Immunohistochemical Localization of Calcitonin Receptor in Mouse Tibiae

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We have raised specific antisera against the extracellular domain of the rat/mouse calcitonin receptor (CTR), consequently immunolocalizing the CTR-positive cells in mouse tibiae. As expected, the immunoreactivity for the CTR was intensely detected in cells identical to tartrate-resistant acid phosphatase (TRAP) positive multinucleated osteoclasts and mononuclear cells, whereas no immunoreactivity was detected in osteoblasts and bone marrow cells. Most osteoclasts on the bone surface possessed the CTR, therefore indicating that bone resorbing osteoclasts could be prompted to respond to endogenous calcitonin. Immuno-electron microscopy revealed CTR-immunoreactivity mainly on the plasma membranes including the pits associated with the cell membranes, and sometimes on intracellular translucent vacuoles and in the vesicles in the vicinity of the Golgi apparatus in the osteoclasts. These results lead to the postulation that CTR is chiefly localized to the cell surface of osteoclast, but is subjected to continuous internalization followed by the receptor-transport to the Golgi apparatus.

Key words: Osteoclast, Calcitonin receptor (CTR), Immunohistochemistry, Immunoelectron microscopy

I. Introduction

A hypocalcemic hormone [2], calcitonin (CT) acts directly to osteoclasts by mediating cell surface-calcitonin receptor (CTR) to inhibit bone resorption [15]. The CTR belongs to the class II subfamily of the 7-transmembrane G-protein-coupled receptors that includes the parathyroid hormone receptor [3]. *In vivo* radioautographic studies [16] demonstrated the CTR on plasma membrane of osteoclasts. Recently, Quinn *et al.* developed polyclonal antibodies specific for the C-terminal intracellular domain of CTR. They have shown that the antibody immunostained predominantly osteoclast cell membranes in histological sections of mouse bone [14]. In contrast to immunohistochemical study, Ikegame *et al.* reported, by electron microscopic radioautography, that silver grains indicative of ^{125}I-elcatonin (eel calcitonin) were localized on plasma membranes of osteoclasts, and which, with time-dependency, were internalized and accumulated in the Golgi apparatus [5]. However, this radioautography technique could not reflect the localization of CTR peptide, and therefore, a high resolution technique using the antibody to the CTR appears to be invaluable for demonstrating the localization and translocation of the CTR. In this study, we developed antisera to the extracellular domain of the rat/mouse CTR, and demonstrated the intracellular localization of the CTR in osteoclasts of mouse tibiae.

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II. Materials and Methods

Antisera were raised in rabbits against the peptide of first extracellular domain of the rat/mouse CTR including 20 amino acids from #113 to #132 (DENGEWFRHPDSNRTWSNYT) in the amino terminal region (Fig. 1). This region was common to C1a and C1b. SDS-PAGE revealed a putative molecular weight (17 kd) corresponding to the amino terminal peptide of CTR expressed in E. coli (Fig. 2).

For histochemistry, four-week-old male BALB/C mice were used. The animals were anesthetized with Nembutal and perfused through the left venticle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min. The

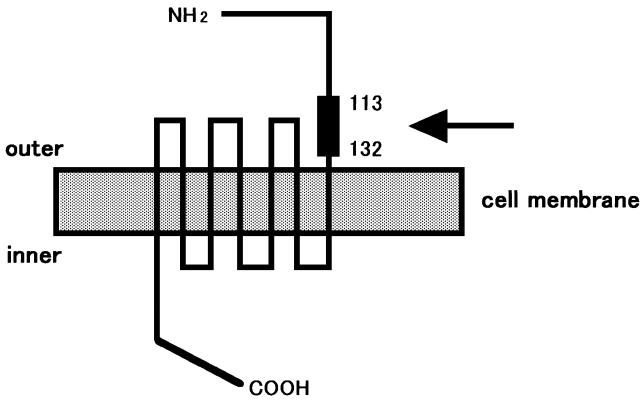

Fig. 1. Diagrammatic representation of the rat calcitonin receptor. The position of synthetic peptides (DENGEWFRHPDSNRTWSNYT) used as antigens is indicated by an arrow.

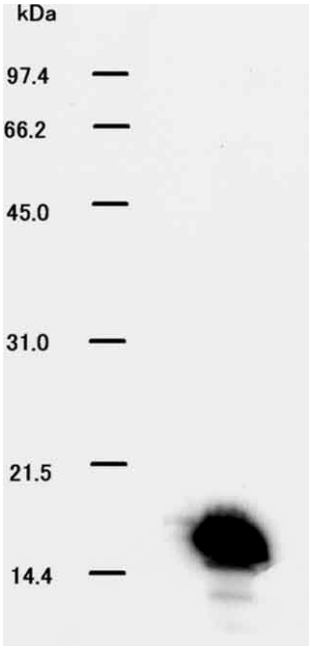

Fig. 2. Western blot analysis showing that the antisera raised for the CTR recognized the amino terminal peptide (17 kd) of CTR expressed in E. coli.

tibiae were dissected, immersed in the same fixative for 2 hr at 4°C and decalcified in 4.13% EDTA (pH 7.4) for 5 days at 4°C. Specimens were immersed in phosphate-buffered saline (PBS) which contained sucrose in gradually strengthening concentrations of 10, 20, and 25%. They were then rapidly frozen by immersion in liquid nitrogen. Frozen 10-μm sections were obtained by Microtome (Damon, Needhan, Mass., USA). Other specimens were dehydrated with an increasing concentration of ethanol and were embedded in paraffin. Eight μm-thick sections were obtained using a sliding microtome.

For immunolocalization of CTR, to inhibit endogenous peroxidase, first both paraffin and frozen sections were treated with PBS containing 0.6 % hydrogen peroxide for 1 hr. After washing with PBS, unlike frozen sections, paraffin sections in 10 mM citrate buffer (pH 6.0) were twice illuminated with microwaves for 5 min. The sections were carefully handled so as not to increase the temperature. The frozen and parafin sections were preincubated in 1% bovine serum albumin in PBS, for 30 min at room temperature in order to prevent non-specific binding, and then incubated with rabbit anti-rat/mouse CTR antibody diluted to 1:50 for 3 hr at room temperature. After washing with PBS, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cappel, Durhan, NC, UK) diluted to 1:100 for 1 hr at room temperature. Following rinsing with PBS, they were immersed in DAB-H_2O_2 solution (0.05% Diaminobenzidine (Wako Pure Chemical Co., Osaka, Japan) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6) for 10 min, at room temperature, and examined under light microscopy after counterstaining with methyl green.

For immunoelectron microscopic observation, after incubation with secondary antibody described above, the frozen sections were refixed with 2.5% glutaraldehyde in PBS. Following rinsing with PBS, they were immersed in DAB-H_2O_2 solution for 10 min, at room temperature, and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr at 4°C. They were dehydrated in a graded ethanol series and embedded in Poly/Bed 812 (Polyscience, Warrington, PA). Ultrathin sections were obtained by Porter-Blum MT-1, and then mounted on copper grids. They were stained with lead citrate, prior to observation under JEM-100CXII electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

Negative control sections were incubated with normal rabbit serum in place of the primary antibody.

For detection of tartrate-resistant acid phosphatase (TRAP), some frozen specimens were incubated with a mixture of 5 mg naphtol AS-BI phosphate (Sigma, St. Louis, MO) as a substrate and 18 mg of fast red violet LB salt (Sigma) diluted in 30 ml 0.1 M acetate buffer (pH 5.2) containing 0.5 mmol/L tartrate (pH adjusted to 5.2 by 1N HCl) at room temperature for 10 min. The sections were observed by light microscopy after faintly counterstained with methyl green.

III. Results

The frozen sections of the metaphysis of the mouse tibia displayed intense CTR-immunopositivities around the termini of the trabecular bones at the junction between cartilage and bone, referred to as the "erosion zone" (Fig. 3). Employing the serial frozen sections, TRAP-positive osteoclasts exhibited similar localization to the CTR-immunopositive cells. When observed at a higher magnification, the CTR-immunostaining was apparently identical to multinucleated osteoclasts that were located on the bone surface (Fig. 4). There were only a few osteoclasts which displayed TRAP activity without the CTR-immunoreactivity. Mononuclear CTR-immunoreactive cells apart from bone surfaces

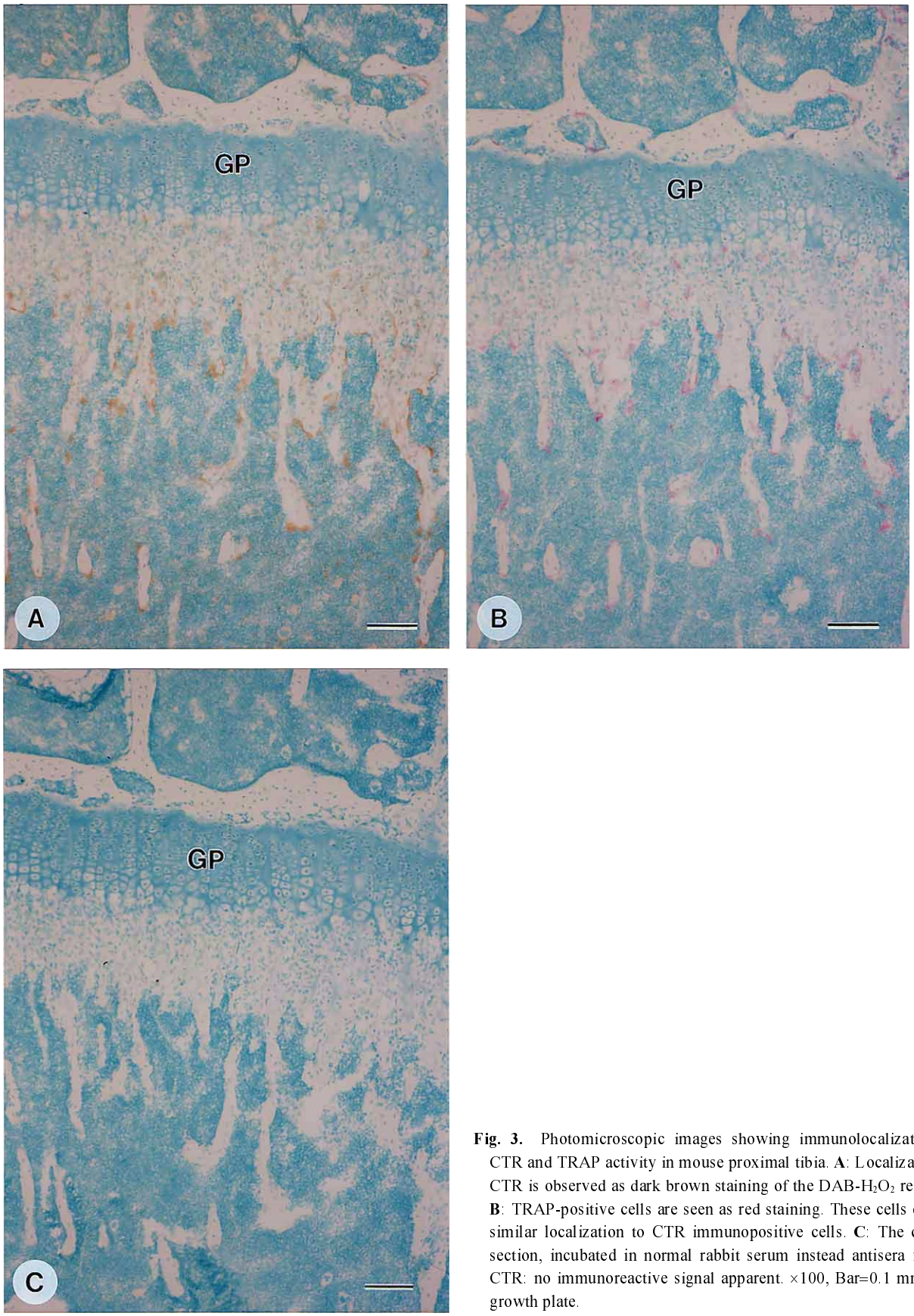

Fig. 3. Photomicroscopic images showing immunolocalization of CTR and TRAP activity in mouse proximal tibia. **A**: Localization of CTR is observed as dark brown staining of the DAB-H_2O_2 reaction. **B**: TRAP-positive cells are seen as red staining. These cells exhibit similar localization to CTR immunopositive cells. **C**: The control section, incubated in normal rabbit serum instead antisera for the CTR: no immunoreactive signal apparent. ×100, Bar=0.1 mm. GP: growth plate.

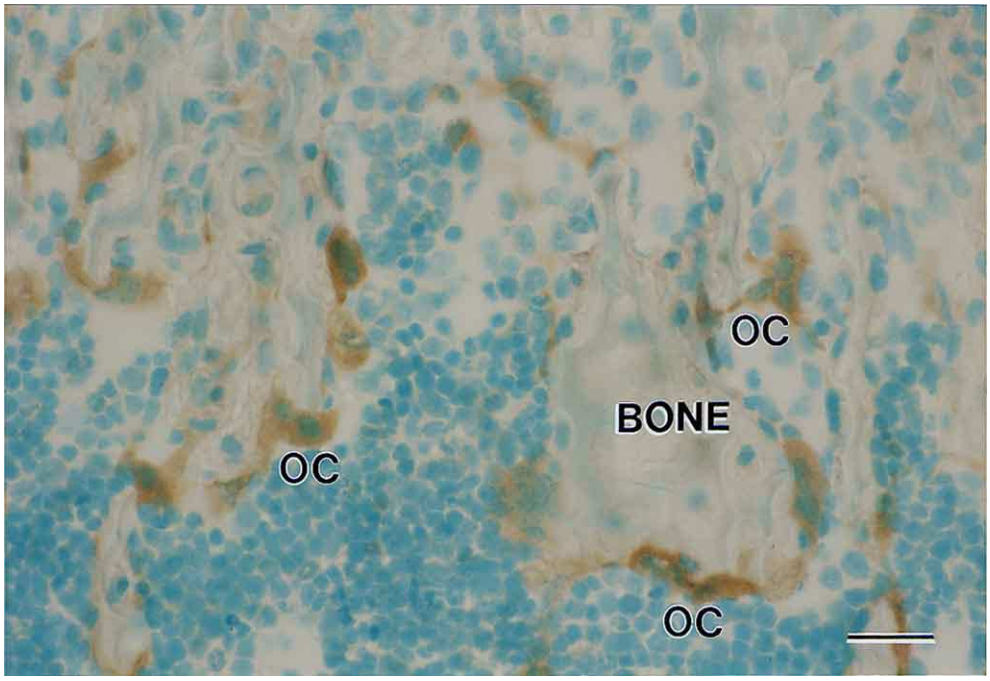

Fig. 4

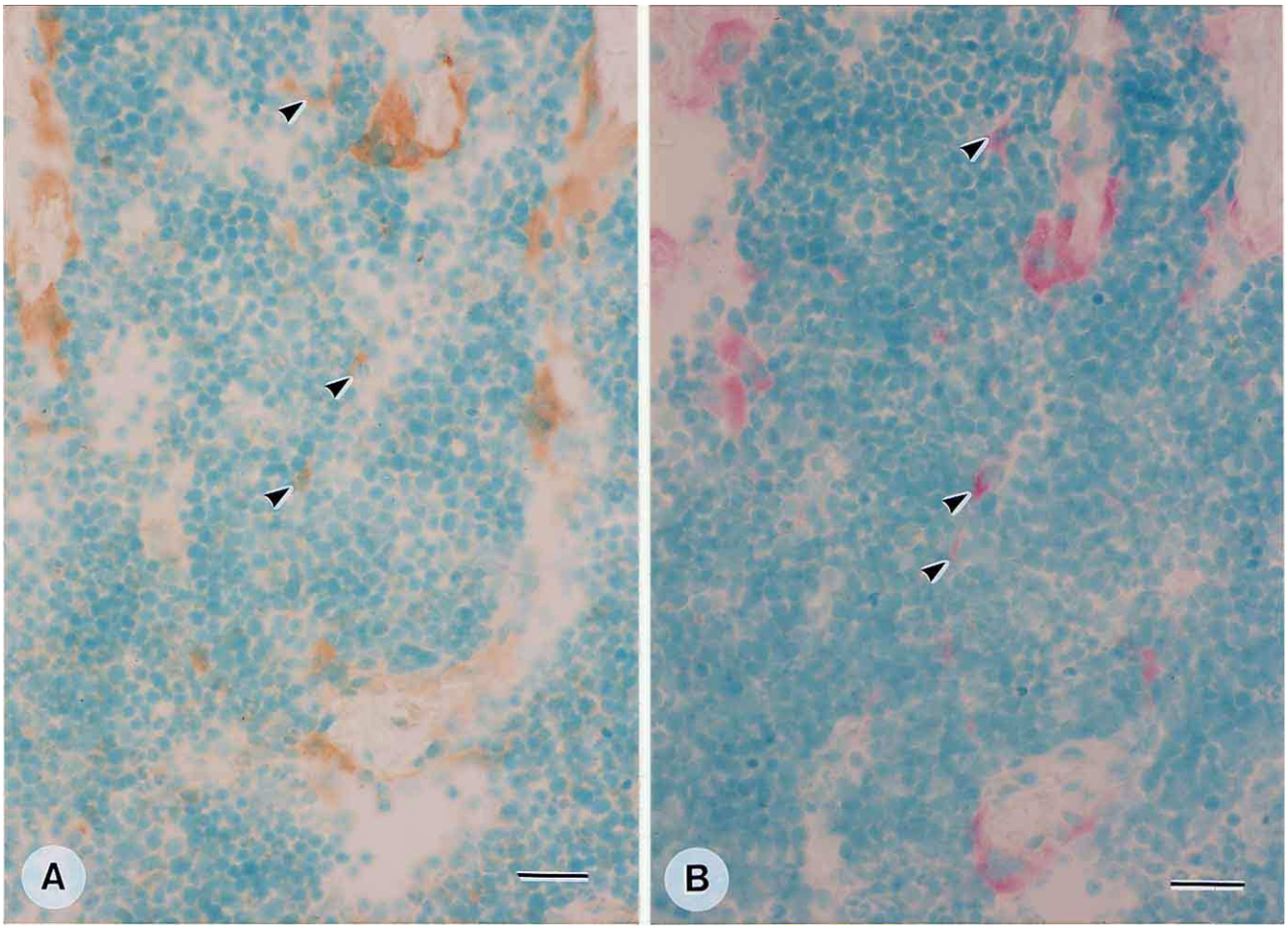

Fig. 5

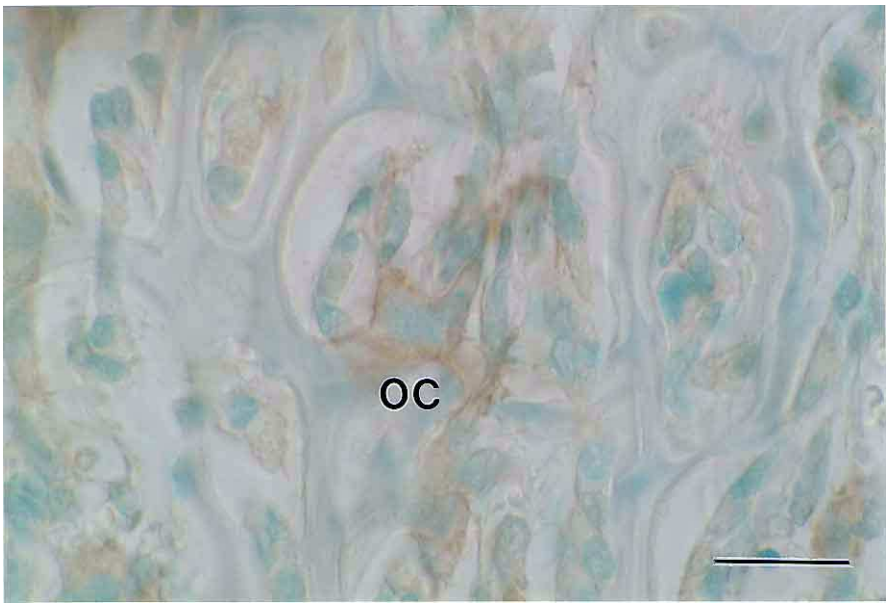

Fig. 6. Photomicroscopic images showing immunolocalization of CTR in osteoclast of a microwave treated section. A osteoclast (OC) shows intense immunoreactivity for CTR on the cell periphery demonstrating the existence of plasma membrane. ×750, Bar=20 μm.

hyp ocal e mi arn[2] 2[, at(2CTa) sTadbanyb[gr [a[ya[ho oaugyfob on[2yb -aRrgruu2ba on[2yb a[gor[ocap 2[hal 2ngyp r] o ac2caby[hyp a [gybCa2l 1 5bygorn[2] 2[, ar al 5nhar a[hoaugyfoba on. [2yb ac2c-ahyp o] og-a[hoay [oynIr [ao7h2G2[oca2l 1 5bygorn. [2] 2[, auygaQl e a1 r2bI, ayba[ho2ganoIIa1 o1 Cgrbo at(2CTa6sT v bI2woay [oynIr [arbcaRgoy [oynIr [2nanoII -ay [oyCIr [c2caby[ago] orIaQl e .2l 1 5bygorn[2] 2[, a2baGy[haRrgruu2barbc ugyfoba on[2yb at4ooa(2C Ta) -a6sT

k Ion[gybal 2ngy nyR2nao7r1 2br[2ybago] orIoca[hoa2l 1 5. bygorn[2] 2[, a2bc2nr[2] oayua[hoaQl e a1 r2bI, aybanoIIa 5gurno 1 o1 Cgrbo ayua[hoay [oynIr [at(2CTaAms-arbca y1 o[2l o ayb [hoaRIr 1 ral o1 Cgrbo ayua[hoaR2[auygl ocayba[hoanoIIal o1 . Cgrbo Ta4y1 oayua2b[grnoII5Irga[grb I5nob[a] rn5yIo ap ogo Ry 2[2] oI, a2l 1 5by [r2boca G, a Ql e arb[2Gyc, at(2CTa Az sT db[ogo [2bCI, -a y1 oa] o 2nIo a2ba[hoa] 2n2b2[, ayua[hoaHyIC2 rRRrgr[5 ac2ca hyp a2l 1 5byIynrI2fr[2ybayuaQl e ayba[ho2g RIr 1 ral o1 Cgrbo at(2CTaAQsT

dba[hoanyb[gyIa on[2yb a2bn5Gr[oca2babygl rIagrGG2[a og51 2b [orcarb[2 ograuyga[hoaQl e -a2l 1 5bygorn[2] 2[, any5Icaby[aGo yG og] ocat(2CTa3QsT

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l onhb2nrII, -a1 2ngyp r] o. 2II51 2br[2ybaybaRrgruu2ba on. [2yb any5Icago] orIagoIo] rb[a2l 1 5byIynrI2fr[2ybayuaQl e -a*l ie i-*

Fig. 4. Photomicroscopic images showing immunolocalization of CTR in a trabecular bone. Multinucleated osteoclasts (OC) associated with bone matrix (BONE) show immunoreactivity for CTR. ×400, Bar=30 μm.

Fig. 5. Photomicroscopic images showing immunolocalization of CTR and TRAP activity apart from bone surface. **A**: Mononuclear cells (arrowheads) apart from bone surfaces show immunoreactivity for CTR. **B**: These cells (arrowheads) also possess TRAP activity. ×400, Bar= 30 μm.

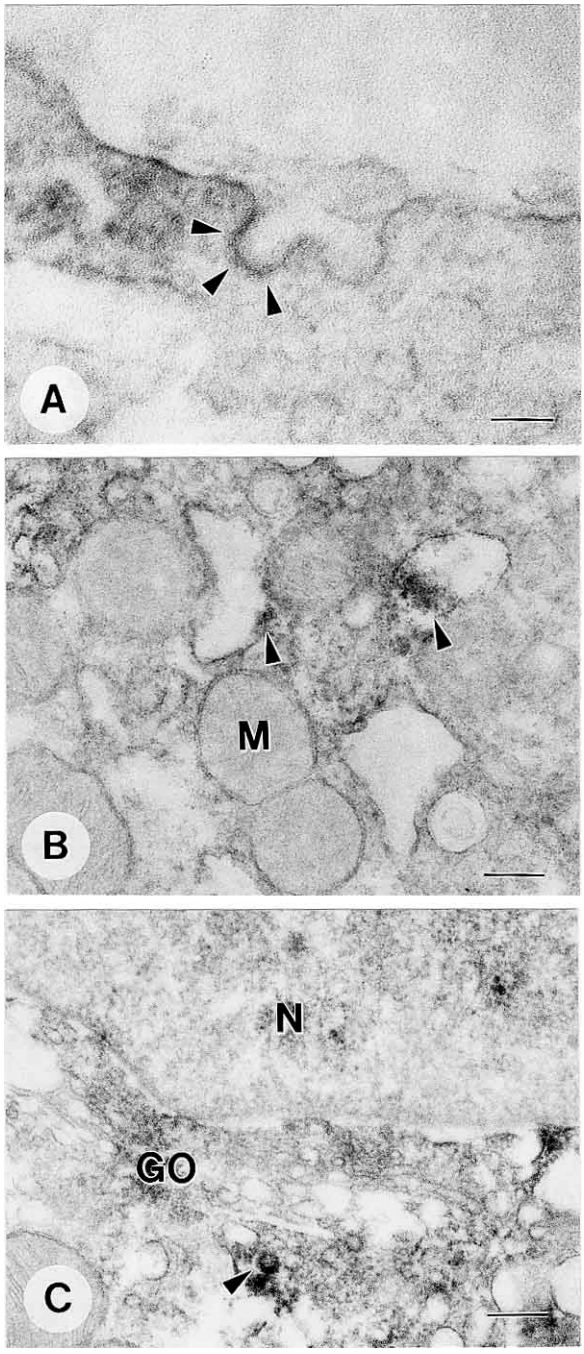

Fig. 7. Electronmicroscopic images showing the immunolocalization of CTR in osteoclasts. **A**: Immunoreactivity for CTR is seen on plasma membranes, especially on the pit formation area (arrowheads). **B**: Intracellular membranous structures (arrowheads) show immunolocalization of CTR. **C**: Some vesicles (arrowhead) near the Golgi apparatus (GO) are positively immunostained by CTR antibody. ×80000 (**A**), ×40000 (**B, C**). Bars=0.1 μm (**A**), 0.2 μm (**B, C**). M, mitchondria; N, nucleus.

[hoaQl e .2l l 5bygorn[2] 2[, aybа[hoanoIIa 5gurnoayuay [oynIr [-Ry 2GI, aG, agoc5n2bCagoc5bcrb[aG2bc2bCayua[hoarb[2 ogrT i rgruu2ba on[2yb a p 2[hy5[a l 2ngyp r] o.[gor[1 ob[a hyp oc hy1 yCoby5 a2l l 5bygorn[2] 2[, a2ba[hoaob[2goan, [yRIr 1 ayu y [oynIr [-arbca[hoa2l l 5bygorn[2] 2[, aybaugyfoba on[2yb ac2c by[ac2uogap 2[harbcap 2[hy5[a l 2ngyp r] oa2Il51 2br[2ybatcr[r by[a hyp bsTal hogouygo-al 2ngyp r] o.[gor[1 ob[al r, aGoa5 ou5I uygagoc5n2bCa[hoabyb. Ron2u2naG2bc2bCayuarb[2Gyc2o Tam ap o hr] oaRgo] 2y5 I, agoRyg[oc-a5 2bCal 2ngyp r] o ap r arbaouu2n2bb[1 o[hycayuaobhrbn2bCaconr In2u2nr[2ybaG, akDl maxA0-a ya[hr[l 2ngyp r] oa l r, aGoaRh, 2nr II, aouu2n2bb[auyga] rg2y5 a[onh. b2q5o a 2bnI5c2bCa uZ7r[2yb-a conr In2u2nr[2yba rbca 2l l 5by. gorn[2ybT

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