Archives of Oral Biology 71 (2016) 134–143

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Heterogeneity of osteoclast activity and bone turnover in different skeletal sites

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 21 June 2016
Accepted 28 June 2016

Keywords:
Osteoclastogenesis
Heterogeneity
Estrogen
Bone turnover
Resorption pits

ABSTRACT

Objective: To compare osteoclasts and bone turnover in the cranial and appendicular skeletons of mice and determine whether estrogen depletion has an impact on these differences.
Design: In vitro osteoclastogenesis (OCG) was performed on osteoclasts precursors derived from calvarial, mandibular and femoral bone marrow. *In vitro,* mature osteoclasts were stained with TRAP in plastic petri dishes and with DAPI and Phalloidin on glass coverslips to identify mature osteoclasts and compare osteoclast surface area and nuclei number in the different bone sites, respectively. Quantification of osteoclast resorption pit (Rpit) volume and surface area from different bone sites was achieved using dentin slices stained with Picrosirius red and confocal microscopy. *In vivo* TRAP, static and dynamic histomorphometric analyses were performed on 5-month-old mouse calvarial, long bone and mandibular trabecular bone to compare bone resorption and formation rates, respectively. Mice were ovariectomized (OVX) at 5 months of age and sacrificed at 6 months of age to establish an osteoporosis model for differences in osteoclasts activity and to monitor the changes in bone turnover rates in the three bone sites upon estrogen depletion.
Result: s Phalloidin stained calvarial osteoclasts were larger compared to long bone and mandibular osteoclasts. Rpits from osteoclasts derived from mandibular bone were smaller and had lower volume values compared to long bone and calvarial bone Rpits. *In vivo* analysis showed significant increases in bone formation rates in calvarial trabecular bone compared to long bone and mandibular trabecular bone. Turnover was enhanced upon estrogen depletion in calvarial trabecular bone. Resorption was increased without a corresponding increase in bone formation in the trabecular metaphysis of long bone. Mandibular trabecular bones do not appear to be affected by OVX.
Conclusion: The cranial and appendicular skeletons differ from one another in that osteoclasts from calvarial bone have the highest resorptive capacity which is coupled to bone formation both pre and post-OVX. Mandibular bones show the lowest turnover rates and are not affected by OVX.

1. Introduction

Osteoclasts are multinucleated bone-resorbing cells that arise from the monocyte-macrophage lineage. Bone resorption is critical in the maintenance and repair of bones in the mammalian skeleton. Osteoclasts are located on bone surfaces in Howship's lacunae also known as Rpits (Teitelbaum, 2000). The lacunae are the end-products of the dissolution of bone mineral via lysosomal enzymes including carbonic anhydrase, matrix metalloproteinases (MMPs) tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK) (Boyle, Simonet, & Lacey, 2003; Kirstein, Chambers, & Fuller, 2006; Rucci, 2008). Recent studies have raised some new questions about the possible existence of different types of osteoclasts at different bone sites. It is unclear however, how osteoclasts arise at different bone locations. It may involve differences in osteoclast precursor populations, and/or different priming by the local bone itself, or a combination of the two (Everts, de Vries, & Helfrich, 2009; Quarto et al., 2010). Since bone remodeling is a homeostatic system coupling bone resorption to bone formation, it could be the result of different local priming by cells of the osteoblast lineage. Another important question is whether disparate embryonic tissue origins impart variable osteogenic potential. For example, bones of the skull arise from the neural crest and paraxial mesoderm and undergo intramembranous ossification. Long bones, on the other hand, arise

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http://dx.doi.org/10.1016/j.archoralbio.2016.06.026
0003-9969/

from embryonic mesoderm and undergo both intramembranous and endochondral ossification (Quarto et al., 2010).

Evidence of different subsets of osteoclasts arose from differences in osteoclast activities between the axial skeleton and the head region, leading to lack of tooth eruption in osteopetrotic rodents (De Souza Faloni et al., 2011). Tooth eruption requires time-limited recruitment of osteoclast precursors, their local formation and subsequent activation. Diseases affecting osteoclast differentiation, or osteoclast function are not the only ones that can result in reduced or delayed tooth eruption, but also conditions in which osteoclast precursor recruitment is insufficient (De Souza Faloni et al., 2011). For example, in RANKL−/− mice, that lack an essential osteoclast differentiation factor, a general osteopetrotic phenotype is seen (Odgren et al., 2003). However, when Odgren et al. performed rescue experiments with CD4-driven RANKL they noticed that the teeth did not erupt in the rescued mice. Their findings indicated continued under-activity of osteoclasts in the jaw, while at the same time osteopetrosis in the long bones was resolved, indicating normalization of osteoclast function. These findings suggest that osteoclasts at different bone sites respond differently to rescue by RANKL presented by CD4-positive immune cells (Odgren et al., 2003).

Many studies focus on comparing long bone to calvarial bone or long bone to mandibular bone *in vitro*. Recent literature comparing long bone to calvarial bone suggests that calvarial osteoclasts are larger and express higher levels of TRAP compared to long bones, and use MMPs as well as CTSK for resorption. Long bones express lower TRAP levels compared to calvarial bone and primarily use CTSK for bone resorption (Perez-Amodio et al., 2006). In addition, anion exchanger 2 (AE2) proved to be essential for resorption by long bone osteoclasts but less important for calvarial bone resorption; calvariae have sodium transporter slc4a4 to compensate for the loss of AE2 (De Souza Faloni et al., 2011; Jansen et al., 2009). Resorptive capacity has not been compared in these bone sites at both the *in vitro* and *in vivo* level. The determination of osteoclast size via *in vitro* TRAP and/or Phalloidin staining is not sufficient enough to prove increased resorptive capacity. We used an Rpit analysis technique to provide the complete *in vitro* picture with respect to osteoclast activity in the bone sites. Further proof of osteoclast activity is quantified via osteoclast number using *in vivo* TRAP histology. Bone histomorphometry is the means by which bone remodeling, modeling and structure can be quantitatively assessed. It is invaluable in determining cellular pathophysiology of osteoporosis. Dynamic histomorphometry is used to define the amount of bone that has been formed within a specific period of time and is a direct measurement of bone formation. This is the first study that has attempted to compare osteoclasts and their resorptive capacity in all three bones at both the cellular and tissue level, in the absence and presence of estrogen. The experimental importance of this study is to further assess differences in osteoclasts arising from different bone locations to eventually develop pharmacotherapeutics for osteoporosis that are targeted to specific locations where they will be most beneficial.

2. Materials and methods

2.1. Animals

All procedures described were performed in accordance with the Guide for the Human Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. For the purposes of this study, 6 groups of C57BL/6 mice (type of mouse) containing 5-month-old wild-type (WT) calvarial, femoral and mandibular bone (groups 1, 2 and 3) as well as 6-month-old post-OVX calvarial, femoral and mandibular bone (groups 4, 5 and 6) were used. Each group consisted of 12 mice for a total of 72 mice to establish statistical significance and guard against losses. Mice were OVX at 5 months of age and then sacrificed at 6 months to establish an osteoporosis model for bone turnover rates at different skeletal sites. In order to investigate whether bone turnover rates differ among different skeletal sites pre and post-OVX, 5-month-old WT calvarial bone, long bone and mandibular bone were compared to each other and to their 6-month-old OVX counterparts. Animals were sacrificed by CO_2 asphyxiation, following animal care protocol established by University of Toronto Animal Care Committee.

2.2. In vitro *OCG*

Long bones, calvarial bone and mandibular bone from 5-month-old mice were dissected aseptically under a laminar flow hood. Bones were crushed using sterile tools and bone marrow was collected using a needle and syringe containing α-MEM (Life Technologies). Cell aggregates were broken by repeated aspiration using the same syringe and a 20-gauge needle. Cells were washed once and re-suspended in α-MEM supplemented with 10% fetal bovine serum (FBS) and 5X antibiotics. To remove stromal cells, bone marrow cells were cultured overnight (in a humidified incubator at 37 °C with 5% CO_2) in tissue culture flasks after which cells in the supernatant were pelleted and re-suspended in 10 mL of α-MEM.

For TRAP staining, OCG was initiated by plating 2×10^6 cells per well in a 6-well tissue culture plate supplemented with 20 ng/ mL of M-CSF (M9170, Sigma, St. Louis, MO, USA) and 100 ng/mL of recombinant purified sRANKL. 1×106 cells were plated in a 48-well tissue culture plate containing dentin slices. Cells were cultured for 6 days, with a change of cell culture medium and cytokine supplementation every other day. On day 6, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA), and stained for tartrate-resistant acid phosphatase (TRAP) (Fig. 1).

To quantify number of nuclei and surface area of osteoclasts via DAPI and Phalloidin staining, OCG was initiated by plating 2×10^6

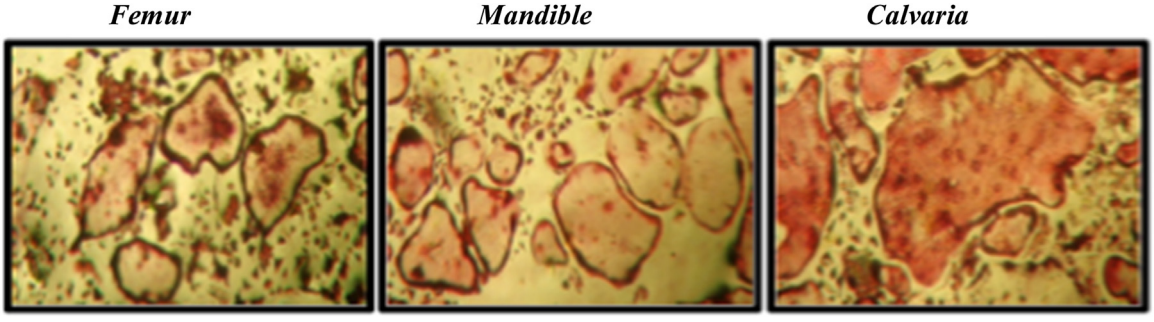

Fig. 1. TRAP staining.