at 37 °C in a humidified 5% CO_2 atmosphere. All experiments were performed in pyruvate- and glutamine-free media buffered at pH 7.4 (3.7 g/l $NaHCO_3$) (complete DMEM), unless stated otherwise. After 2 h, medium was discarded and replaced with complete DMEM added with RANKL [50 ng/mL] and M-CSF [10 ng/mL] (Peprotech) (pro-osteoclastogenic medium), or with complete DMEM without pro-osteoclastogenic factors (control medium), depending on the experimental conditions. In order to verify osteoclast differentiation, after 5–7 days of cell culture, cells were analyzed for tartrate-resistant acid phosphatase (TRAP) activity by cytochemistry (Acid Phosphatase Leukocyte assay, Sigma) and stained with Hoechst 33258. Only TRAP+ cells with 3 or more nuclei were considered as osteoclasts. The quantification of osteoclast formation was obtained by counting the total number of osteoclasts in 6 random optical fields (20 x lens). The experiment was repeated with cells obtained form three different donors.

2.2. Immunoflorescence assays

Immunofluorescence assays were performed on PBMC cultured on glass coverslips with pro-osteoclastogenic medium or control medium. Cells were fixed in 2% paraformaldehyde. Permeabilization was performed with 0.5% Triton X-100 in HEPES and blocked with 1% BSA. We used anti-mitochondria MAb against the surface of intact mitochondria (clone 113-1, MAB1273, Merck Millipore, 1:50), anti- Pyruvate kinase isozymes M2 (PKM2) polyclonal antibody (Cell Signaling, 1:200); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) MAb (Santa Cruz, 1:50). As secondary antibodies we used anti-mouse or anti-rabbit antibodies Alexa Fluor 488 nm (Life Technologies). For the staining of polymeric actin (F-actin) we used phalloidin-TRITC (Sigma, 0.5 μg/mL). Nuclei were stained with Hoechst 33258. Mitochondrial mass index was quantified as the ratio of the area with a fluorescent signal to the total area of the single cell by using NIS Element image software BR4.00.00 (Nikon). Cells were counted in 28 different random fields. Mitochondria networks were observed by confocal microscope (Nikon TI-E).

2.3. Electron microscopy

CD14+ cells were isolated from the mononuclear cell population obtained from buffy coats with the method described above and according to the previously described protocol (Avnet et al., 2013). We used an immunomagnetic cell separation technique (MACS, Miltenyi Biotech). Cells were washed with MACS buffer (PBS pH 7.2 supplemented with 0.5% BSA and 2 mM ethylene diamine tetracetic acid), and clumps were removed by passing cells through a 30 μm pre-filter. Cells were then centrifuged at 400 x *g*. The cell pellet was resuspended in MACS buffer and counted. 10^7 cells were mixed with anti-CD14 MACS antibody-coated microbeads, and incubated at room temperature. The cell suspension was applied to an LS-positive selection column previously washed with MACS buffer, and placed in a magnetic separation unit. The column was rinsed and removed from the magnetic separation unit, and positive bound cells were flushed. The collected cell population was then used for ultrastructural analysis. Pellets of CD14 + cells from PBMC and glass-adherent osteoclasts were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, postfixed with 1% osmium tetroxide (Electron Microscopy Science), dehydrated in a graded series of ethanol, and embedded in Epon (Electron Microscopy Science). The embedded samples were sectioned by an ultramicrotome (Ultracut E, Richert-Jung, Leica Microsystem). Thin sections (90 nm thick) were collected on 300 mesh nickel grids and stained with uranyl acetate (Electron Microscopy Science) and lead citrate. Samples were observed by using a Zeiss EM 109 apparatus (Zeiss). Images were captured with Nikon digital camera Dmx 170 1200F and ACT-1 software.

2.4. Western blotting

PBMC were seeded on cell culture dishes, and maintained in pro-osteoclastogenic medium or control medium until osteoclast differentiation was obtained. When multinucleated TRAP+