Bone 51 (2012) 353–361

Contents lists available at SciVerse ScienceDirect

Bone

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

Original Full Length Article

A specific subtype of osteoclasts secretes factors inducing nodule formation by osteoblasts

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ARTICLE INFO

Article history:
Received 5 March 2012
Revised 7 June 2012
Accepted 9 June 2012
Available online 19 June 2012

Edited by: J. Aubin

Keywords:
Coupling
Osteoclasts
Osteoblasts
Osteopetrosis
Resorption inhibitors

ABSTRACT

Osteoclasts are known to be important for the coupling process between bone resorption and formation. The aim of this study was to address when osteoclasts are anabolically active.

Human monocytes were differentiated into mature osteoclasts by treatment with M-CSF and RANKL. Conditioned medium was collected from macrophages, pre-osteoclasts, and mature functional or non-resorbing osteopetrotic osteoclasts on either bone, plastic, decalcified bone or dentine with or without diphyllin, E64 or GM6001. Osteoclasts numbers were measured by TRACP activity. Bone resorption was evaluated by CTX-I and calcium release. The osteoblastic cell line 2 T3 was treated with 50% of CM or non-CM for 12 days. Bone formation was assessed by Alizarin Red extraction.

CM from mature osteoclasts induced bone formation, while CM from macrophages did not. Non-resorbing osteoclasts generated from osteopetrosis patients showed little resorption, but still an induction of bone formation by osteoblasts. Mimicking the reduction in bone resorption using the V-ATPase inhibitor Diphyllin, the cysteine proteinase inhibitor E64 and the MMP-inhibitor GM6001 showed that CM from diphyllin and E64 treated osteoclasts showed reduced ability to induce bone formation compared to CM from vehicle treated osteoclasts, while CM from GM6001 treated osteoclasts equaled vehicle CM. Osteoclasts on either dentine or decalcified bone showed strongly attenuated anabolic capacities.

In conclusion, we present evidence that osteoclasts, both dependent and independent of their resorptive activity, secrete factors stimulating osteoblastic bone formation.

Introduction

Bone remodeling is an essential process for maintaining bone strength. Bone remodeling is a complex process most likely initiated by the osteocytes sensing stress, which then activate the osteoclasts leading to bone resorption [1,2]. After bone resorption bone formation by the osteoblasts is initiated and the removed bone is completely replaced, a process originally identified and termed coupling by Harold Frost and co-workers [1,3–6]. Further evidence that coupling was to some extent controlled locally in bone was published by the Baylink group, who showed that bone organ cultures with activated resorption, secreted a bone anabolic molecule [7].

Alterations in the balance between bone resorption and bone formation result in pathologies, of which the most common is osteoporosis, where the osteoblasts are unable to counter the excessive bone resorption taking place [8]. Another situation, where the balance is destroyed, is the much rarer cases of osteoclast-rich forms of osteopetrosis [9]. In these cases bone resorption is strongly reduced, often due to defective acid secretion by the osteoclasts, while osteoclast numbers are increased [9]. Importantly, bone formation by the osteoblasts is often increased [8]. Furthermore, it was shown that the number of mature bone forming osteoblasts in these patients correlated to the number of non-resorbing osteoclasts [10,11]. These findings highlight that anabolic signaling from the osteoclasts is not strictly mediated by bone resorption, but most likely also involves the presence of osteoclasts [8]. Additionally, a study conducted in adult mice indicated that when the mature non-resorbing osteoclasts are present, this leads to increased bone formation resulting in stronger bones [12]. Furthermore, studies conducted in c-fos deficient mice, which have macrophages but no osteoclasts, showed reduced bone formation, as well as failure to provide an anabolic response to PTH, while c-src deficient mice, which have non-resorbing osteoclasts, showed a full anabolic response to PTH treatment

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doi:10.1016/j.bone.2012.06.007

[13–15]. Finally, these data are supported by early findings in humans with no osteoclasts, where the bone phenotype is milder than that observed in osteoclast-rich osteopetrosis, despite both phenotypes having no bone resorption [16,17].

Within recent years, several studies have highlighted that an osteoclasts are different depending on the circumstances, a phenomenon termed osteoclast subtypes [8]. These studies have indicated that resorptive activity, acid transport and proteolytic machinery and more parameters are highly influenced by the origin of the osteoclast as well as the interaction with the matrix [18–27]. However, how the differences in osteoclast subtype influence the anabolic activity of the osteoclasts is presently unclear.

Following the paradigm shift in the understanding of the coupling between osteoclasts and osteoblasts, several studies have studied local communication between osteoclasts and osteoblasts, and how different parameters, such as bone resorption and osteoclast subtype, regulate this process [8]. Several studies have indicated different molecular candidates, including TGF-β, the IGFs, Wnt10b, BMP-6, S1P, TRACP, Cardiotrophin-1 and bidirectional signaling through the ephrin-eph system, and several of these are independent of bone resorption [6,28–30].

When it comes to how much of the coupling signal that is derived from osteoclasts and how much that originates from bone resorption, this is still unclear. However, it was shown that cathepsin K inhibitors lead to increased secretion of bone matrix derived anabolic molecules, such as IGF-1, as the inhibitors prevented degradation of these factors [31], although it presently is unclear whether this has any relevance *in vivo* [32,33].

With these things in mind, this study focused on providing a thorough investigation of when the osteoclasts secrete anabolic molecules. Importantly, we utilized naturally non-resorbing osteoclasts, as well resorption inhibitors to investigate the role resorption in this process. Furthermore, we investigated whether seeding the osteoclasts on different matrices, including the non-remodeled matrix dentine and the "artificial" matrix decalcified bone, modulated the ability of the osteoclasts to secrete anabolic molecules.

Experimental procedures

Chemicals and other reagents

Diphyllin was from Bioduro, Beijing, China, GM6001 and E64 from Calbiochem. All other materials were from Sigma-Aldrich unless otherwise specified.

CD34+ Cell isolation

Samples of peripheral blood from an Infantile Malignant Osteopetrosis patient or umbilical cord blood from normal deliveries were obtained after informed consent under protocols approved by institutional ethical boards. Mononuclear cells from these cell sources were isolated on Ficoll gradient, and subsequently $CD34^+$ cells were separated from the mononuclear cell fraction using MACS columns (Miltenyi Biotec) as previously described [34]. Cells were cultured in SFEM StemSpan medium (StemCell Technologies), with the following human recombinant cytokines: M-CSF (50 ng/ml), GM-CSF (30 ng/ml), SCF (200 ng/ml), IL-6 (10 ng/ml) and Flt3L (50 ng/ml) all from R&D Systems. CD34+ cells were plated at a density of 5×10^4 cells in 1 ml medium using 24-well bacteriological plates and incubated for a week at 37 °C before collection and replating at a density of 1×10^5/well. From day 7 the medium was exchanged every 2–3 days by demi-depletion.

CD14+ cell isolation

Human CD14+ monocytes were isolated using magnetic bead sorting as previously described [35,36]. Briefly, buffycoats diluted 1:1 in PBS were layered onto ficoll gradients and centrifuged, and the lymphocyte fraction was collected at the interphase. The lymphocytes were then washed and incubated with anti-CD14 beads (DYNAL Biotech) for 20 min, and finally isolated using a magnetic device.

Osteoclastogenesis, resorption and collection of conditioned medium

The monocytes were counted and seeded at a cell density of 150,000 /cm^2 and cultured for 10 days in αMEM containing 10% of heat-inactivated fetal bovine serum (FBS) and 25 ng/mL M-CSF and 25 ng/mL RANKL (R&D Systems) with a medium change every 2nd or 3rd day to induce the formation of mature osteoclasts.

Mature human osteoclasts were lifted by trypsin digestion and subsequent scraping, and then reseeded on either bone slices or plastic at a density of 600,000 cells/well in 12-well plates. The osteoclasts were then cultured in medium containing RANKL, M-CSF and 10% heat-inactivated FBS for three weeks with a change of medium every second or third day. During each medium change the supernatants were collected and stored at −20°C until further use. Corresponding non-CM (non-CM) was collected from wells without osteoclasts, and used in experiments as negative controls. Before use in the osteoblast cultures the CM and non-CM was filtered through a 0.45 μm low-protein binding filter to remove aggregates, cell fragments etc. from the solution.

Bone slices

Bone and dentin slices with a diameter matching either 96-well or 12-well plates were prepared as previously described [29]. Decalcified bone slices were prepared by decalcification until all calcium was removed as described by [37].

Measurement of CTX-I in the conditioned medium

The release of the c-terminal type I collagen fragments (CTX-I) from mineralized bone slices was determined using the CrossLaps for Culture kit (IDS Nordic), which was used according to the manufacturer's instructions.

Measurement of calcium levels in supernatants

The concentration of total calcium was measured in culture supernatants after resorption, by using a colorimetric assay and a Hitachi 912 Automatic Analyzer (Roche Diagnostics) following the assay method validated and warranted by Roche Diagnostics [38].

Measurement of TRACP activity in conditioned medium

The TRACP activities were measured using a colorimetric assay. The conditioned media were tested for TRACP acitivity by addition of 6 mM 4-nitrophenylphosphate and 25 mM sodiumtartrate at pH5.5. The reaction products were quantified by measuring absorbance at 405 nm with 650 nm as reference using an ELISA reader.

Immunoblotting

Immunoblotting was done as previously described [39]. Briefly, cells were harvested into RIPA lysis buffer. Protein concentrations were measured using the Bio-Rad protein measurement assay (Bio-Rad). 20 μg of total protein in SDS sample buffer was loaded onto a 4–12% gradient gel, followed by blotting onto a nitrocellulose membrane. Membranes were then blocked in TBS, 0.1% tween20 with 5% skim milk powder for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4 °C in TBS, 0,1% tween20, 5% skim milk powder, with the following dilutions: mouse anti-TCIRG1 (Abnova) 1:1000, mouse anti-Cathepsin K (Chemicon) 1:1000, and p38 MAPK (Cell Signalling Technology) 1:1000, which was used as constant control. The blots were then washed 3×10 min and incubated with the corresponding