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Submitted 10 July 2003; accepted in final form 20 May 2004

An osteoclastic protein-tyrosine phosphatase may play a role in differentiation and activity of human monocytic U-937 cell-derived, osteoclast-like cells

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An osteoclastic protein-tyrosine phosphatase may play a role in differentiation and activity of human monocytic U-937 cell-derived, osteoclast-like cells. Am J Physiol Cell Physiol 287: C874–C884, 2004; 10.1152/ajpcell.00294.2003.—This study investigated if an osteoclastic protein-tyrosine phosphatase (PTP), PTP-oc, plays a role in the functional activity and differentiation of osteoclastic cells by determining the effects of overexpression of wild-type (WT-) or phosphatase-deficient (PD-) PTP-oc on bone resorption activity and differentiation of human promonocytemonocytic U-937 cells, which could be induced to differentiate into “osteoclast-like” cells by phorbol ester/1,25(OH)2D3 treatment. U-937 cells overexpressing WT- or PD-PTP-oc were produced with a transposon-based vector. The size and depth of resorption pits created by WT-PTP-oc-overexpressing osteoclast-like cells were greater, while those by PD-PTP-oc-overexpressing osteoclast-like cells were less, than those created by control osteoclast-like cells. Overexpression of WT-PTP-oc also enhanced, while overexpression of PD-PTP-oc suppressed, their differentiation into osteoclast-like cells. Overexpression of WT-PTP-oc increased apoptosis and proliferation of U-937 cells, and overexpression of PD-PTP-oc reduced cell proliferation. Cells overexpressing WT-PTP-oc has also led to greater c-Src and NF-κβ activation, whereas cells overexpressing PD-PTP-oc resulted in less c-Src and NF-κβ activation. c-Src activation and NF-κβ activation each correlated with resorption activity and differentiation into osteoclast-like cells. In summary, these results show that 1) PTP-oc regulates both the activity and the differentiation of osteoclast-like cells derived from U-937 cells; 2) PTP-oc enzymatic activity is important to these processes; 3) high PTP-oc enzymatic activity caused an increase in U-937 cell apoptosis and proliferation, leading to no significant changes in the number of viable cells; and 4) some of the PTP-oc actions are mediated in part by the c-Src and/or NF-κβ pathways.

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We previously cloned a structurally unique transmembrane protein-tyrosine phosphatase (PTP) from rabbit osteoclasts, termed PTP-oc (39). Unlike most transmembrane PTPs, it lacks an extracellular domain, does not have a signal peptide proximal to the NH2 terminus, contains only a single PTP catalytic domain, and is relatively small (39). With the exception of an additional 28 amino acid residues near the NH2 terminus of PTP-oc, PTP-oc shows sequence identity with the intracellular domain of a renal receptor-like PTP, glomerular epithelial protein 1 (GLEPP1), which is also known as PTP-U2, PTPRO, or PTP-δ (28). While PTP-oc has been referred to as the truncated variant of GLEPP1 (3), we recently showed that the expression of PTP-oc is driven by an alternative, intronic, tissue-specific promoter (5). GLEPP1 and PTP-oc are each expressed in a tissue-specific manner; GLEPP1 is primarily expressed in the kidney and the brain (28, 38), whereas PTP-oc is expressed predominantly in several hematopoietic cell types, including B lymphocytes and cells of monocyte-macrophage lineage (which are precursors of osteoclasts), and mature osteoclasts (3, 28, 34, 39). GLEPP1 has important functions in the kidney, because targeted disruption of GLEPP1 in mice led to an altered podocyte structure associated with hypertension and low glomerular filtration rate (37). We previously showed that suppression of PTP-oc expression in mature osteoclasts by a PTP-oc antisense oligonucleotide reduced both basal and stimulated osteoclastic resorption (34), indicating that PTP-oc is an important regulator of the functional activity of mature osteoclasts. However, it is unclear whether PTP-oc also has a regulatory role in the differentiation, proliferation, and/or apoptosis of osteoclasts and precursor cells. We also do not know whether its enzymatic activity is essential. This issue is important because some studies have suggested that many PTKs and PTPs activate signaling mechanisms by serving as “docking proteins” to recruit downstream effectors for activation rather than through their own enzymatic activity (24, 27).

The objective of this study was threefold: 1) to determine whether PTP-oc plays a regulatory role in the functional activity, differentiation, proliferation, and/or apoptosis of precursors of osteoclastic cells; 2) to determine whether the enzymatic activity of PTP-oc is essential for these processes; and 3) if so, to gain insights into the molecular mechanisms by which PTP-oc acts to regulate osteoclast formation and/or activity. Our experimental approach was to determine and compare the effects of overexpression of wild-type (WT)-PTP-oc and phosphatase-deficient (PD)-PTP-oc in “osteoclast-like” cells on their differentiation, apoptosis, and bone resorption activity. Primary osteoclasts are terminally differentiated and do not proliferate, and they are not suitable for transgenic overexpression studies. Thus this study used human promonocytemonocytic leukemic U-937 cells as the cell model, because 1) these cells express PTP-oc (30); 2) they have been used in the past in a number of laboratories as the cell model of osteoclast precursors (6, 7, 10, 12, 25); 3) we have developed a protocol to induce the differentiation of these cells into osteoclast-like cells, which stained strongly for tartrate-resistant acid phosphatase (TRAP) and expressed known osteoclast marker genes, i.e., TRAP, calcitonin receptor, receptor

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activator of NF-κB (RANK), cathepsin K (CatK), and matrix metalloproteinase (MMP)-9, and the derived osteoclast-like cells were capable of resorbing bone in vitro; and 4) at the time this study was initiated several years ago, there were no convenient preosteoclast cell line models available or suitable for this work.

**MATERIALS AND METHODS**

**Cell cultures.** Human U-937 cells (ATCC CRL-1593,2) were obtained from American Type Culture Collection (Manassas, VA) and were maintained in suspension cultures in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). Cell medium was changed every 2–3 days, and cells were passaged weekly.

**Resorption pit formation assay.** U-937 cells (5,000 cells) were plated on a circular (70 mm in diameter) dentin disk in 48-well plates. 12-O-tetradecanoylphorbol 12-myristate 13-acetate (TPA) (10–7 M; Sigma, St. Louis, MO) was added 24 h later, and the cells were incubated for 48 h. The cell medium was then replaced with fresh RPMI 1640 containing 10–7 M 1,25(OH)2 D3 (Biomol Biomolecules Research Laboratory, Plymouth Meeting, PA). The cells were incubated for 2–5 days with medium containing 1.25(OH)2 D3 changed daily. This TPA/1,25(OH)2 D3 sequential treatment converted U-937 cells into osteoclast-like cells. The medium was then changed to fresh RPMI 1640 without effectors, and the resorption pit formation was measured 48 h later as previously described (31). The resorption activity per osteoclast (an index of mean osteoclast activity) was determined by dividing the total resorption pit area by the number of resorption pits.

The average resorption pit depth was determined by confocal microscopy using an Olympus IX70 inverted confocal microscope workstation (Olympus, Melville, NY). Briefly, after the total area and number of acid hematoxylin-stained resorption pits on the dentin disk were measured, each dentin disk was mounted on a glass slide in 50% glycerin (vol/vol). The coverslip was sealed with nail polish and dried for 72 h, and the dried slide was mounted upside down on the microscope. The resorption pits were viewed on the Olympus microscope fitted with a confocal argon-krypton mixed gas laser. Serial images of the pits at 2-μm intervals from the top surface of the dentin disk to the bottom of the pit were obtained using a z-plane motorized stage. Each image was obtained using appropriate excitation and emission filters. Six randomly selected pits per disc and four replicate discs per group were analyzed. The average depth is calculated from the number of 2-μm slices needed to reach the bottom of the pit.

**Stable U-937 cell pools overexpressing PTP-oc protein.** The pDNA 3.1(+) expression vector (Invitrogen, Carlsbad, CA) was initially used to produce stable U-937 cells. The full-length rabbit PTP-oc cDNA was cloned into the pDNA 3.1(+) vector. The PD-PTP-oc expression construct was prepared by polymerase chain reaction (PCR)-based, site-directed mutagenesis with WT-PTP-oc pcDNA 3.1(+) as the template using the QuickChange kit (Stratagene, La Jolla, CA), in which the catalytic cysteine (Cys225) was mutated to serine. The mutant lacked appreciable PTP activity (data not shown). U-937 cells were transected with each PTP-oc expression vector or empty vector with Effectene (Qiagen, Valencia, CA). However, “stable cells” generated with these constructs were unstable (apparently because of gene silencing) and did not overexpress PTP-oc after 2–3 mo.

We subsequently used our recently developed transposon-based “Prince Charming” (pPC) gene transfer vector (13) for this work. This vector system has two important advantages: 1) cell clones produced with the pPC vector are extremely stable (13, 40), and 2) the pPC-PTP-oc expression vector does not require continuous selection pressure, which allows experimentation with these stable cells in the absence of antibiotics (13). To construct the pPC-PTP-oc expression vector, linkers corresponding to the NotI and PmeI restriction sites were added in frame to the 5' and 3' ends, respectively, of the full-length WT- and PD-PTP-oc cDNA. The resulting plasmids were cloned into the pPC cassette at the NotI-PmeI sites to generate respective pPC-PTP-oc expression vector. The U-937 cells were then transfected with each pPC-PTP-oc expression vector as well as the pPC-RFP vector (a control vector) (13) using Effectene. After selection and expansion in G418 for 4 wk, stable cell pools overexpressing WT- or PD-PTP-oc were isolated. Three different stable pools for each group were used in these studies. The cell pools produced with the pPC-PTP-oc vectors were extremely stable: the PTP-oc protein expression level in each cell pool has not changed for more than 1 year, even in the absence of G418 (data not shown).

**Measurement of cellular PTP-oc protein levels.** The cellular PTP-oc protein level was measured by Western immunoblot assay using a guinea pig polyclonal antibody against the unique region of the NH2 terminus of rabbit PTP-oc as described previously (9). The blot was stripped and rebotted against an anti-actin antibody for normalization against protein loading.

**Differentiation of U-937 cells into osteoclast-like cells.** U-937 cell differentiation was monitored by counting the number of TRAP-positive, multinucleated (i.e., 2 or more nuclei) osteoclast-like cells formed after the TPA/1,25(OH)2 D3 treatment. Briefly, U-937 cells (10,000 cells/well) were plated in RPMI 1640 medium. The cells were treated with TPA/1,25(OH)2 D3 (10–7 M TPA/1,25(OH)2 D3) for 6 days, changes of medium containing 1,25(OH)2 D3 every 2–3 days. A large number of attached cells were then fused to form TRAP-positive, multinucleated cells. The number of TRAP-positive, multinucleated cells was counted in eight representative fields with a bright-field microscope.

U-937 cell differentiation was also quantitated by measuring the TRAP specific activity. Briefly, U-937 cells (50,000 cells/well) were plated in 24-well plates in serum-free RPMI 1640 medium. After the TPA/1,25(OH)2 D3 treatment, the attached cells were extracted with 0.5 ml of 0.1% Triton X-100. The TRAP activity in the extract was measured with a TRAP assay kit (Sigma) and normalized against cellular protein determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Expression of osteoclastic genes in the U-937 cell-derived, osteoclast-like cells. Total RNA was extracted from parental U-937 cells or U-937 cell-derived, osteoclast-like cells using the RNaseasy kit (Qiagen). The RNA was digested with 0.4 U/μl DNase I (Invitrogen), and the DNase was heat-inactivated at 80°C for 10 min. Complementary DNA (cDNA) was synthesized from the DNase-treated RNA sample using Superscript II reverse transcriptase (GIBCO-BRL, Grand Island, NY). PCR amplification of calcitonin receptor (CTR), TRAP, RANK, CatK, MMP-9, and β-actin (cDNA loading control) was performed individually with HotStar Taq DNA polymerase using the primer sets shown in Table 1. The reaction was subjected to a hot start at 95°C, followed by 35 amplification cycles comprising denaturing for 1 min at 95°C, annealing for 1 min at 55°C, and extending for 1 min at 72°C per cycle. The PCR products were analyzed on 1.2% agarose gels.

**Assay of cell apoptosis.** Apoptosis was evaluated with a DNA ladder-based apoptosis assay (Suicide-Track; Oncogene Research Products, Boston, MA) and an ELISA-based apoptosis assay kit (Cell Death Detection ELISA; Roche Diagnostics, Mannheim, Germany).

**Assays of cell proliferation, cell cycle, and viable cell number.** U-937 cell proliferation was assessed by [3H]thymidine incorporation. Briefly, U-937 cells (10,000 cells in 1 ml of serum-free RPMI) were plated in 24-well plates. After 24 h, 1.5 μCi of [3H]thymidine (Amersham, Arlington Heights, IL) was added for 6 h and the cells were transferred to a test tube, collected by centrifugation, and washed three times with ice-cold phosphate-buffered saline. After the final wash, the cells were obtained with a
cotton swab moistened in 12.5% trichloroacetic acid to trap the radioactivity. [3H]thymidine incorporation was determined by liquid scintillation counting.

Cell cycle analysis was performed using fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, San Jose, CA) with propidium iodide to label cell DNA. The relative number of viable cells was assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based assay (Roche Diagnostics, Penzberg, Germany). In this assay, MTT is taken up and reduced only by metabolically active cells, in part by the action of dehydrogenases.

Measurement of c-Src Tyr527 phosphorylation level. The relative levels of Tyr527-phosphorylated (PY-527) c-Src and total c-Src level were measured by performing the Western immunoblot assay as described previously (34) using a phospho-specific polyclonal antibody against PY-527 of c-Src (Biosource International, Camarillo, CA) and an anti-c-Src antibody (Upstate Biotechnology, Lake Placid, NY), respectively.

Measurement of Ikβα degradation. The Ikβα degradation was assessed by determining the amounts of cellular Ikβα in each cell pool. A decrease in cellular Ikβα level compared with control indicates an increase in degradation. Briefly, equal amounts of cell extracts, prepared by extraction in the radioimmunoprecipitation assay (RIPA) buffer, were separated by 10% SDS-PAGE and transblotted onto nitrocellulose membrane. The cellular Ikβα level was determined by Western immunoblot assay using a specific anti-Ikβα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and normalized against actin content for protein loading.

NF-κB nuclear translocation assay. Nuclear translocation of NF-κB is an index of NF-κB activation (4). To assess NF-κB nuclear translocation, the nuclei of U-937 cells were isolated as described previously (17), washed, and extracted with the RIPA buffer. The same amount of nuclear proteins from each extract was fractionated on 10% SDS-PAGE and transblotted onto a nitrocellulose membrane. The nuclear NF-κB level was measured by Western immunoblots using a specific anti-p65 RelA antibody (BD Transduction Laboratories, San Diego, CA). There is no convenient nuclear housekeeping protein suitable for normalization of protein loading. Thus no rebloot-ting against a housekeeping protein was done.

Table 1. Nucleotide sequence of primers used for reverse transcription-polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Sequence (5' → 3')</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-TRAP</td>
<td>GCAAGACATGTGCAAGGTG</td>
<td>335</td>
</tr>
<tr>
<td>Forward</td>
<td>GCCATCGCTGCAAGGTG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GCTCATCGCTGCAAGGTG</td>
<td></td>
</tr>
<tr>
<td>h-CTR</td>
<td>GATCCGCACTGTCCTGAGTA</td>
<td>659</td>
</tr>
<tr>
<td>Forward</td>
<td>AAATCGGAGATGGAGAGTC</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>h-RANK</td>
<td>GGAGGCATGGTCCTGAGTA</td>
<td>287</td>
</tr>
<tr>
<td>Forward</td>
<td>AAATCGGAGATGGAGAGTC</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>h-MMP-9</td>
<td>GCAAGATGCGGTGAGAGTC</td>
<td>312</td>
</tr>
<tr>
<td>Forward</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>h-Cathepsin K</td>
<td>AGGTTTGGCTGCTACCTGTT</td>
<td>513</td>
</tr>
<tr>
<td>Forward</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>h-β-actin</td>
<td>AGGTTCATCTACAGGCTGTG</td>
<td>497</td>
</tr>
<tr>
<td>Forward</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTGTACACCGGAGGGGAAG</td>
<td></td>
</tr>
</tbody>
</table>

h-TRAP, human tartrate-resistant acid phosphatase; h-CTR, human calcitonin receptor; h-RANK, human receptor activator of NF-κB; h-MMP9, human matrix metalloproteinase-9.

NF-κB transcriptional activation assay. A firefly luciferase (luc) reporter gene-based pNF-κB-transcription activity assay kit (BD Bioscience, Palo Alto, CA), which uses a construct (pNF-κB-luc) containing multiple copies of NF-κB consensus sequence fused to the luc reporter gene, was used to assess NF-κB transcriptional activity. Briefly, cells were transiently transfected with 1 μg of pNF-κB-luc plasmid or with the promoterless pTAL-Luc (control) with Effectene. Two days later, the luc activity in the cell extract was measured with an assay kit from Promega (Madison, WI). To control for transfection efficiency, cells in each experiment were also transfected with pGL3-control plasmids. The NF-κB activation was normalized against SV-40 promoter activity and reported as relative changes of parental cells.

Statistical analysis. Results are shown as means ± SE or SD with three to six replicates or repeat measurements. Statistical significance was determined with two-tailed Student’s t-test, and the difference was significant when P < 0.05.
Figure 4 shows that the average bone resorption activity (i.e., resorption pit area/pit) of osteoclast-like cells derived from WT-PTP-oc-overexpressing U-937 cells was significantly higher (by ~50%; \( P < 0.01 \)) than that of osteoclast-like cells derived from the vector control cells. The average depth of resorption pits created by osteoclast-like cells derived from WT-PTP-oc-overexpressing cells was also higher (by ~40%; \( P < 0.001 \)) than that of cells derived from vector control cells (Fig. 5). Conversely, the average pit area/pit of PD-PTP-oc-overexpressing cell-derived, osteoclast-like cells was significantly reduced (by ~30%; \( P < 0.05 \)) compared with that of the control osteoclast-like cells (Fig. 4). The average resorption pit depth created by these cells was also significantly less (by ~46%; \( P < 0.001 \)) than that of control cell-derived, osteoclast-like cells (Fig. 5). These findings indicate that the enzymatic activity (and not the protein level) of PTP-oc regulates the bone resorption activity of U-937 cell-derived, osteoclast-like cells.

Effects of PTP-oc overexpression on differentiation of U-937 cells.

Upon the sequential treatment with TPA for 2 days and 1,25(OH)\(_2\)D\(_3\) for 2–5 additional days, there were significantly more and apparently larger TRAP-positive, multinucleated cells formed in WT-PTP-oc-overexpressing cells than in vector control transfected cells (Fig. 6) or in parental U-937 cells (data not shown). Consistent with the interpretation that more cells stably overexpressing WT-PTP-oc (\( \geq 3\)-fold) were differentiated into osteoclast-like cells, the TRAP specific activity of WT-PTP-oc-overexpressing cells was significantly higher (\( \sim 7\)-fold) than that of control cells. Conversely, there were...
significantly fewer (~50%) osteoclast-like cells as well as significantly lower TRAP specific activity (by ~45%) in cells overexpressing PD-PTP-oc than there were in control cells after TPA/1,25(OH)2D3 treatment. Thus the enzymatic activity of PTP-oc may have a role not only in the bone resorption activity of the derived osteoclast-like cells but also in the differentiation of U-937 cells into osteoclast-like cells.

Effects of PTP-oc overexpression on U-937 cell apoptosis. Overexpression of GLEPP1 in U-937 cells enhanced the apoptosis of terminally differentiated U-937 cells (30). Thus we tested whether overexpression of PTP-oc also enhanced apoptosis of U-937 cells. A DNA ladder-based apoptosis assay revealed that cells overexpressing WT-PTP-oc showed significant amounts of DNA fragmentation, an index of increased apoptosis (Fig. 7A). Neither control cells nor PD-PTP-oc-expressing cells showed detectable levels of DNA fragmentation. Quantitation of apoptosis by an ELISA-based assay (Fig. 7B) confirmed that WT-PTP-oc-overexpressing cells showed an approximately fourfold increase (P < 0.001) in apoptosis compared with control cells and that overexpression of PD-PTP-oc had no significant effect on apoptosis. It is unclear why PD-PTP-oc overexpression did not affect apoptosis. Nevertheless, these findings indicate that supraphysiologically high levels of cellular PTP-oc activity enhance U-937 cell apoptosis.

Effects of PTP-oc overexpression on U-937 cell proliferation. The cell cycle analysis revealed that cells stably overexpressing WT-PTP-oc contained a higher number of cells at the S phase (with a corresponding decrease in the number of cells at the G0/G1 phase) than did the control cells, suggesting increased proliferation. Cells overexpressing PD-PTP-oc had a greater number of cells at the G2/M phase than did the control cells (Fig. 8A), which could prolong the cell cycle, resulting in a reduction in cell proliferation. Thus we next examined the effects of PTP-oc overexpression on the proliferation of U-937 cells by performing the [3H]thymidine incorporation assay and found that WT-PTP-oc-overexpressing cells exhibited a signif-

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**PTP-OC AND OSTEOCLAST ACTIVITY AND DIFFERENTIATION**

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**Fig. 3.** Overexpression of an osteoclastic protein-tyrosine phosphatase (PTP-oc) in stable U-937 cell pools transduced with pPC-wild-type (WT)-PTP-oc or pPC-phosphatase-deficient (PD)-PTP-oc vectors. Cellular PTP-oc protein level was analyzed using Western blot analysis as described in MATERIALS AND METHODS. The blot was stripped and reblotted against actin for protein loading. The level of PTP-oc expression in both the WT-PTP-oc and PD-PTP-oc stable cell pools was ~6-fold higher than in the parental U-937 cells.

**Fig. 4.** Effects of overexpression of WT-PTP-oc or PD-PTP-oc on the bone resorption activity of U-937 cell-derived, osteoclast-like cells. U-937 cells overexpressing WT-PTP-oc or PD-PTP-oc mutant (1,000 cells each) were plated on thin dentin disks and treated with TPA for 2 days and 1,25(OH)2D3 for 4 days. The bone resorption activity of osteoclast-like cells, which, after differentiation, attached to the surface of the dentin disk, was measured by the resorption pit formation assay. Images at top show representative resorption pits created from osteoclast-like cells of each stable cell pools. Graph summarizes average bone resorption activity, which is reported as average resorption pit area per pit number (means ± SE; n = 4 each). Dashed line represents 100% of vector-alone controls.

**Fig. 5.** Effects of overexpression of WT-PTP-oc or PD-PTP-oc on the average depth of resorption pits created by U-937 cell-derived, osteoclast-like cells. Average resorption pit depth was determined by confocal microscopy as described in MATERIALS AND METHODS. Top 3 rows show confocal microscopic images of representative resorption pits created by osteoclast-like cells derived from control vector-treated cells (vector control), cells overexpressing WT-PTP-oc, and cells overexpressing PD-PTP-oc. The slice number represents the number of 2-μm sections from the top surface of the dentin disk needed to reach the depth of each corresponding image. The brightness and contrast of each image were individually adjusted to show clearly the resorption pits. Graph summarizes the average depth of resorption pits created by each group of derived osteoclast-like cells. Results are shown as means ± SD. Each group contained 4 individual dentin disks, and 6 randomly selected pits were measured from each dentin disk. Dashed line represents 100% of vector-alone controls.
icient (50%) increase in [3H]thymidine incorporation compared with U-937 cells. PD-PTP-oc-overexpressing cells showed significant (55%) inhibition in [3H]thymidine incorporation (Fig. 8B). However, the increased cell death in cell pools overexpressing WT-PTP-oc would likely release large amounts of thymidine, which would reduce significantly the specific radioactivity of the added [3H]thymidine. Thus it is likely that the [3H]thymidine incorporation assay could have underestimated the proliferation rate of WT-PTP-oc-overexpressing cells. In any event, these findings clearly indicate that the enzymatic activity of PTP-oc may also have a role in U-937 cell proliferation.

Because overexpression of PTP-oc affected both proliferation and apoptosis of U-937 cells, we also assessed the effects of PTP-oc on the overall number of viable cells in each cell population. Figure 8B shows that the relative number of viable cells.
cells (assessed using MTT assay) in WT-PTP-oc-overexpressing cells was only slightly (7%) greater than that of parental cells and that the relative number of viable cells in PD-PTP-oc-overexpressing cell pools was also only slightly (10%) less than that of the parental cell population. Thus the PTP-oc-dependent increase in cell proliferation was sufficient to counterbalance the PTP-oc-mediated increase in cell death, resulting in only a small change in the overall number of viable cells. These findings suggest that the observed differences in the differentiation and bone resorption activity of U-937 cell-derived, osteoclast-like cells are probably not due to different numbers of viable cells among various cell populations.

Effects of PTP-oc overexpression on PY-527 level of c-Src in U-937 cells. Because we have preliminary evidence that PTP-oc acts to stimulate osteoclast activity by activating the c-Src PTK activity through the PTP-oc-mediated dephosphorylation of PY-527 of c-Src (19, 34), we measured the steady-state c-Src PY-527 level in each test cell pool. Figure 9 shows that the steady-state c-Src PY-527 level (normalized against either total c-Src or actin) in cells stably overexpressing WT-PTP-oc was reduced to $\approx 25%$ ($P < 0.01$) of that of the vector control cells. Cells overexpressing PD-PTP-oc had a $25%$ ($P < 0.05$) increase in c-Src PY-527 levels. The c-Src PY-527 level correlated negatively with the resorption activity of the derived osteoclast-like cells ($r = -0.998$, $P < 0.01$ for pit area/pit; $r = -0.961$, $P < 0.05$ for pit depth), suggesting that the bone resorption activity of U-937 cell-derived, osteoclast-like cells is associated with the PTP-oc-mediated c-Src activation. The c-Src PY-527 level also correlated negatively with osteoclast-like cells formed in response to the TPA/1,25(OH)$_2$D$_3$ sequence.
tial treatment ($r = -0.996$, $P < 0.01$ for osteoclast-like cell number).

Effect of PTP-oc overexpression on NF-κβ activation in U-937 cells. We next evaluated whether overexpression of PTP-oc would also lead to NF-κβ activation, because activation of NF-κβ is important for the activation and differentiation of osteoclasts (15, 31). Because NF-κβ activation involves degradation of IkBα and nuclear translocation of NF-κβ (4), we assessed NF-κβ activation by measuring IkBα degradation and nuclear NF-κβ translocation. There was a marked decrease ($P < 0.001$) in cellular IkBα level in cells overexpressing WT-PTP-oc compared with that of parental cells (Fig. 10A). Cells stably overexpressing PD-PTP-oc showed a small (25%) increase in cellular IkBα level, although the increase did not reach a statistically significant level because of the small sample size. Nuclear NF-κβ translocation was determined by measuring the nuclear level of NF-κβ with a specific anti-p65 NF-κβ antibody (Fig. 10B). Cells overexpressing WT-PTP-oc had twice as much NF-κβ in the nucleus as the parental cells did, whereas the nuclear NF-κβ level in cells overexpressing PD-PTP-oc was 32% of that in parental cells. To confirm the NF-κβ activation, the NF-κβ transcriptional activity of each of the test cell pools was also measured with the pNF-κβ reporter gene assay (Fig. 10C). The NF-κβ transcriptional activity of WT-PTP-oc-overexpressing cell pools was significantly higher (263.3 ± 38.6% of control, $P < 0.001$), and that of PD-PTP-oc-overexpressing cell pools was significantly lower (25.0 ± 5.9% of control, $P < 0.001$), than that of control cell pools, confirming the results of the NF-κβ nuclear translocation experiments (Fig. 10B).

The NF-κβ activation correlates significantly with bone resorption activity ($r = 0.997$, $P < 0.01$ for pit area/pit; $r = 0.999$ for pit depth) and with the differentiation of U-937 cells ($r = 0.971$, $P < 0.05$ for osteoclast-like cell number), suggesting that PTP-oc overexpression-mediated increase in the bone resorption activity and/or differentiation of U-937 cell-derived, osteoclast-like cells may be associated with PTP-oc-induced activation of the NF-κβ pathway. The fact that the cellular c-Src PY-527 level correlated positively with the cellular IkBα level ($r = 0.974$, $P < 0.05$) and negatively with nuclear NF-κβ ($r = -0.989$, $P < 0.01$) supports an association between the NF-κβ activation and PTP-oc-mediated c-Src activation.

**DISCUSSION**

Our decision to use the U-937 cell model was based on our findings that U-937 cells could be differentiated into TRAP-positive, multinucleated, osteoclast-like cells capable of resorption of bone using the TPA/1,25(OH)2D3 sequential treatment and that the derived osteoclast-like cells expressed many of the known osteoclastic marker genes (i.e., RANK, CTR, MMP-9, CatK, TRAP). We are mindful that U-937 cells are not bona fide osteoclast precursors and that, unlike normal osteoclast precursors, they do not respond to RANKL. However, because overexpression of PTP-oc in U-937 cells enhanced the formation and the bone resorption activity of the

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**Fig. 10. Effects of overexpression of PTP-oc on IkBα degradation (A), NF-κβ nuclear translocation (B), and NF-κβ transcriptional activity (C). A: steady-state cellular IkBα levels normalized against actin level in the parental U-937 cells (parental) and in cells overexpressing WT-PTP-oc or PD-PTP-oc (means ± SE, $n = 3$ replicates for each). B: effect of PTP-oc overexpression on nuclear translocation of NF-κβ (means ± SE, $n = 3$ replicates for each). Dashed line represents 100% of vector controls. C: effect of PTP-oc overexpression on NF-κβ transcriptional activity (means ± SE, $n = 4$ replicates for each). NF-κβ transcriptional activity is normalized against transcriptional activity of pGL3-control plasmid and is shown as %pGL3-control, which showed similar luciferase activity in each test cell population (57,000–61,000 arbitrary light units). Dashed line represents 100% of vector controls.**
derived osteoclast-like cells, we think that information gathered from the U-937 cell model may still be useful in understanding the potential roles of PTP-oc in the osteoclasts.

This study presents strong evidence that PTP-oc may have some regulatory roles in the formation and resorption activity of osteoclast-like cells in the U-937 cell model. To that end, we have demonstrated that overexpression of WT-PTP-oc increased the bone resorption activity and the formation of osteoclast-like cells in response to TPA/1,25(OH)$_2$D$_3$ sequential treatment. By contrast, overexpression of PD-PTP-oc in U-937 cells suppressed differentiation into osteoclast-like cells in response to TPA/1,25(OH)$_2$D$_3$ sequential treatment and the bone resorption activity of the derived osteoclast-like cells. Importantly, the fact that overexpression of PD-PTP-oc not only did not enhance, but also significantly suppressed, the bone resorption activity and the differentiation of U-937 cell-derived, osteoclast-like cells led us to conclude that the enzymatic activity (but not the protein level) of PTP-oc is important for these processes. This further led us to speculate that PTP-oc functions in osteoclasts and precursor cells to dephosphorylate key tyrosyl-phosphorylated proteins rather than to serve as docking proteins. The fact that PTP-oc overexpression stimulates the differentiation of U-937 cells is not unexpected, because there is circumstantial evidence that members of GLEPP1/PTP-oc family have enhancing effects on the differentiation of several cell types, including U-937 cells (9, 28, 29, 36).

Consistent with the previous finding that overexpression of WT-GLEPP1 in U-937 cells induced apoptosis of the terminally differentiated monocytes/macrophages (30), we found that overexpression of WT-PTP-oc in U-937 cells also increased apoptosis. Similarly to PD-GLEPP1 (30), overexpression of PD-PTP-oc had no significant effect on U-937 cell apoptosis. Although the reason for the lack of an effect of PD-PTP-oc on apoptosis is unclear, these findings raise the interesting possibility that high, ultraphysiological levels of cellular enzyme activity of PTP-oc (or GLEPP1) enhanced the apoptosis of U-937 cells. Consistent with this speculation are our previous findings that suppression of PTP-oc expression with PTP-oc antisense oligodeoxynucleotide did not appear to affect the life span of mature rabbit osteoclasts (34). Moreover, we have preliminary evidence that the enhancing effect of PTP-oc on cell apoptosis might be unique to U-937 cells, because WT-PTP-oc overexpression in murine RAW267.4 cells (an osteoclast precursor cell model) did not enhance apoptosis (unpublished observation).

The mechanism whereby high levels of active PTP-oc (or GLEPP1) induce apoptosis is unknown. While we suspect that some of the PTP-oc actions are associated with the PTP-oc-mediated NF-$\kappa$B activation (see below), we do not think that the induced U-937 cell apoptosis involved NF-$\kappa$B activation, for the following three reasons: 1) activation of NF-$\kappa$B in other cell types (including RAW264.7 cells) has antiapoptotic effects; 2) overexpression of PD-PTP-oc, which led to a marked inhibition of NF-$\kappa$B, did not show a corresponding inhibition of U-937 cell apoptosis; and 3) while the actions of NF-$\kappa$B on apoptosis are mediated largely through caspase-dependent pathways, a specific caspase inhibitor could only slightly inhibit the GLEPP1-induced apoptosis in U-937 cells (30). Thus it is possible that GLEPP1 and/or PTP-oc acts primarily through caspase-independent pathways to induce apoptosis in U-937 cells.

One of the most surprising findings of this study is that overexpression of WT-PTP-oc also led to the stimulation of cell proliferation in U-937 cells as reflected by an increase in [$^3$H]thymidine incorporation and an increase in the number of cells at the S phase, with a corresponding decrease in the number of cells at the G$_0$/G$_1$ phase. Because there was not a large difference in the number of viable cells between the test cell populations, the increase in apoptosis in response to WT-PTP-oc overexpression might have been counterbalanced by the PTP-oc-associated stimulation in cell proliferation. On the other hand, although PD-PTP-oc overexpression did not affect apoptosis, it appeared to reduce cell proliferation as reflected by a reduction in [$^3$H]thymidine incorporation and a significant increase in the number of cells at G$_0$/G$_1$ phase without a corresponding reduction in the number of cells at G$_0$/G$_1$ phase. However, the significance of this finding is unknown at this time.

Regarding the potential mechanisms whereby PTP-oc regulates the bone resorption activity of osteoclasts, we have strong circumstantial evidence in rabbit osteoclasts that the PY-527 of c-Src could be a substrate of PTP-oc and that PTP-oc may act through activation of the c-Src signaling pathway by dephosphorylating the PY-527 residue of c-Src to activate bone resorption activity (19, 34). In this study, we have shown that overexpression of WT-PTP-oc in U-937 cells, which led to an increase in bone resorption activity, also activated c-Src PTK activity by reducing c-Src PY-527 levels, whereas overexpression of PD-PTP-oc, which caused an inhibition of bone resorption activity, also reduced c-Src PTK activity by increasing c-Src PY-527 levels. That the c-Src PY-527 level correlated strongly with the bone resorption activity of the U-937 cell-derived, osteoclast-like cells provided additional associative evidence for the involvement of c-Src activation in the PTP-oc-mediated activation of osteoclast-like cells.

The findings that overexpression of WT-PTP-oc resulted in the activation of NF-$\kappa$B and that the bone resorption activity of the derived osteoclast-like cells also correlated with NF-$\kappa$B activation raise the interesting possibility that PTP-oc-mediated osteoclast activation may involve NF-$\kappa$B activation. The mechanism whereby PTP-oc activates the NF-$\kappa$B pathway and the relationship between PTP-oc-mediated c-Src activation and the associated NF-$\kappa$B activation is largely unknown. Under unstimulated conditions, NF-$\kappa$B is retained in the cytoplasm as a heterodimer with an inhibitory protein, I$\kappa$B (41). I$\kappa$B$\alpha$ is the most important I$\kappa$B member in cells of hematopoietic origin, including osteoclasts (8). Activation of NF-$\kappa$B is usually initiated by phosphorylation of I$\kappa$B at Ser$^{32}$ and/or Ser$^{36}$, which prompts the dissociation of I$\kappa$B$\alpha$ from NF-$\kappa$B, marking it for ubiquitination and rapid degradation via the ubiquitin-proteasome pathway (5). The liberated NF-$\kappa$B is, in turn, rapidly phosphorylated and translocated to the nucleus to function as a transcriptional complex to activate specific gene expression. An important discovery relevant to the relationship between NF-$\kappa$B activation and c-Src activation is that I$\kappa$B$\alpha$ is also phosphorylated at Tyr$^{32}$ in certain cell populations (e.g., osteoclasts, lymphocytes) (1, 2, 14, 21, 32). The protein-tyrosine phosphorylation of I$\kappa$B$\alpha$ is mediated by members of the c-Src PTK family, such as c-Lck in T lymphocytes (1) and c-Src in cells of the osteoclast lineage (2). The protein-tyrosine phos-
phosphorylation of IκBα also leads to the rapid degradation of IκBα (21, 32) and nuclear translocation of NF-κB for specific gene transcription. Consistent with the premise that phosphorylation of IκBα at Tyr42 in osteoclasts plays a regulatory role in the activation of NF-κB, administration of a dominant negative IκBα mutant protein in which Tyr42 was deleted blocked both NF-κB activation and osteoclast activation (1). Therefore, we postulate that PTP-oc dephosphorylates Tyr42 of c-Src in osteoclasts, resulting in the activation of its PTK activity, which in turn phosphorylates IκBα at Tyr42, leading to degradation of IκBα and nuclear translocation and activation of NF-κB pathways.

Inasmuch as our proposed mechanism focuses only on PTP-oc-mediated NF-κB activation, we by no means conclude that NF-κB activation is the primary mechanism by which the PTP-oc-c-Src pathway acts to regulate osteoclast activity. To the contrary, there is abundant evidence that c-Src regulates osteoclast activity through multiple mechanisms. Accordingly, the SH2 domain of c-Src has been shown to function as an adaptor protein to regulate c-Src trafficking and membrane localization, to mediate interactions with downstream signaling proteins, and/or to recruit key signaling proteins to membrane receptor PTKs for phosphorylation (26). Activated c-Src in osteoclasts has also been reported to migrate to mitochondria to phosphorylate cytochrome c oxidase, which is required for osteoclast function (20). There is also evidence for the involvement of PYK2 in the c-Src-induced spreading and activation of osteoclasts (18). Thus it is clear that the mechanism whereby the PTP-oc-c-Src pathway regulates osteoclast activity is complex and involves multiple signaling pathways. Further work is needed to delineate the relationship between these pathways and PTP-oc-mediated NF-κB activation.

Finally, we are somewhat surprised to note the highly significant correlation between PTP-oc-dependent c-Src activation and the differentiation of U-937 cells into osteoclast-like cells. There is compelling evidence in the literature against a role for c-Src in osteoclast differentiation (33). Consequently, we think that the PTP-oc-dependent osteoclast differentiation probably does not involve the c-Src pathway. Much additional work is needed to determine the exact mechanism by which PTP-oc enhances osteoclast differentiation.

In summary, we have shown in U-937 cells that J) PTP-oc stimulated the bone resorption activity of the U-937 cell-derived, osteoclast-like cells and enhanced the differentiation of U-937 cells into osteoclast-like cells; 2) the enzymatic activity (but not the protein level) of PTP-oc was essential for these processes; 3) high levels of enzymatically active PTP-oc led to an increase in apoptosis as well as proliferation; and 4) PTP-oc stimulated the bone resorption activity and differentiation of U-937 cell-derived, osteoclast-like cells through c-Src-dependent and c-Src-independent NF-κB activation, respectively. However, because U-937 cells are not bona fide osteoclast precursors, these findings must be confirmed with primary osteoclasts and/or precursors before the role of PTP-oc in osteoclast activity and differentiation can be ascertained.

ACKNOWLEDGMENTS

We thank Belinda Nestor for excellent technical assistance. Present address of S.-M. Suhr: Dept. of Biochemistry, Yonsei Univ., Seoul, Korea.

GRANTS

This work was supported in part by National Institutes of Dental and Craniofacial Research Grant R01 DE-13097 to K.-H. W. Lau. All work was performed in facilities provided by the Department of Veterans Affairs.

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