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Co-Detection of PTH/PTHrP Receptor and Tartrate Resistant Acid Phosphatase in Osteoclasts

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Abstract Serial sections of rat metaphyses were prepared from paraffin embedded tissue blocks and analyzed in sets of three. The central section was stained for tartrate resistant acid phosphatase (TRAP) in order to identify osteoclasts, one adjacent section was immunostained with an affinity purified antibody to a 15 amino acid sequence unique to rat PTH/PTHrP receptor, and the other adjacent section in the set served as an immunostaining control. This allowed each of the 110 osteoclasts examined to be identified by TRAP and to be tested for the presence or absence of PTH/PTHrP receptor. All antibody solutions and rinses contained 1% donkey serum and 0.5% Tween 20 to ensure antibody integrity and good rinsing procedure. Confocal microscopy was used to evaluate fluorescence intensity of the immunostained osteoclasts. Pixel intensities of 58 osteoclasts from young (4 month) rats and 52 osteoclasts from old (15 month) rats were obtained. Pixel intensities were similar ($P = 0.89$) for both young and old animals. However, the number of PTH/PTHrP receptor deficient osteoclasts was greater for the older animals (14.29% vs. 7.24%). This provides direct evidence of PTH/PTHrP receptors in osteoclasts. J. Cell. Biochem. 89: 902–908, 2003.

Key words: PTH/PTHrP receptor; osteoclasts; immunodetection; rat metaphysis

For several decades, following the discovery by Albright et al. [1929] that PTH stimulates bone resorption, the hormone was presumed to act directly on osteoclasts. However, when it became possible to isolate osteoblast-like and osteoclast-like cells, it was found that PTH had a pronounced effect on osteoblast-enriched cell populations isolated from calvaria [Wong et al., 1977]. Many studies now show that osteoblasts express an abundance of PTH/PTHrP receptors (PTH/PTHrP-R), and when the receptor is occupied by PTH or PTHrP, osteoblasts are activated and, in turn, stimulate osteoclasts [review: Bringhurst and Strewler, 2002].

As predicted by Rodan and Martin [1981], osteoblasts are a major target for PTH and it is widely held that other bone cells do not respond directly to PTH or PTHrP. However, several experimental approaches indicate direct interaction of PTH with osteoclasts. These approaches include immunocytochemical staining of PTH on osteoclasts [Rao et al., 1983], binding of radiolabeled PTH [Teti et al., 1991], plasma membrane binding and clearance of fluorescein-tagged PTH by living osteoclasts [Agarwala and Gay, 1992], induction of a superoxide anion burst in isolated osteoclasts following PTH stimulation [Datta et al., 1996], and PTH stimulation of acid formation by isolated osteoclasts [Hunter et al., 1988; Gay et al., 1993; May and Gay, 1997a]. Pertussis toxin was found to block PTH-stimulated acidification through $Gi_{\alpha\text{-}3}$, a G-protein abundantly present in osteoclasts but undetectable in osteoblasts [May and Gay, 1997b]. Because of these indications that PTH has direct effects on osteoclasts, we embarked on the project of localizing PTH/PTHrP-R in sections of rat metaphysis in which multinucleate osteoclasts were identified by tartrate resistant acid phosphatase (TRAP). Since initiating this project [Gay et al., 2001], PTH/PTHrP-R has been detected by immunostaining in osteoclasts in sections of human bone [Langub et al., 2001], in deer antler [Faucheux et al., 2002], in isolated rat osteoclasts [Watson et al., 2002], and in the osteoclast cell line,

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RAW264.7 [Watson et al., 2002]. Detection by in situ hybridization of mRNA has also been reported [Langub et al., 2001].

In the present study, we identify osteoclasts in sections of rat metaphysis by TRAP staining and show that the TRAP stained cells also bind antibodies to PTH/PTHrP-R. Reasons for the difficulty in showing that osteoclasts express PTH/PTHrP-R are discussed.

METHODS

Animals and Tissue Preparation

Fischer 344 virgin female rats of two ages (4 and 15 months) were obtained through the National Institute on Aging (NIA) colonies from Harlan Sprague-Dawley (Bethesda, MD). Femurs were dissected from CO_2 overdosed rats (three young and three old rats) and quickly placed into fixative consisting of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 on ice. Within 1 h the distal femur was cut from the shaft, then cut sagittally with a dental saw to expose trabecular bone and placed in freshly made fixative for 24 h at 4°C. After fixation, the tissues were decalcified in several changes of 15% ethylenediaminetetraacetate (EDTA), 0.5% PFA in 0.1 M PBS (pH 8, 3 weeks, 4°C). Following decalcification, the tissues were rinsed in PBS then dehydrated in a graded series of ethanol (30, 50, 70, 80, 95%, 1 h each; 100%, 2 × 1 h), ethanol:xylene 1:1 (30 min), infiltrated with xylene (30 min), followed by a 1:1 xylene-paraffin mixture (15 min, 56°C) and finally paraffin only (4 h, 56°C). Samples were embedded in paraffin and 5 μm serial sections were collected on charged, precleaned microscope slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA). Serial sections were divided into groups of three: the central section was stained for TRAP, one adjacent section was immunostained, and the other adjacent section served as an immunostaining control (1° antibody omission). Only osteoclasts present at the same x,y locus in all three sections were examined. For staining, the slides were heated at 56°C for 2 h to firmly attach sections to slides, then deparaffinized in xylene (2 × 10 min) followed by sequential rehydration in 100% ethanol (2 × 2 min), 95–30% ethanol (2 min each) and PBS (2 min). Slides were re-fixed in 4% PFA for 15 min and rinsed in PBS (3 × 5 min). The serial section approach was used because double staining with TRAP and the PTH/PTHrP-R antibody failed due to the former masking the fluorescence of the latter.

Immunostaining

After rinsing in PBS (3 × 5 min), the sections were blocked with 6% BSA in PBS (45 min, 20°C), rinsed in PBS (2 × 5 min), and rinsed a final time in 1% BSA in PBS for 5 min. Subsequently all rinses and antibody dilutions were with 1% BSA in PBS. Slides were incubated with rabbit anti-PTH/PTHrP-R (PRB-635P; BAbCO, Berkeley, CA) diluted 1:50 or 1:100 (4 h, 20°C) and rinsed thoroughly (20°C, 30 min; 4°C, overnight; 20°C, 30 min). The polyclonal antibody was produced in rabbits against the sequence CLVDADDVFTKEEQI, which is present near the C-terminus of the PTH/PTHrP-R. This sequence has 100% identity in mouse, rat and rabbit and is different by one amino acid from opossum, zebrafish, dog, and human (93% identity). The antibody was purified by affinity chromatography by BAbCO, making it a highly specific antibody. Nearby muscle cells were devoid of specific fluorescence. A secondary antibody, goat anti-rabbit IgG conjugated with Alexa Fluor 568 (Molecular Probes, Eugene, OR) was applied to the sections (1:200 dilution, 2 h, 20°C) and then rinsed (3 × 30 min). Finally a third antibody, donkey anti-goat IgG conjugated with Alexa Fluor 568 was applied to the sections (1:200 dilution, 2 h, 20°C). Alexa Fluor fluoroprobes are exceptionally photostable and intensely fluorescent; the double application of Alexa Fluor enhanced antigen detection. Sections were rinsed in 1% BSA in PBS (3 × 30 min), briefly rinsed in distilled H_2O and mounted with Fluoromount-G mounting media (Southern Biotechnologies, Birmingham, AL). Slides were examined using a Bio-Rad 1024 confocal microscope (Bio-Rad, Hercules, CA). Images were collected using the 568 nm laser line and transmission detector. Control slides consisted of staining adjacent serial sections but omitting primary antibody or applying non-immune rabbit IgG antibody (Sigma, St. Louis, MO) at a dilution of 1:50 and 1:100 in 1% BSA in PBS.

TRAP

One section from the arrays of serial sections was selected for TRAP staining. Briefly, these sections were deparaffinized, rehydrated, washed in PBS and re-fixed in buffered formalin

(10 min). The slides were then incubated for 1 h at 37°C in a Naphthol AS-BI phosphoric acid/ Fast Garnet/tartrate solution (Sigma). After rinsing in distilled H_2O (3×1 min), the slides were mounted with Fluoromount-G mounting media. Slides were first examined using the transmission detector optics of the Bio-Rad 1024 Confocal microscope. Fields containing TRAP stained osteoclasts were identified and compared to identical fields of adjacent PTH/ PTHrP-R immunostained sections.

Image Analysis

After collecting images, polygon areas were selected in PTH/PTHrP-R stained cells. The same cells were found in the unstained control (1° antibody omission) adjacent serial section and matching polygon area selected. Pixel intensities were obtained. The polygon areas included much of the cytoplasm and cell surface region, but excluded nuclei. Pixel intensities from 58 PTH/PTHrP-R stained osteoclasts from the young rats were averaged and compared with pixel intensity averages of 52 PTH/PTHrP-R stained osteoclasts from old rats. Student's t-test ($P = 0.089$) indicated no statistically significant differences. Total area examined for PTH/PTHrP-R stained sections from young rat metaphysis was 1282.07 μm^2 and for control sections from the same tissue blocks the total area measured was 1073.53 μm^2. Total area examined for sections from old rat metaphysis stained for PTH/PTHrP-R was 996.34 μm^2, and for control sections it was 882.61 μm^2. Pixel intensities of 972 μm^2 muscle adherent to bone in the same sections used for the osteoclast analysis were obtained for both immunostained and unstained control sections.

RESULTS

Localization of PTH/PTHrP Receptor

Figure 1A,B,C shows regions of three adjacent serial sections with the central section immunostained for PTH/PTHrP-R, one adjacent section stained for TRAP and the other adjacent section serving as the immunostaining control (omission of primary antibody). A PTH/ PTHrP-R expressing osteoclast and a PTH/ PTHrP-R negative osteoclast are present in this particular set of sections. Panels D, E, and F show another example of a PTH/PTHrP-R positive osteoclasts. Panels G, H, and I show the level of staining encountered in cuboidal osteoblasts lining a segment of trabecular bone.

Evaluation of Pixel Intensities of PTH/PTHrP Receptor Positive Osteoclasts

The data in Figure 2 was obtained by first finding TRAP stained osteoclasts, then locating the same osteoclasts in the immunostained adjacent serial section; pixel intensity values were then obtained for immunofluorescence. To obtain background levels of autofluorescence of the same osteoclast, a third section from the array of serial sections was selected and pixel intensities determined. The background section had been identically immunostained, except that primary antibody was omitted. Figure 2 shows levels of fluorescence obtained from a total of 58 osteoclasts from 4-month old rats and 52 osteoclasts from 15-month old rats for both immunostained and control sections. Pixel intensities of the immunostained osteoclasts were ~2.2 times higher than the background fluorescence of the same osteoclasts in adjacent sections for both age groups. Also shown in Figure 2 are pixel intensities of muscle adjacent to the bone. Levels of fluorescence in muscle are lower than that of unstained (control) osteoclasts, indicating that only weak autofluorescence and non-specific binding of antibody occurred in muscle. Autofluorescence of muscle was expected to be lower than for osteoclasts due to a lower abundance of mitochondria.

Table I shows that most, but not all, osteoclasts expressed detectable levels of PTH/ PTHrP-R, i.e., 7.24% of osteoclasts in young rat samples and 14.29% in old rat samples lacked detectable levels of the receptor.

DISCUSSION

This study shows that PTH/PTHrP-R can be detected by immunostaining in most TRAP-positive osteoclasts in both cytosol and plasma membrane. The occurrence of receptor in the cytosol reflects reserve receptor for cycling into the plasma membrane. The levels of receptor are similar in osteoclasts of both young adult and old rats; however the proportion of unstained osteoclasts was higher in the old rats than in the young (14.29% vs. 7.24%), suggesting that a greater proportion of osteoclasts in older animals were in an unresponsive state. Amplification steps in the immunostaining procedure to detect the PTH/PTHrP-R were

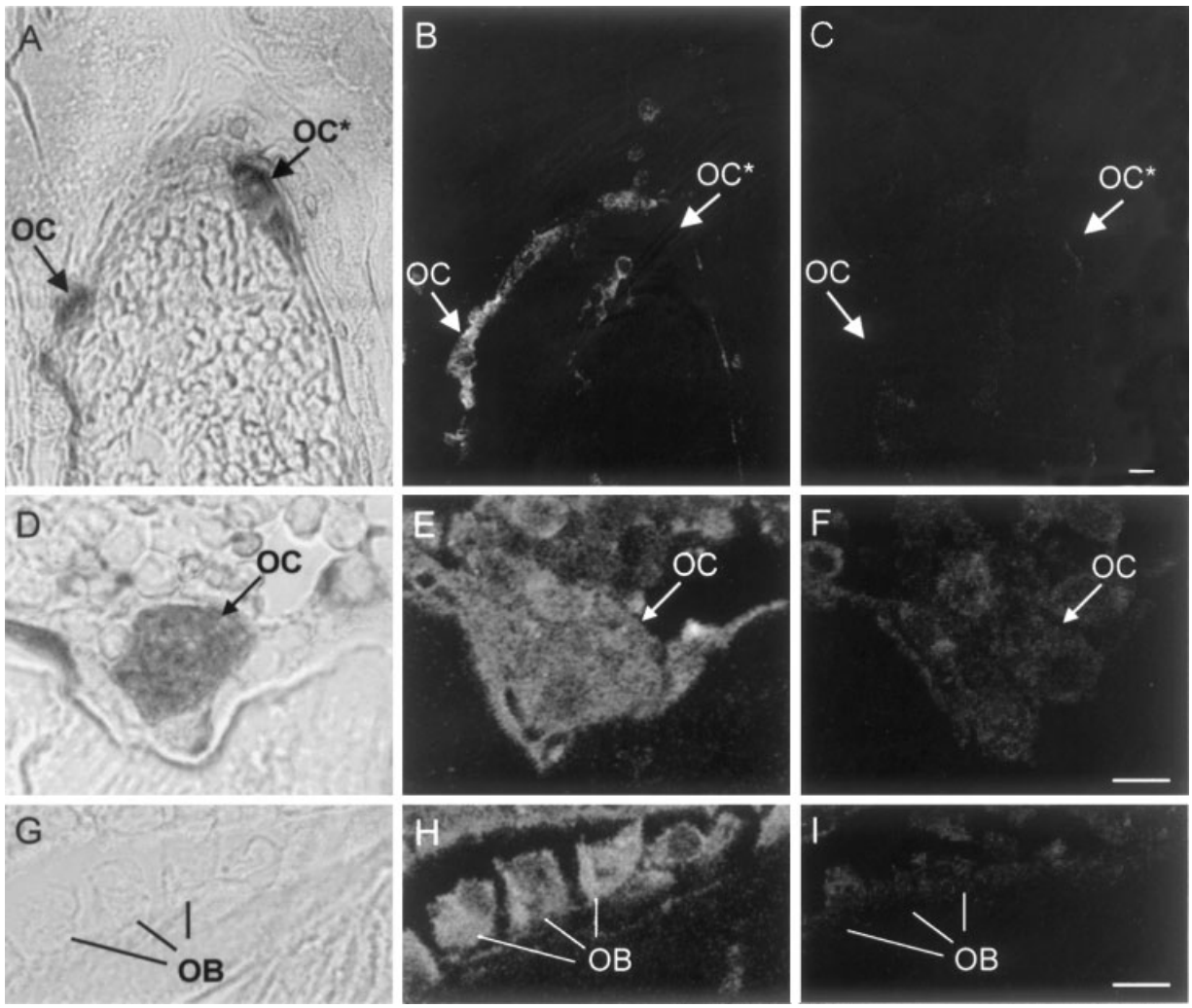

Fig. 1. Representative views in sections of metaphysis of 4-month old rat showing TRAP stained osteoclasts (**A**, **D**). The same osteoclasts are shown in adjacent serial sections stained by PTH/PTHrP-R antibody (**B**, **E**). The osteoclast identified by the asterisk is an example of absence of staining for PTH/PTHrP-R in some osteoclasts (see Table I). Pronounced staining of osteoblasts was also found (**G**, **H**). Controls of primary antibody omission (**C**, **F**, **I**) show the absence of non-specific staining. Magnification bar = 10 μm.

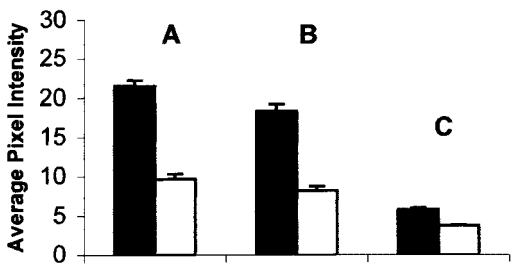

Fig. 2. Average pixel intensities of PTH/PTHrP-R immunostained sections (solid bars) for (**A**) 58 osteoclasts from young rat metaphyses, (**B**) 52 osteoclasts from old rat metaphyses, and (**C**) an equivalent area of muscle. Mean pixel intensities of the same osteoclasts and muscle in adjacent control sections in which primary antibody is omitted are also shown (open bars). Open bars reveal levels of autofluorescence.

needed, indicating that the expression level of PTH/PTHrP-R in osteoclasts is low. The amplification steps included application of a triple antibody protocol and a fluorescent tag, Alexa Fluor, that has 10–20× brighter fluorescence than the more commonly used fluoroprobes. The use of a confocal microscope also enhanced image detection and, additionally, provided pixel intensity comparisons to show that immunostained cells were consistently and substantially more intense than pixel intensities of background fluorescence detected in control sections.

Detection of PTH/PTHrP-R mRNA by in situ hybridization is another definitive way to show that osteoclasts express the receptor. The recent

TABLE I. Number of Osteoclasts Identified by TRAP Staining Versus Number Immunostained for PTHrP Receptor

Age of rat	No. TRAP stained cells	PTHrP-R positive	PTHrP-R negative	Unstained (%)
4 mo. (n = 3)	69	64	5	7.24
15 mo. (n = 3)	77	66	11	14.29

no., number; mo., months.

study by Langub et al. [2001] reported PTH-1 receptor message by in situ hybridization in osteoclasts in sections of iliac crest biopsies. In samples from normal individuals 65.7% of osteoclasts contained PTH-1 receptor mRNA and in patients with secondary hyperparathyroidism 98% of osteoclasts contained message. Interestingly, immunostaining revealed the PTH-1 receptor protein only in osteoclasts from the diseased tissue, suggesting that the expression level of the receptor protein is much less under normal circumstances and perhaps occurs intermittently.

As mentioned in the introduction, several studies indicate that PTH/PTHrP-R is present in osteoclasts. A number of these studies also indicate that the receptors are functional. Mears [1971] reported rapid plasma membrane depolarization (within minutes) in isolated rabbit osteoclasts. Rapid changes in osteoclast morphology occurred following PTH injection into Japanese quail; there was notable development of ruffled border in 20 min [Miller and Kenney, 1985]. When fluorescently-tagged PTH was introduced to cultures of osteoclasts, it was found that PTH bound to cell surfaces was rapidly cleared (in ~20 min); the binding was both specific and saturable [Agarwala and Gay, 1992]. This phenomenon has also been reported for cultured renal cells [Niendorf et al., 1986, 1989]. Rapid clearance of occupied receptors is important for readying the cell for subsequent re-stimulation. Rapid clearance also contributes to the difficulty of localizing the receptor by conventional staining methods. Regulation of PTH receptor internalization has been examined in a human embryonic kidney (HEK-293) cell line [Malecz et al., 1998; Ferrari et al., 1999; Huang et al., 1999]. In a study on isolated rat osteoclasts, PTH stimulated a rapid (within 20 s) superoxide (O_2^-) burst [Datta et al., 1996]. The burst was proportional to the number of osteoclasts, but not to the number of osteoblasts present, indicating a direct PTH effect on osteoclasts. Other studies revealed direct PTH stimulation of osteoclasts caused enhanced acidification [Hunter et al., 1988; Gay et al., 1993; May and Gay, 1997a]. These cell preparations contained greater than 95% osteoclasts and less than 2% osteoblasts. Further, pertussis toxin blocked PTH-stimulated acidification [May and Gay, 1997a], an effect that is transmitted through G_i. The $G_{i\alpha\text{-}3}$ isoform was abundant in osteoclasts and was not detected in osteoblasts [May and Gay, 1997b]. The differences in the G-protein profile in osteoblasts and osteoclasts indicate that the PTH/PTHrP-R signal pathways are distinct in those cell types. These studies indicate that PTH/PTHrP-R occupancy on osteoclasts activate the resorption machinery of the osteoclasts, which culminates in acid secretion. All of this early work utilized full length PTH and so it is possible that the activation was a function of the C-terminus, rather than the N-terminus of the PTH molecule. However, studies utilizing C-terminal portion of PTHrP-(107-111) or PTH-(7-84) found that bone resorption was inhibited [Fenton et al., 1991; Divieti et al., 2002]. It is possible that the N-terminus of PTH or PTHrP is involved in osteoclast activation while the C-terminus has the opposite effect.

How functional PTH/PTHrP-R in osteoclasts is integrated into coupled bone resorption and formation is currently unknown. It is intriguing that PTHrP is secreted by both osteoblasts [Amizuka et al., 1996] and osteoclasts [Kartsogiannis et al., 1998]. PTHrP, being a locally produced peptide, acts as a paracrine/autocrine factor in many tissues [Wysolmerski et al., 2002]. It is interesting to speculate that PTHrP may be part of the coupling mechanism between osteoblasts and osteoclasts, that is, that PTHrP secreted by osteoblasts influences osteoclasts, and conversely. In addition to its N-terminal homology to PTH, through which it can bind to the same receptor, PTHrP has a centrally located nuclear targeting sequence [Henderson et al., 1995]. Thus, in addition to its actions as a paracrine/autocrine factor, PTHrP may also

affect the cell that synthesizes it as an intracrine factor [Massfelder et al., 1997; Aarts et al., 1999], further expanding its possible roles in osteoblast-osteoclast interactions [Nguyen and Karaplis, 1998]. PTHrP is also synthesized by vascular endothelium of a broad range of species [Clemens and Broadus, 2002]; this is relevant because of the extensive blood supply found in the metaphysis.

Osteoclast biology can be conveniently subdivided into two domains, osteoclast development and osteoclast function. Osteoclast development clearly requires the involvement of osteoblasts and their PTH/PTHrP receptors. Osteoclast function may be regulated both indirectly by osteoblasts and directly through their PTH/PTHrP receptors. A substantial number of studies, as outlined above, show that osteoclasts can bind and respond directly to PTH. It would be valuable to learn the stage(s) of the osteoclast life cycle in which expression of functional PTH/PTHrP-R on osteoclast surfaces occurs.

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