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Features of the clear zone of odontoclasts in the Chinook salmon (*Oncorhynchus tshawytscha*)

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Keywords: Odontoclast, Clear zone, Actin,

Immunohistochemistry, Chinook salmon

Abstract

This study aims to clarify the features of the clear zone of odontoclasts on shedding teeth of a teleost fish, Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), using a light microscope to determine the orientation between a cell body and a resorptive lacuna, followed by transmission electron microscopy (TEM). Ultrathin sections of LR White embedded material were incubated in rabbit anti-actin polyclonal antibody and then were incubated with 15 nm gold-conjugated goat anti-rabbit IgG. The clear zones of odontoclasts showed a variable structure with electron dense structures on sections, but distinct clear zones were not always seen on odontoclasts. In odontoclasts sectioned in the direction perpendicularly to the surface of a resorptive lacuna, some cells showed a wide clear zone, but two types of clear zones were usually observed: a part composed of some cytoplasmic processes and one composed of several complicatedly interwoven processes. Gold particles were localized on the clear zones, especially in electron dense structures; very few gold particles were detected in ruffled borders. These results show that the clear zone of odontoclasts in Chinook salmon contains actin. Our results suggest that the clear zone of an odontoclast in Chinook salmon is not always a wide annular structure.

Introduction

An active osteoclast resorbing bone or odontoclast resorbing teeth is characterized by the ruffled border and the wide annular zone encircling it, known as the clear zone on the basis of the paucity of cellular organelles as observed by transmission electron microscopy (TEM) (Scott and Pease, 1956; Schenk et al., 1967; Holtrop et al., 1974; Marks and Popoff, 1988; Pierce et al., 1991). Although termed the clear zone, electron dense striations are often observed (Malkani et al., 1973) and it is known that the clear zone contains actin-filaments because the electron dense striations bind to heavy meromyosin (King and Holtrop, 1975). By fluorescent microscopy (FM), annular structures with F-actin were immunocytochemically observed in the peripheral area of cultured osteoclasts (Turksen et al., 1988; Zambonin-Zallone et al., 1988; Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1991). Yoshida et al. (1989) observed immunocytochemically annular structures with F-actin in cultured osteoclasts by FM and then examined the same structures again by TEM, and reported that an annular structure of osteoclasts observed by FM was consistent with the TEM clear zone.

It is well known that the annular structures of cultured osteoclasts observed by FM contain proteins: vinculin, α-actinin, and talin, which are involved in the regulation of actin, and they associate with the membrane in adhesive structures such as focal contacts and focal adhesions (Aubin, 1992; Väänänen and Horton, 1995; Akisaka et al., 2001). As the cell membranes in this region come into close proximity to the bone surfaces (Holtrop et al., 1974; Holtrop and King, 1977), it has been accepted that the clear zone of an osteoclast or odontoclast has the function of sealing off the ruffled border compartment and forming a diffusion barrier between the resorptive lacuna and extracellular fluid (Marks and Popoff, 1988; Väänänen and Horton, 1995).

Osteoclasts are found in teleost fish, and the appearance of many odontoclasts associated with tooth replacement is well known. Some investigators examined TEM features of teleost osteoclasts and reported that they were similar to those of mammalian osteoclasts showing ruffled border and clear zone (Sire et al., 1990; Huysseune and Sire, 1992, 1998); however there are few TEM observations of the clear zone of teleost odontoclasts (Van der heyden et al., 2000). There is no report of immunolocalization of actin in the clear zone of teleost osteoclasts or odontoclasts. Therefore, the features of the clear zone in teleost odontoclasts remain unclear. The present study aims to examine the ultrastructure of the clear zone and the immunolocalization of actin in odontoclasts on shedding teeth of a teleost fish, Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and determine the features of the clear zone observed there.

Materials and Methods

All animal experimentation followed the Guide for the Care and Use of Laboratory Animals, Hokkaido University Graduate School of Dental Medicine, which is based on the Guide for the Care and Use of Laboratory Animals (NIH, 1985).

Preparation for light microscopy (LM), TEM, and immunohistochemistry

Four Chinook salmon which were reared in fresh water at 10°C and were 60–62 mm standard length (SL) and of about 6 months of age, were used in this study. Because the animals did not reach sexual maturity, we did not check gender. Prior to decapitation, the animals were subjected to an overdose of MS222 (SIGMA, St. Louis, MO). After decapitation, the maxillae and mandibles were immediately fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) overnight at 4 °C for TEM observations, and with 4% formaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) overnight at 4 °C for immunohistochemistry. Specimens were decalcified with 5% EDTA (pH 7.4) for two weeks at 4 °C, and samples from each fish were used for TEM and immunohistochemical observations.

LM and TEM

Specimens were postfixed with 1% osmium tetroxide in 0.05M sodium cacodylate buffer (pH 7.4) for 3 hr at room temperature. They were block-stained with 4% uranyl acetate for 30 min, dehydrated in a graded series of ethanol, and then embedded in Epon 812. The specimens were sectioned in the frontal direction with diamond knives on a Reichert-Nissei ultramicrotome into 0.5-μm semithin sections. The semithin sections were stained with toluidine blue, and observed with a LM

(NIKON FX-A, Japan) at a magnification of x125 under oil-immersion. The specimens were then sectioned with diamond knives on an ultramicrotome into 0.08-μm ultrathin sections. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a TEM (HITACHI H-7000) at an operating voltage of 75kV.

Immunohistochemistry

Specimens were dehydrated in a graded series of ethanol at 4 °C, and then embedded in LR White (London Resin Company Ltd, UK). Polymerization was performed under ultraviolet irradiation for 24 h at 4 °C. The specimens were sectioned in the frontal direction with diamond knives on a Reichert-Nissei ultramicrotome into 0.5-μm semithin sections. The semithin sections were stained with toluidine blue, and observed by LM. After observing the orientation between a cell body of an odontoclast and a resorptive lacuna, the specimens were sectioned in the frontal direction with diamond knives on an ultramicrotome into 0.1-μm ultrathin sections, and mounted on nickel grids. Sections were immersed in 0.2M Tris-buffered saline containing 0.5% bovine serum albumin (BSA) for 5 min, incubated in rabbit anti-actin polyclonal antibody (Biomedical Technologies Inc, USA) diluted 1:100 for 12 h at 4 °C, and finally incubated with 15 nm gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, UK) diluted 1:25 for 40 min at room temperature. They were then washed with phosphate-buffered saline (PBS), distilled water, and air-dried, then stained with uranyl acetate and lead citrate and examined with a TEM. Control sections were incubated with normal rabbit IgG or without a primary antibody.

The determination of the orientation between a cell body and a resorptive lacuna

On ultrathin sections, the distinction between a clear zone and a ruffled border was determined based on the features of them in previous reports (Holtrop et al., 1974; Holtrop and King, 1977). In either side of a ruffled border, when the cell membrane of cell bodies or cytoplasmic processes came into close proximity to the dentine surfaces, the region of cell body or cytoplasmic processes was identified as a clear zone.

The clear zone of an osteoclast completely encircles the ruffled border facing a resorptive lacuna, and on the side facing resorptive lacunae, ruffled borders, vacuoles, and nuclei are usually observed in sequence from the periphery towards the center of an osteoclast (Holtrop et al., 1974; Holtrop and King, 1977). According to these features, the orientation of cell was determined on semithin sections as follows; a cell which had brush border, vacuoles, and nuclei, was classified as a cell where the cell body was sectioned in the direction perpendicularly to the surface of a resorptive lacuna. A cell which had nuclei and vacuoles but most of the cell body was encircled by the dentine, was classified as a cell where the cell body was sectioned in the direction parallel to the surface of a resorptive lacuna. A cell which had brush border and vacuoles but no nuclei was classified as a cell where the cell body was sectioned in the direction obliquely to the surface of a resorptive lacuna.

Results

LM and TEM

On semithin sections, odontoclasts were multinucleated giant cells, and they were observed in the dental pulp on the inner surfaces of shedding teeth and on pedicels. On semithin sections, odontoclasts could be observed sectioned obliquely to the surface of a resorptive lanuna. On ultrathin sections, these odontoclasts had ruffled borders toward the resorptive lacunae on the dentine surfaces. However, on either lateral side of the ruffled borders, distinct clear zones were not always seen on the cells. Many cytoplasmic processes were observed there and the cell membrane of several processes came into close proximity to the dentine surfaces. Electron dense structures were seen in some of these cytoplasmic processes (Fig. 1).

On the dentine surface, there were some odontoclasts which had nuclei without brush borders on semithin sections. On ultrathin sections, the cells showed clear zones only opposite to the dentine surfaces (data not shown).

There were odontoclasts which had a brush border, vacuoles, and nuclei in sequence on resorptive lacuna on semithin sections (Figs. 2, 3, 5, and 6). These cells were regarded as cells sectioned in the direction perpendicular to the surface of a resorptive lacuna. On ultrathin sections, some odontoclasts showed clear zones with wide width on the lateral side of the ruffled borders. Figures 2 and 3 show the usual images of clear zones of odontoclasts. Based on the distinction between a clear zone and a ruffled border, two types of clear zones were observed: a clear zone composed of some cytoplasmic processes (Fig. 2) and one composed of several processes complicatedly interwoven with each other (Fig. 3). On the border between clear zones and ruffled borders, there were some cytoplasmic processes which could not be precisely assigned to either the clear zone or the ruffled border. The processes of the

clear zone contained electron dense structures, and these structures were observed both in the processes which were near and/or far from the dentine surface. The cell membrane of the clear zones came into close proximity to the dentine surfaces, and these clear zones appeared free of cellular organelles.

Figure 4 shows clear zones of these odontoclasts on an ultrathin section. The clear zones appeared free of cellular organelles, but the electron density was irregular and electron dense structures were present. Complicated infoldings of the cell membranes were often seen in the clear zone. The electron dense structures showed dot-, patch-like, linear, or mesh shapes. There were filament-like structures in these electron dense structures.

Immunohistochemistry

Figures 5 and 6 show clear zones of odontoclasts sectioned in the direction perpendicular to the surface of a resorptive lacuna. Gold particles (gold-labeled actin) were detected on the clear zones composed of some cytoplasmic processes (Fig. 5) and on wide clear zones (Fig. 6). Gold particles were often localized in the area of clear zones in the proximity of a resorptive lacuna. Very few gold particles were detected in the processes of the ruffled borders (Fig. 5). Figure 7 shows a clear zone of an odontoclast sectioned in the direction parallel to the surface of a resorptive lacuna. Gold particles were detected throughout the clear zones, but the gold particles were localized predominantly in the electron dense structures and the density of particles here was larger than that in the clear zone outside the electron dense structures.

Discussion

Holtrop et al. (1974) examined TEM features of clear zones of osteoclasts, and observed that every section with a ruffled border always had wide clear zones on either side and that some osteoclasts showed clear zones only opposite to the bone surfaces. According to these findings, Holtrop et al. (1974) proposed that the clear zone of an osteoclast completely encircles the ruffled border. The same TEM features of clear zones have been observed in osteoclasts or odontoclasts of various species: mammalian osteoclasts (Schenk et al., 1967; Marks and Popoff, 1988), teleost osteoclasts (Sire et al., 1990; Huysseune and Sire, 1992), frog odontoclasts (Yaeger and Kraucunas, 1969), kitten odontoclasts (Ten Cate and Anderson, 1986), and human odontoclasts (Furseth, 1968). The present study showed that some odontoclasts showed clear zones only opposite to the dentine surfaces, but that there were not always distinct clear zones on either side of ruffled borders of odontoclasts in Chinook salmon. This result indicates that the clear zone of an odontoclast in Chinook salmon may not be a wide annular structure. When interpreting the structure, the orientation of the cell body with respect to the resorptive lacuna should be taken into consideration.

In the present results, odontoclasts, which were sectioned in the direction perpendicular to the surface of a resorptive lacuna, usually showed two types of clear zones; a clear zone composed of wide cytoplasmic processes and one composed of several complicatedly interwoven processes. There were some odontoclasts with wide clear zones. In odontoclasts sectioned in the direction parallel to the surface of a resorptive lacuna, there were complicated infoldings of the cell membranes in the clear zones. These findings mean that a clear zone of an odontoclast in Chinook salmon is composed of two parts: a part composed of a wide cytoplasmic process or some processes and a part composed of several complicatedly interwoven processes.

It is reported that the anti-actin polyclonal antibody binds to both F-actin (filamentous) and G-actin (nonfilamentous or unpolymerized) in cultured osteoclasts (Akisaka et al., 2001). Using anti-actin polyclonal antibody, the present study showed the immunohistochemical localization of actin in odontoclasts in a teleost fish, Chinook salmon, and indicated that many gold particles (gold-labeled actin) were localized in the clear zones. This shows that the clear zone of odontoclasts in Chinook salmon as well as that in mammalian osteoclasts contains actin. The present study furthermore showed that the density of gold particles in the clear zones was larger within than outside the electron dense structures. This indicates that the electron dense structures are rich in actin, and suggests that the filament-like structures in the electron dense structures in clear zones in this study are microfilaments. As it is well known that exposed epitopes of antibody and many other macromolecules can be attached to colloidal gold on the section surface, there is a possibility that other proteins are present in the electron dense structures of the clear zone. It is reported that the annular structures of cultured osteoclasts contain actin, vinculin, α-actinin, and talin (Väänänen and Horton, 1995). Therefore, it may be that gold particles in electron dense structures of a clear zone of an odontoclast in Chinook salmon are attached to such proteins, however this can not be proven here as in this study we detected immunohistochemically only the localization of actin.

In this study the cell membrane of the part composed of a wide cytoplasmic process or some processes in a clear zone came into close proximity to the dentine surface. This TEM finding is similar to that of a clear zone in mammalian osteoclast (Holtrop et al., 1974; Holtrop and King, 1977). The present study also showed the part composed of several complicatedly interwoven processes in a clear zone. This arrangement indicates that this part of the clear zones faces the dentine surface not at

“areas” but at “points”, and suggests a possibility that sealing off the ruffled border compartment in this part may be incomplete, different from parts of the clear zone composed of a wide cytoplasmic process or some processes. Actin possibly plays a role in sealing off the ruffled border compartment tighter.

In the present results, on the border between clear zones and ruffled borders there were some cytoplasmic processes which could not be precisely assigned to a clear zone or a ruffled border. This finding may suggest that a frequent transformation between clear zone and ruffled border occurs on either lateral side of a ruffled border in an odontoclast of Chinook salmon as speculated by Malkani et al. (1973), who did not study Chinook salmon but examined guinea-pig.

Väänänen and Horton (1995) have reported a change of organization of the cytoskeleton of osteoclasts during the resorption cycle as follows: in the first stages of the resorption cycle, actin is distributed throughout podosomes, and towards the resorption stage these podosomes coalesce to the specific area, and then the accumulation of actin forms a dense continuous band around the future resorptive area and this corresponds to the clear zone. The present study showed that the part composed of several complicatedly interwoven processes in a clear zone faced the dentine surface at “points”. Accordingly, this part may be interpreted as an aggregation of cytoplasmic processes toward the resorption stage and may show the structure of a clear zone before forming an active ruffled border.

In teleost fish, the characteristics of osteoclasts are reported in detail (Witten et al., 2000, 2001; Witten and Hall, 2003), and there are some reports which refer to clear zone of osteoclasts (Sire et al., 1990; Huysseune and Sire, 1992). It may be that the differences between our observations and literature reports in the structure of the clear zones are due to differences between odontoclasts resorbing teeth and osteoclasts

resorbing bone, however, this can not be substantiated as there is little data. The characteristics of clear zones of odontoclasts and osteoclasts in teleost fish remain unclear. To clarify it, further study will be needed.

Acknowledgements

We wish to thank Mr. N. Satoh and Ms. N. Aruga, Sapporo Salmon Museum (Sapporo, Japan), for providing Chinook salmon. We are also grateful to Mr. Y. Honma and Dr. Y. Nodasaka, Hokkaido University Graduate School of Dental Medicine, Department of Oral Health Science, for their technical assistance; and to C.M.T. van de Sande-Rijkers, Laboratory of Molecular Biology, Leiden University Medical Centre, the Netherlands, for stimulating suggestions to this study. This study was supported by grants from the Japanese Ministry of Education, Science, Sports, Culture and Technology (Grant numbers: 19390548, 15659432, 16591819), and by a grant from the Ministry of Education, Science, Sports, Culture and Technology to promote multidisciplinary research projects (2003).

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Legends

Abbreviations: CZ: clear zone; D: dentine; Nu: nucleus; RB: ruffled border; V: vacuole

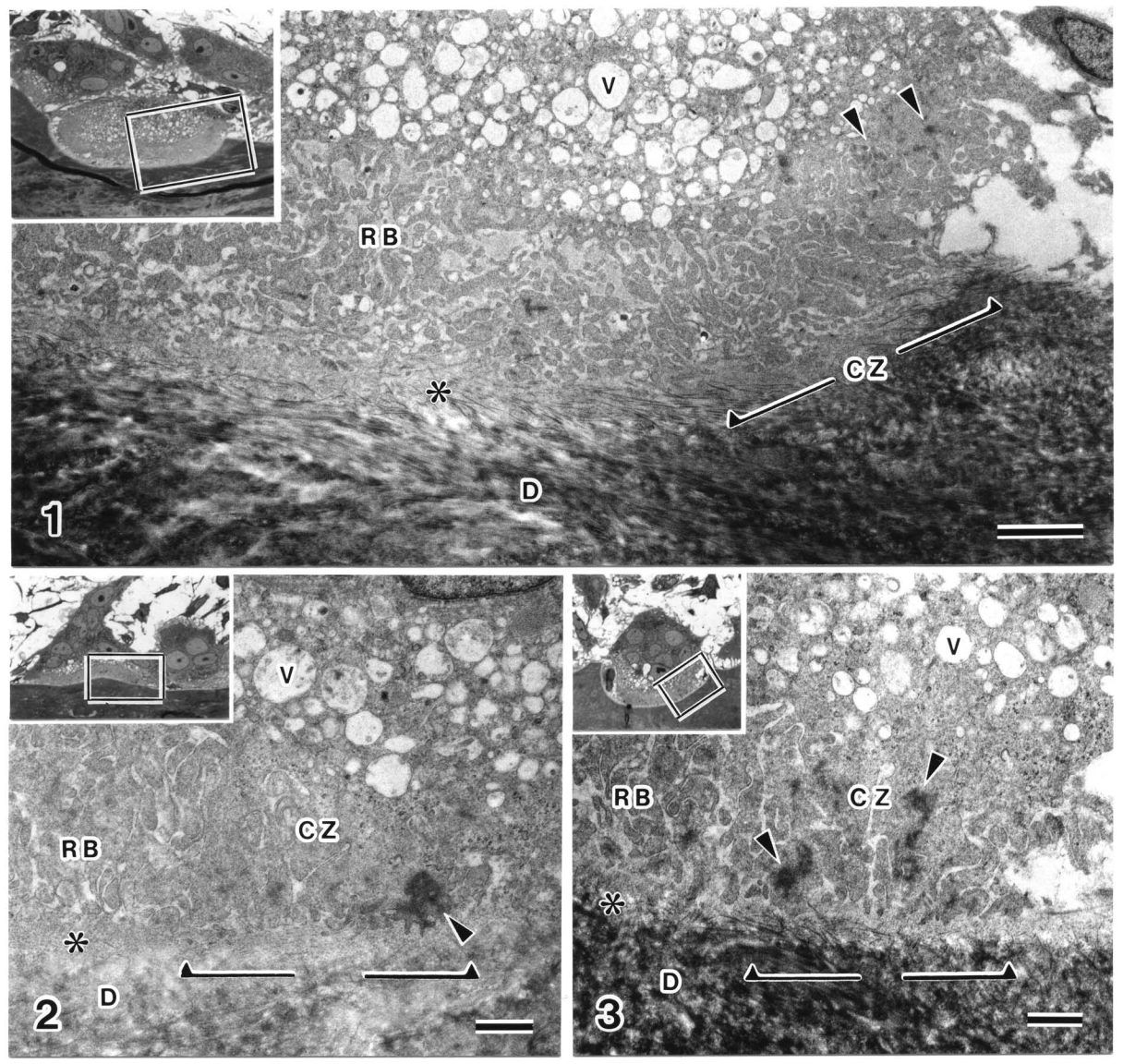
Fig. 1. Electron micrograph showing an odontoclast sectioned in a direction obliquely to the surface of a resorptive lacuna (asterisk). On either lateral side of a ruffled border, a distinct clear zone (brackets) is not seen on the cells, and complicated interwoven cytoplasmic processes are observed here. Electron dense structures (arrowheads) are seen in the cytoplasmic processes. Insert: boxed area is enlarged on Figure 1. x 4,200. bar = 3 μm, Insert: x 200.

Figs. 2 and 3 Electron micrographs showing odontoclasts sectioned in a direction perpendicularly to the surface of a resorptive lacuna (asterisks). (2) An odontoclast with a clear zone (brackets) composed of some cytoplasmic processes encircling the ruffled border. Electron dense structures (arrowhead) are present here. Insert: boxed area is enlarged on Figure 2. (3) An odontoclast with a clear zone composed of several complicatedly interwoven cytoplasmic processes. Electron dense structures are present in these processes. Insert: boxed area is enlarged on Figure 3. x 8,000, bar = 1 μm, Insert: x 180.

Fig. 4. Electron micrographs showing clear zones of two adjacent odontoclasts (Ocl 1, Ocl 2) sectioned in a direction parallel to the surface of a resorptive lacuna. (a) The clear zones appear free of cellular organelles, but their electron density is irregular and electron dense structures (arrowheads) are present. The boxed area 4b and 4c are enlarged on Figures 4b and 4c, respectively. Insert: boxed area is enlarged on Figure 4a. (b) The electron dense structures are seen as patch-like, linear, or mesh-like structures.

Complicated infoldings of the cell membrane are seen in the clear zone (arrows). (c) There are filament-like structures (small arrows) in the electron dense structures. (a): x 6100, bar = 2 μm, Insert: x 200, (b): x 12,000, bar = 1 μm. (c): x 27,000, bar = 0.4 μm.

Figs. 5-7. Electron micrographs showing the immunolocalization of actin in odontoclasts. Clear zones sectioned in the direction perpendicular to a resorptive lacuna (5 and 6): (5) Clear zone composed of some cytoplasmic processes. There are gold particles (gold-labeled actin, arrows) on the clear zone, and only few particles are detected in the ruffled border. Insert: boxed area is enlarged on Figure 5. (6) Gold particles are localized on the area of the wide clear zone facing to the dentine surface (D). Insert: boxed area is enlarged on Figure 6. (7) A clear zone sectioned in the direction parallel to the resorptive lacuna: Gold particles (arrows) are localized in the electron dense structures (arrowheads) and the density of particles here is larger than that in the clear zone outside the electron dense structures. Insert; boxed area is enlarged on Figure 7. (5): x 24,000, bar = 0.5 μm, Insert: x 180, (6): x 55,000, bar = 0.2 μm, Insert: x 3,000. (7): x 26,400, bar = 0.4 μm, Insert: x180.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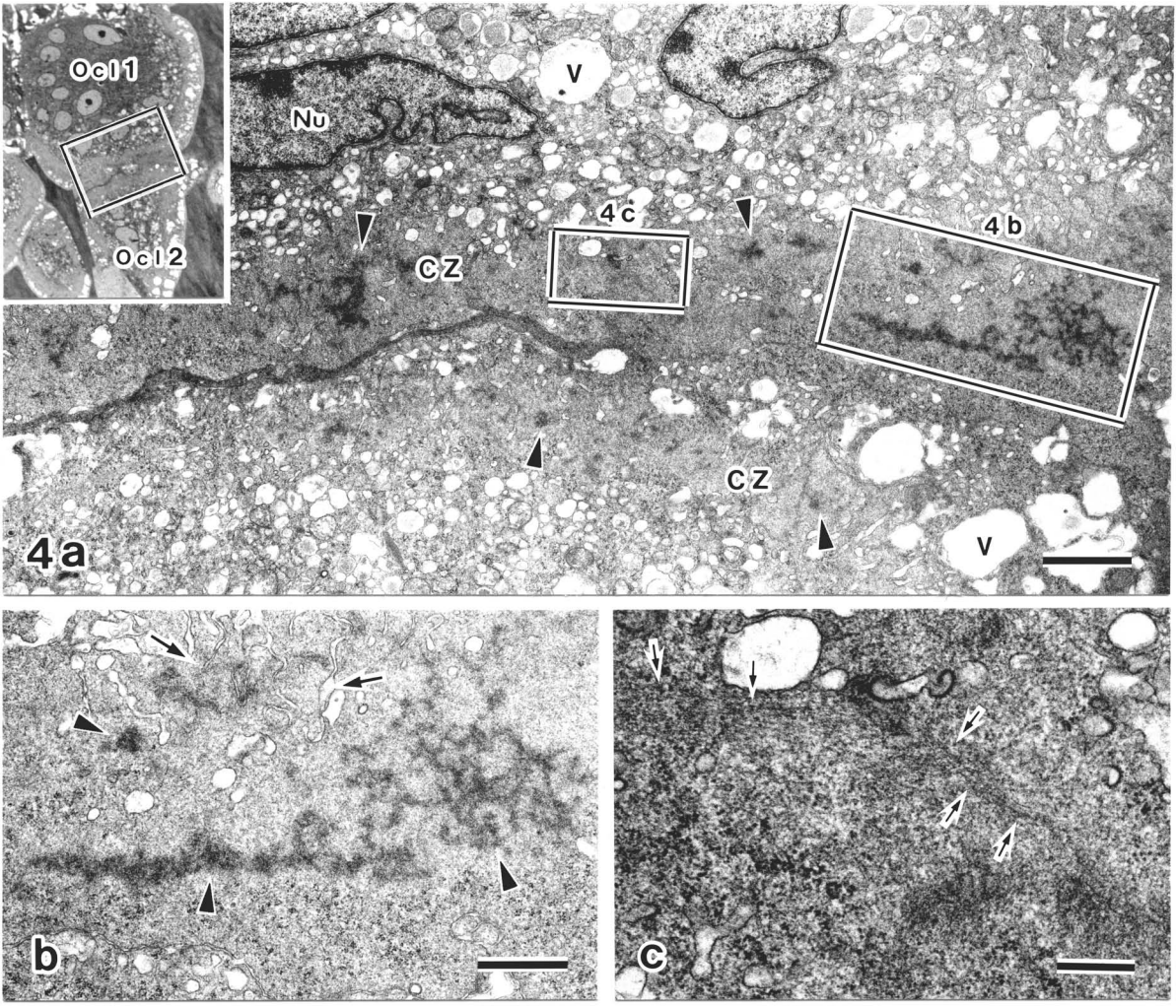
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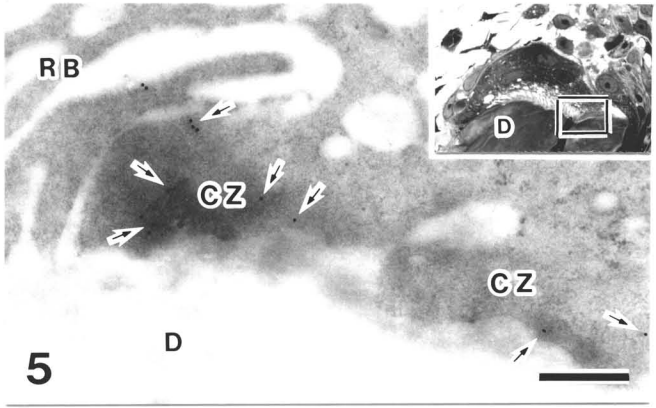
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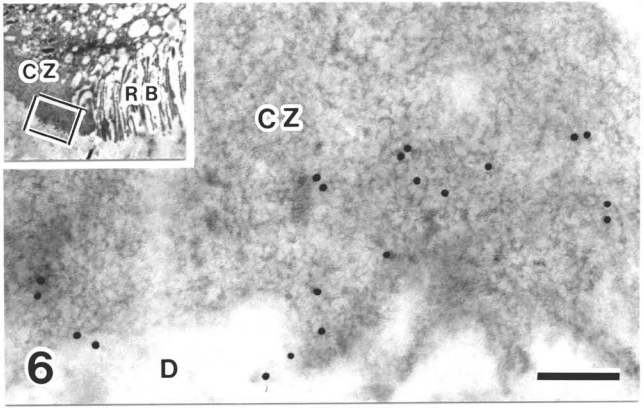
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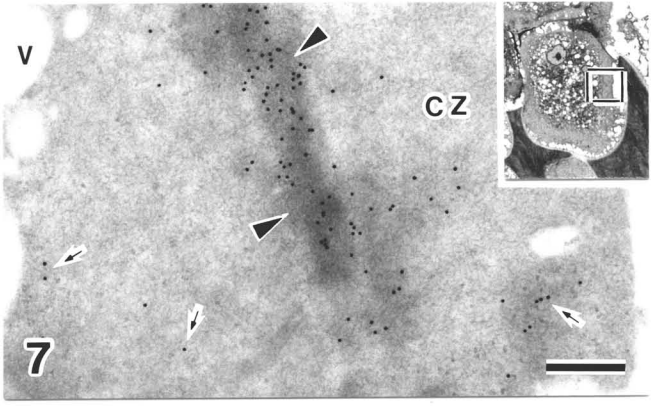
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