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A transmembrane osteoclastic protein-tyrosine phosphatase regulates osteoclast activity in part by promoting osteoclast survival through c-Src-dependent activation of NFκB and JNK2

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Abstract

This study evaluated the effects of overexpression of wild-type (WT) or phosphatase-deficient (PD) mutant of an osteoclastic protein-tyrosine phosphatase (PTP-oc) in RAW/C4 cells. Osteoclast-like cells derived from WT-PTP-oc overexpressing clones increased, while those derived from PD-PTP-oc expressing clones decreased, their resorption activity. WT-PTP-oc clones had lower apoptosis, lower caspase 3/7 activity, reduced c-Src tyr-527 phosphorylation (PY527) and IκBα cellular levels, and increased NFκB activation and JNK phosphorylation. Overexpression of PD-PTP-oc or PTP-oc siRNA treatment increased apoptosis, caspase 3/7 activity, PY527 and IκBα levels, and decreased NFκB and JNK2 activation. Inhibition of the c-Src kinase blocked the PTP-oc-mediated NFκB and JNK2 activation. Blocking the NFκB activation had no effect on the JNK2 activation. Inhibiting the NFκB and/or JNK2 pathway prevented the PTP-oc-mediated reduction in apoptosis. In conclusion, PTP-oc activates osteoclast activity in part by promoting osteoclast survival through the PTP-oc-mediated c-Src-dependent activation of NFκB and JNK2.
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Keywords: Osteoclasts; Protein-tyrosine phosphatase; Survival; c-Src; JNK; Apoptosis

There is compelling evidence that c-Src is important for osteoclast activity, since targeted disruption of the c-Src gene in mice led to a form of osteopetrosis, characterized by inactive osteoclasts [1], and the lack of c-Src expression in osteoclasts resulted in complete failure of formation of ruffled borders [2]. The c-Src encodes a cytosolic protein-tyrosine kinase (PTK)[1] [3]. Active osteoclasts expressed strong c-Src PTK activity on ruffled borders [4], and the c-Src PTK activity correlated with the number and activity of resorbing osteoclasts in vivo [2] and in vitro [5]. Inhibition of c-Src PTK activity in osteoclasts abolished their bone resorption activity in vitro and in vivo [6–8]. However, the contention that the c-Src PTK activity is essential for osteoclast activity was challenged by the partial rescue of osteopetrotic phenotype in c-Src deficient mice via transgenic expression of kinase-deficient c-Src mutants [9]. It was suggested that c-Src in osteoclasts acts predominantly as an adaptor molecule to recruit signaling proteins necessary for osteoclast attachment and migration [9]. On the other hand, the c-Src-dependent phosphorylation of downstream signaling proteins is clearly essential for resorption [10] and transgenic expression of a truncated c-Src mutant lacking the PTK domain induced osteopetrosis in wild-type or c-Src$^{+/-}$ mice and worsened osteopetrosis in c-Src$^{-/-}$ mice [11]. Thus, both the PTK activity and the adaptor

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[1] *Abbreviations used:* PTK, protein-tyrosine kinase; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein.

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function of c-Src are essential for osteoclastic resorption [12].

The c-Src PTK activity is inactivated by phosphorylation of its tyr-527 residue (PY527) and activated by the dephosphorylation [3]. Since c-Src deficiency yielded a phenotype of inactive osteoclasts, we postulate that one or more osteoclast-specific protein-tyrosine phosphatases (PTPs) are responsible for the dephosphorylation of PY527 and activation of c-Src signaling pathway. Consistent with this speculation are the findings that (1) 1,25$(OH)_2D_3$ activated the c-Src PTK activity in avian osteoclast precursors by increasing the expression of a PTP [13], (2) PTH activated, while calcitonin inhibited, the c-Src PTK activity in osteoclasts [5], (3) mice deficient in the receptor type PTP-ε had an increased trabecular bone mass due to defective osteoclast activity [14], and (4) osteoclasts derived from PTP-ε deficient bone marrow precursors showed low bone resorbing activity in vitro [14].

We have cloned from a rabbit osteoclast cDNA library an osteoclastic transmembrane PTP, termed PTP-oc [15]. PTP-oc is structurally unique in that it has a very short extracellular domain, lacks a signal peptide, and contains only a single PTP catalytic domain. PTP-oc is expressed primarily in hematopoietic cells [B lymphocytes and cells of monocyte–macrophage lineage (precursors of osteoclasts)] and mature osteoclasts [15,16]. Our recent studies suggested that PTP-oc may function as a positive regulator of osteoclasts in part by activating c-Src PTK via PY527 dephosphorylation. Accordingly, we have shown that: (1) suppression of PTP-oc expression in osteoclasts by a PTP-oc antisense oligodeoxynucleotide reduced their bone resorption activity, which was accompanied by an increase in c-Src PY527 level [16]; (2) osteoclast-like cells derived from U937 cells overexpressing wild-type (WT)-PTP-oc produced larger and deeper resorption pits than those derived from control cells; whereas osteoclast-like cells derived from phosphatase-deficient PTP-oc (PD-PTP-oc) mutant yielded smaller and shallower pits in vitro [17]; (3) overexpression of WT-PTP-oc also activated, and overexpression of PD-PTP-oc inhibited, the c-Src signaling pathway in these transgenic U937 cells [17]; (4) upregulation of PTP-oc expression by certain resorption activators in osteoclasts led to c-Src PTK activation [18]; and (5) the c-Src PY527 is a potential cellular substrate of PTP-oc [18].

Because transgenic overexpression of a truncated c-Src mutant lacking the PTK domain induced apoptosis in osteoclasts and precursors [11], and specific inhibition of the c-Src PTK kinase activity also induced osteoclast apoptosis both in vivo and in vitro [8], we postulate that the PTP-oc-mediated activation of c-Src PTK would reduce apoptosis of osteoclast precursors and/or osteoclasts, leading to an increased survival of functionally mature osteoclasts, which may in part be responsible for the PTP-oc-mediated increase in the osteoclastic activity. Thus, the objective of this study were 3-fold: (1) to determine the effects of overexpression of WT-PTP-oc or PD-PTP-oc mutant on bone resorption activity of osteoclast-like cells derived from the stable transgenic RAW/C4 cell clones; (2) to determine whether overexpression of PTP-oc would decrease apoptosis in c-Src-dependent manner; and (3) to determine molecular mechanisms responsible for the PTP-oc/c-Src-mediated effects on apoptosis in RAW/C4 cells.

Materials and methods

Cell cultures

A subclonal line of RAW264.7 cells (RAW/C4) was obtained from Dr. A. Ian Cassady of the University of Queensland (Brisbane, Australia) and used for these studies. The RAW/C4 clone was isolated through limiting dilution and screened for their ability to undergo RANKL-induced osteoclastic differentiation [19]. This subclone line showed an approximately 3-fold greater efficiency in RANKL-induced differentiation into osteoclast-like cells compared to the parental RAW264.7 cells [19]. There were no other apparent differences between RAW/C4 cells and the parental RAW264.7 cells in expression of osteoclastic genes, ability to resorb bone in vitro, and responsiveness to resorptive effectors. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY).

Production of stable PTP-oc overexpressing RAW/C4 cell clones

The PD-PTP-oc mutant construct, in which the catalytic cysteine (C325) was mutated to serine, was prepared by PCR-based site-directed mutagenesis with the Quikchange kit (Stratagene, La Jolla, CA) using our previously cloned full-length rabbit WT-PTP-oc cDNA in pcDNA 3.1(+) vector [17] as the template. The coding sequence of PD-PTP-oc construct was confirmed by DNA sequencing. The recombinant PD-PTP-oc protein, produced in *Escherichia coli*, was deficient in PTP activity (data not shown). The WT- and PD-PTP-oc cDNAs along with the cDNA for red fluorescent protein (RFP) (as a control) were each subcloned into our single-plasmid, *Tc1*-like transposon-based, "Prince Charming" (pPC) gene transfer vector [20]. This expression vector allows stable incorporation of the transgene into host chromosome at TA dinucleotide sites [21]. The RAW/C4 cells were transfected with pPC-WT-PTP-oc, pPC-PD-PTP-oc, or pPC-RFP expression vector using Effectene (Qiagen Inc., Valencia, CA). Stable transfectant clones were selected by G418 (800 ng/ml) for 4 weeks. Several stable clones each transfected with pPC-RFP, pPC-WT-PTP-oc, or pPC-PD-PTP-oc vectors were isolated, propagated, and stored at −80 °C. Two stable clones from each group were used in this study. The cell clones produced with the pPC-PTP-oc vectors were extremely stable, as the PTP-oc expression level in each clone has not changed for more than 3 years in the absence of the G418 selection pressure.

Measurement of PTP-oc expression levels

The PTP-oc mRNA expression level was assessed by RT-PCR. Briefly, total RNA was extracted with the RNeasy Mini kit (Qiagen). The integrity of RNA was determined by the bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RT-PCR was carried out using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Superscript II reverse transcriptase was used in RT reactions, and platinum Taq DNA polymerase was used in the PCR amplification. The primer sets for the rabbit PTP-oc transgene, the endogenous murine PTP-oc and housekeeping gene controls are shown in Table 1. The amplification condition consisted of an initial 2-min hot start at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing and extension for 1 min each at 54 °C and 72 °C, respectively, followed by a final cycle of 72 °C for 10 min. The PCR products were analyzed on 1% agarose gels.

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