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Peroxidase enzymes inhibit osteoclast differentiation and bone resorption

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ABSTRACT

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are heme-containing enzymes, well known for their antimicrobial activity, are released in abundance by innate immune infiltrates at sites of inflammation and injury. We have discovered new and previously unrecognised roles for heme peroxidases in extracellular matrix biosynthesis, angiogenesis, and bone mineralisation, all of which play an essential role in skeletal integrity. In this study we used *in vitro* models of osteoclastogenesis to investigate the effects of heme peroxidase enzymes on osteoclast differentiation and bone resorbing activity, pertinent to skeletal development and remodelling. Receptor activator of nuclear factor kappa B-ligand (RANKL) stimulates the formation of tartate-resistant acid phosphatase (TRAP) positive multinucleated cells and increases bone resorption when cultured with human peripheral blood mononuclear cells (PBMCs) or the RAW264.7 murine monocytic cell line. When RANKL was added in combination with either MPO or EPO, a dose-dependent inhibition of osteoclast differentiation and bone resorption was observed. Notably, peroxidases had no effect on the bone resorbing activity of mature osteoclasts, suggesting that the inhibitory effect of the peroxidases was limited to osteoclast precursor cells. Mechanistically, we observed that osteoclast precursor cells readily internalize peroxidases, and inhibited the phosphorylation of JNK, p38 MAPK and ERK1/2, important signalling molecules central to osteoclastogenesis. Our findings suggest that peroxidase enzymes, like MPO and EPO, may play a fundamental role in inhibiting RANKL-induced osteoclast differentiation at inflammatory sites of bone fracture and injury. Therefore, peroxidase enzymes could be considered as potential therapeutic agents to treat osteolytic bone disease and aberrant bone resorption.

1. Introduction

Bone remodelling is a dynamic metabolic process requiring the precise balance between the activities of osteoclasts and osteoblasts (Raggatt and Partridge, 2010). Multinucleated osteoclasts are derived from hematopoietic progenitor cells of the monocyte/macrophage lineage and are responsible for bone resorption (Chambers and Horton, 1984; Lorenzo et al., 2008). Osteoblasts are responsible for the production of bone matrix constituents and function in concert with osteoclasts to regulate bone regeneration (Sims and Vrahnas, 2014). An imbalance between the numbers and/or activity of osteoclast and osteoblast cells can result in a number of skeletal diseases including Paget's disease, osteoporosis, rheumatoid arthritis and cancer-associated bone loss (Theill et al., 2002; Boyle et al., 2003; Lajeunesse et al., 1996; Roodman, 2004). In most instances, the causative effect of this imbalance is driven by abnormalities in inflammatory cytokines, growth factors, and hormones, which can tip the balance towards either bone resorption or new bone formation (Weitzmann, 2013).

Osteoclast differentiation and bone resorption is primarily driven by two cytokines, namely macrophage colony stimulation

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Abbreviations

EPO	Eosinophil peroxidase
MPO	Myeloperoxidase
SBP	Soybean peroxidase
HRP	Horseradish peroxidase
ECM	Extracellular matrix
PBMC	Peripheral blood mononuclear cells
TRAP	Tartrate-resistant acid phosphatase
M-CSF	Macrophage colony-stimulating factor
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANK	Receptor activator of nuclear factor kappa-B receptor
LPO	Lactoperoxidase
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinases
ERK1/2	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
ROS	Reactive oxygen species
BSA	Bovine serum albumin
PBS	Phosphate buffered saline

factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Chambers, 2000; Teitelbaum, 2000; Li et al., 2000), which are found at elevated levels in bone destructive disorders (Mcinnes and Schett, 2007; Bartold et al., 2005). M-CSF promotes survival and proliferation of osteoclast precursors, while RANKL simulates osteoclast formation and activity (Soysa et al., 2012; Lee et al., 1994). Mechanistically, RANKL activates the MAPK family signalling cascade, including JNK, p38 MAPK and ERK1/2, which are essential for the differentiation of osteoclasts (Li et al., 2002; He et al., 2011; Wei et al., 2002) Thus, novel therapeutic agents that suppress RANKL and its downstream signalling events have the potential to inhibit osteoclast-mediated bone loss in a variety of bone destructive diseases.

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are heme-containing enzymes with pro-oxidative properties and play an important role in the oxidative defence against invading pathogenic microorganisms. (Klebanoff, 2005). Peroxidases are found in the cytoplasmic granules of circulating neutrophils and eosinophils respectively, which infiltrate bone fracture and injury sites during the healing process (Bastian et al., 2011; Serra et al., 2012). Our laboratory has recently discovered new and previously unrecognised roles for members of this superfamily of heme enzymes, including mammalian MPO and EPO, plant derived soybean peroxidase (SBP) and horseradish peroxidase (HRP). These roles include the regulation of extracellular matrix (ECM) biosynthesis (Denichilo et al., 2015), angiogenesis (Panagopoulos et al., 2015), and/or bone mineralisation (Denichilo et al., 2016). Collectively, these functional processes play a vital role in bone biology. In view of our findings, and recent reports which show that osteoclasts readily uptake HRP (Sasaki et al., 1985), we hypothesized that inflammatory peroxidases may also regulate osteoclast function.

Here we report, for the first time, that MPO and EPO, as well as plant derived peroxidase enzymes, SBP and HRP can inhibit RANKL-induced osteoclast differentiation and activity of human peripheral blood mononuclear cells (PBMC) and RAW264 cells *in vitro*. These findings highlight a previously unrecognised role for this group of enzymes in osteoclast biology and raise the possibility that peroxidases may possess therapeutic potential for the treatment of pathologies associated with osteoclast-mediated bone loss.

2. Materials and methods

2.1. Peroxidases

Native human eosinophil peroxidase (EPO) was obtained from Lee Biosolutions Inc. (St. Louis, USA). Recombinant human myeloperoxidase (rhMPO) was purchased from R&D Systems (Minneapolis, USA). Soybean peroxidase (SBP) was obtained from Bio-Research Products Inc (North Liberty, USA). Horseradish peroxidase (HRP) was purchased from Sigma Aldrich (St. Louis, USA).

2.2. Cells and reagents

Human peripheral blood mononuclear cells PBMC and the RAW264.7 murine monocytic cell line, which differentiate into osteoclast-like cells in the presence of RANKL and M-CSF were used as model systems of osteoclastogenesis. M-CSF and RANKL were obtained from Peprotech EC (London, UK). Osteoclast-like cells isolated from Giant Cell Tumours (GCT) of bone specimens, were used as a model of bone resorption activity of mature osteoclasts. Charcoal stripped FBS (fetal bovine serum) was purchased from Invitrogen (Carlsbad, USA). Antibodies against phospho-JNK, total JNK, phospho-p38 MAPK, total p38 MAPK, phospho-ERK1/2, total ERK1/2, were purchased from Cell Signalling Technology (Danvers, USA).

2.3. In vitro PBMC osteoclast differentiation and resorption assays

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats acquired from the Australian Red Cross Blood Service. The cells were diluted in Hank's balanced salt solution (HBSS) and separated by gradient centrifugation with Lymphoprep (Axis Shield). Isolated cells (2.5×10^5 cells/well) were then plated in minimum essential medium (α-MEM), (Sigma-Aldrich), supplemented with 10% charcoal stripped FBS L-glutamine (2 mM), HEPES (20 mM), recombinant human M-CSF (25 ng/ml); Millipore, Cat. No. GF053), 1α, 25 $(OH)_2$ vitamin D3 (10 nm; Wako Industries) and dexamethasone (10 nM; Hospira) directly into 96-well plates for tartate resistant acid phosphatase (TRAP) staining, or osteologic slides (BD Biosciences) for bone resorption assays. The following day media from each well was removed and replaced with fresh media supplemented with recombinant RANKL (50 ng/ml) alone serving as the positive control, and in the presence of increasing concentrations of peroxidases. Cells were fixed after 6 days and stained histochemically for TRAP (Sigma-Aldrich), and TRAP-positive cells were visualised by light microscopy. To assess bone resorption, osteologic slides were stained with Von Kossa stain and resorption pits were counted using light microscope.

2.4. In vitro RAW264.7 osteoclast differentiation assay

Murine RAW264.7 cells were seeded at a density of 0.8×10^4 cells per well in α-MEM, supplemented with 10% charcoal stripped FBS into 96-well plates and cultured overnight. Next day the media from each well was removed and replaced with fresh media supplemented with recombinant RANKL (100 ng/ml) alone serving as the positive control, and in the presence of increasing concentrations of MPO and EPO. Cells were cultured and maintained at 37 °C, 5% CO_2 for 5 days. Cells were then fixed and stained histochemically for TRAP, and TRAP-positive cells were visualised by light microscopy.