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Long bone osteoclasts display an augmented osteoclast phenotype compared to calvarial osteoclasts

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ABSTRACT

Osteoclasts are multinucleated cells specialized in degrading bone and characterized by high expression of the enzymes tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CtsK). Recent studies show that osteoclasts exhibit phenotypic differences depending on their anatomical site of action.

Using immunohistochemistry, RT-qPCR, FPLC chromatography and immunoblotting, we compared TRAP expression in calvaria and long bone. TRAP protein and enzyme activity levels were higher in long bones compared to calvaria. In addition, proteolytic processing of TRAP was more extensive in long bones than calvaria which correlated with higher cysteine proteinase activity and protein expression of CtsK. These two types of bones also exhibited a differential expression of monomeric TRAP and CtsK isoforms. Analysis of $CtsK^{-/-}$ mice revealed that CtsK is involved in proteolytic processing of TRAP in calvaria. Moreover, long bone osteoclasts exhibited higher expression of not only TRAP and CtsK but also of the membrane markers CD68 and CD163.

The results suggest that long bone osteoclasts display an augmented osteoclastic phenotype with stronger expression of both membranous and secreted osteoclast proteins.

1. Introduction

The mammalian skeleton is formed by two different modes of ossification, i.e., endochondral and intramembranous. While long bones are formed by a combination of the two, other bones such as calvaria are solely formed by intramembranous ossification. Moreover, the matrix composition of these two types of bones differs to a considerable extent [1–6]. Not surprisingly, there is a growing body of evidence suggesting that not only matrices but also cells of different types of bones exhibit compositional/phenotypic differences [7–10].

Osteoclasts are multinucleated cells of the skeletal system with a unique capacity of demineralizing and degrading bone matrix. Recent studies suggest that osteoclasts of different bone types exhibit differences regarding their number of nuclei, cell size and machinery they employ for matrix degradation (for an extensive review, see [9]). Intriguingly, osteoclast heterogeneity appears to occur even within the same bone [11,12].

Tartrate-resistant acid phosphatase (TRAP) is highly expressed by osteoclasts and a long-known histochemical and biochemical marker for these cells. Synthesized as an inactive proenzyme, the proteolytic processing of the enzyme by cysteine proteinases gives rise to the enzymatically active form which has been proposed to participate in osteoclast-mediated bone resorption through its phosphatase or/and ROS-generating activities [13–15]. The major cysteine proteinase expressed by osteoclasts, i.e., cathepsin K (CtsK) was, in addition to a crucial role in bone collagen degradation, shown to be involved in proteolytic processing of TRAP in long bones [12]. Besides CtsK, other cysteine proteinases such as cathepsin L (CtsL) and B (CtsB) have also been shown to process and activate monomeric TRAP in vitro [16–18].

In this study, we conducted a comparative analysis of calvaria vs. long bone expression of TRAP and cysteine proteinase protein and activity.

2. Materials and methods

2.1. Materials

The antisera against total TRAP (recognizing both the monomeric and the cleaved variants of TRAP), monomeric TRAP and CtsK was produced as described in [19–21]. HiTrap™ Heparin HP columns were purchased from GE Healthcare (Uppsala, Sweden). Mouse monoclonal anti-rat ED1 (CD68) and ED2 (CD163) antibodies were purchased from Serotec (Serotec Ltd, Oxford, UK). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from

Abbreviations: TRAP, tartrate-resistant acid phosphatase; FPLC, fast protein liquid chromatography; AMC, 7-amino-4-methylcoumarin; CtsK, cathepsin K.

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Sigma (St. Louis, MO). Biotin-conjugated horse anti-mouse/anti-rabbit IgG antibodies were purchased from Vector Laboratories, Inc. (Burlingame, CA).

2.2. Experimental animals

Three-week-old Sprague–Dawley rats of mixed gender were used in the experiments. The use of the rats in this study was approved by the Local Animal Ethical Committee of the Karolinska Institutet.

CtsK-deficient mice was generated as described in [12]. Samples of calvaria from $Ctsk^{+/+}$ and $Ctsk^{-/-}$ of mixed gender were collected at the age of 1 month. The experimental procedures were approved by the Institutional Committee for Animal Welfare, University of Turku, Finland and were reviewed and approved by the Southern Stockholm Regional Board for Animal Welfare.

2.3. Quantitative RT-PCR

Total RNA was extracted from bones using Rneasy Mini kit (Qiagen). cDNA was produced from 2 μg total RNA using Omniscript® kit (Qiagen) and oligoDT primers (Qiagen). RT-qPCR analysis was performed using iCycler® (BioRad, Herkules, CA) and 1× Platinum qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA).

The qPCR thermocycling program consisted of 95 °C for 3 min for polymerase activation, followed by 40 cycles at 95 °C for 30 s, with annealing/elongation at 60 °C for 30 s for TRAP and Ctsk, and 62 °C for 30 s for GAPDH. The relative quantification of the genes was calculated using the "comparative C_t method" as in User Bulletin 2 from Applied Biosystems (Applied Biosystems, Foster City, CA). For primer sequences see Supplementary Table 1.

2.4. Preparation of bone lysates

TRAP was purified from the long bones and calvaria of rats and the calvaria of $CtsK^{+/+}$ and $CtsK^{-/-}$ mice. The dissected bones were freed from soft tissue and cartilage ends, cut into small pieces and placed in lysis solution (0.15 M KCl, 0.1% Triton X-100). After homogenization on ice by a Polytron rotor–stator homogenizer, the homogenate was cleared by centrifugation at 12,000*g* for 15 min at 4 °C. A small aliquot was saved for cysteine proteinase activity assay and proteinase inhibitor cocktail Complete (Roche Diagnostic GmbH, Mannheim, Germany) was added to the remaining homogenate.

2.5. FPLC separation of TRAP isoforms

Separation of monomeric and cleaved forms of TRAP from lysates or media was performed according to [12,22] using an ÄKTApurifier™ 10 FPLC system (GE Healthcare, Sweden) at 4 °C.

2.6. Protein quantification and tartrate-resistant acid phosphatase activity assay

Protein concentrations were determined using the BCA method (Pierce, Rockford, IL, USA), using BSA as the reference protein. TRAP activity was measured using *p*-nitrophenyl-phosphate (pNPP) as the substrate as described previously [12]. Parallel incubations with 100 μM molybdate was also included and molybdate-sensitive activity was regarded as a measure of tartrate-resistant acid phosphatase activity.

2.7. Endoglycosidase digestion of TRAP

For digestion with PNGase F, Heparin FPLC fractions containing 10 mU TRAP were boiled for 10 min in 0.5% SDS and 0.04 M dithiothreitol. Deglycosylation reactions were performed as described in [22].

2.8. Western blotting

Western blots were performed as described in [22].

2.9. Immunohistochemistry

Tissue preparation and immunohistochemistry was essentially performed as described earlier [23]. Antigens were unmasked by microwave treatment. All proteins were detected using antibodies at a dilution of 1:500. Visualization was achieved with DAB staining.

2.10. Determination of cysteine proteinase activity

For measuring cathepsin activity, 5 μg of undiluted lysate (2–5 μl) were mixed with 200 μl substrate buffer (100 mM acetate buffer, pH 5.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Chaps 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, and 20 μM of the substrates [Z-Leu-Arg]-AMC, [Z-Phe-Arg]-AMC or [Z-Arg-Arg]-AMC (Bachem) in a 96-well black plate (Corning Incorporated, Corning, NY, USA) with or without 5 μM of the cysteine proteinase inhibitor E64. After incubation at 37 °C for 20–30 min, substrate hydrolysis was determined by measuring the $\lambda_{emission}$ at 460 nm using spectrofluorometry (Spectramax Gemini, Molecular Devices, Sunnyvale, CA, USA) and E64-sensitive activity was taken as a measure of cysteine proteinase-specific activity in the lysates.

2.11. Statistical analysis

Statistical analyses were performed with Student's *t*-test. A probability value (*P*) of less than 0.05 was considered significant.

3. Results

3.1. Long bone osteoclasts exhibit higher expression of TRAP protein and activity than calvarial osteoclasts

TRAP mRNA levels and enzyme activity were measured in bone extracts from calvaria and femur of 3-week-old rats (Table 1). TRAP mRNA levels quantified by RT-qPCR and normalized to the reference gene GAPDH did not differ significantly between these two types of bones. However, TRAP enzyme activity normalized to either total protein or bone weight was 5- to 7-fold higher in femur

Table 1
Expression of TRAP mRNA and TRAP activity in long bone and calvarial homogenates of 3-week-old rats.

	n	Calvaria	*n*	Femur	Mean fold change
TRAP mRNA expression ($2^{-\Delta C_t}$ relative to GAPDH)	5	1.30 ± 1.35	5	2.03 ± 0.61^{NS}	1.6
TRAP activity in lysates (mU/mg protein)	8	242 ± 98	6	1179 ± 240***	4.9
TRAP activity in lysates (U/g bone weight)	6	3.96 ± 1.82	6	26.85 ± 4.09***	6.8

Data are presented as means ± SD.
NS, not significant.
n, numbers of animals.
*** $P < 0.001$ vs. calvaria.