The Histochemical and Cytochemical Changes from Formative to Resorptive Osteocytes

Kazuharu Irie¹, Hidehiro Ozawa² and Toshihiko Yajima¹

¹Department of Oral Anatomy, Health Sciences University of Hokkaido School of Dentistry, Ishikari-Tobetsu, Hokkaido 061-0293 and ²Department of Oral Anatomy, Niigata University School of Dentistry, 2-5274 Gakkocho-dori, Niigata 951-8514

Received August 18, 2000; accepted November 8, 2000

Based on their ultrastructural features, osteocytes have been classified into three phases: formative, resorptive, and degenerative. However, the mechanism of the morphological changes from formative osteocyte to resorptive osteocyte is still unclear. Therefore, we labeled bone matrix with calcein and examined chronologically the histochemical changes in osteocytes from two distinct areas in the mandible, focusing on the relationship between osteocytes and osteoclastic bone resorption. Tartrate-resistant acid phosphatase (TRAP) activity was detected only in osteocytes located near sites of osteoclastic bone resorption. Osteocytes far from osteoclasts lacked TRAP activity, even those that had been enclosed in bone matrix as long

as the osteocytes that did show TRAP activity. Furthermore, glycogen granules evidenced by periodic acid-Schiff and the periodic acid-thiocarbohydrazide-silver protein staining in osteocytes disappeared when the cell acquired TRAP activity. These findings suggest that the acquisition of TRAP activity in an osteocyte is closely synchronized with bone resorption. Disappearance of glycogen in osteocytes in the same area may also be related to the acquisition of TRAP activity or bone resorbing activity. The osteocytes characterized by glycogen accumulation must be in the phase that follows the formative phase, in which osteocytes display neither formation nor resorption activity.

Key words: Bone resorption, Glycogen, Osteocytes, Tartrate-resistant acid phosphatase (TRAP)

I. Introduction

An osteoblast encloses itself in bone matrix to become an osteocyte [18]. Of the three types of bone cells, osteocytes have the widest interface with bone matrix and are therefore considered to play an important role in mineral homeostasis [16]. Based on their ultrastructural features, osteocytes have been classified into three phases: formative, resorptive, and degenerative [10–14]. Formative and resorptive osteocytes are also referred to as young and mature osteocytes, respectively. Formative osteocytes have similar features to osteoblasts on the bone surface, such as a well developed rough endoplasmic reticulum (rER) and Golgi complex in their cytoplasm. After a certain period of time in the bone matrix, the formative osteocytes “mature” and become resorptive osteocytes. The resorptive osteocytes have been characterized by

having less rER and a characteristic quantity of lysosomes. These osteocytes are considered to have bone-resorbing activity. The activity of tartrate-resistant acid phosphatase (TRAP), one of the enzymes intensely expressed in osteoclasts [5, 6, 9, 15, 23], has been demonstrated in osteocytes near the site of osteoclastic bone resorption [4], suggesting a possible function of osteocytes in osteoclastic bone resorption. However, the morphological changes occurring in formative osteocytes that differentiate into osteocytes associated with osteoclastic bone resorption are still unclear.

In the bone covering the enamel organ of the rat incisor (ECB), bone formation on the periosteal surface is coupled with bone resorption on the enamel organ (EO) facing surface, which results in expansion of the bone towards the outside with controlled thickness [8]. In other words, the matrix on the periosteal surface would be resorbed after being on the EO facing surface during modeling. Thus the osteocytes in this bone would meet with osteoclastic bone resorption after being in the bone matrix for a certain period of time.

Correspondence to: Kazuharu Irie, D.D.S., Ph.D., Department of Oral Anatomy, Health Sciences University of Hokkaido School of Dentistry, Ishikari-Tobetsu, Hokkaido 061-0293, Japan.

In this study, we labeled the bone matrix with calcein and examined chronologically the histochemical and cytochemical changes in osteocytes in ECB in comparison to those in the medial cortical part of the mandible (MCB). We focused on the relationship of these changes with osteoclastic bone resorption and confirmed that the acquisition of TRAP activity in an osteocyte is closely synchronized to bone resorption near the osteocyte.

II. Materials and Methods

For chronological labeling, three male Wistar rats were subcutaneously injected with calcein (10 mg/kg; Wako, Osaka, Japan) 4 and 5 weeks after birth and then sacrificed at 6-weeks old. The mandibles were removed and fixed in 70% ethanol for 7 days and stained with Villanueva bone stain (Maruto, Tokyo, Japan) for 10 days. The specimens were then dehydrated with ethanol and embedded in methylmethacrylate (Wako). Frontal ground sections at the mesial root of the first molar were obtained and observed with a confocal laser scanning microscope (CLSM; LSM-GB200, Olympus, Tokyo, Japan).

For histochemical and ultrastructural studies, six male Wistar rats 6 weeks old were used. Following intracardiac perfusion with a fixative, the mandibles were removed and immersed in the same fixative for 1.5 hr. The specimens were decalcified with 4.13% EDTA for 3 weeks at 4°C before undergoing the following procedures.

For histochemistry, 4% paraformaldehyde in a 0.1 M cacodylate buffer (pH 7.4) was used as the fixative. The decalcified mandibles were cut into frontal sections about 3 mm thick at the mesial root of the first molar and dehydrated in ethanol and embedded in Technovit 7100. Semithin sections were prepared for periodic acid-Schiff (PAS; [19]) or TRAP staining. The sections for TRAP staining were incubated for 20 min at 37°C in the medium described by Hoshi *et al.* [7].

For electron microscopic studies, a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.067 M cacodylate buffer (pH 7.4) was used as the fixative. The decalcified specimens were cut into blocks about 5 mm thick at the mesial root of the first molar. Frontal sections of about 100 μ m thickness were obtained from the block with a Microslicer (D.S.K. DTK-1000). Some sections were postfixed with 1% osmium tetroxide, dehydrated in ethanol and embedded in Epon. Ultrathin sections were obtained and stained for neutral glycoconjugates using the periodic acid-thiocarbohydrazide-silver protein (PA-TCH-SP) method described by Takagi *et al.* [22]. The others were used for the detection of acid phosphatase (ACP) activity. These sections were incubated for 15 min at 37°C in the medium described by Smith [21] using β -glycerophosphate as the substrate. Following the incubation, the sections were rinsed and postfixed with 1% osmium tetroxide containing 1.5% potassium ferrocyanide. The specimens were then dehydrated in ethanol and embedded in Epon 812. The sections used for

cytochemical studies were stained with lead citrate and observed with an electron microscope (100CX, JOEL).

The two distinct areas of the mandible observed in this study, ECB and MDB, are illustrated in Fig. 1.

III. Results

Two linear calcein labels were observed both in ECB and in MCB. The labels were irregular at the site of vascular penetration in ECB, and were much clearer in MCB (Fig. 2A, C). In ECB, TRAP-positive large cells were prominent on the bone surface facing the EO (Fig. 2B). The outer (#1) and inner (#2) calcein labels represent the bone matrix calcified at 1 and 2 weeks, respectively, before the experimental animals were sacrificed. In the bone matrix of MCB, the outer (#1) and the deeper (#2) labels also indicate the bone matrix calcified at 1 and 2 weeks, respectively, before the experimental animals were sacrificed. Only faint activity of TRAP was detected on the endosteal surface of MCB (Fig. 2D).

Three areas defined by the two calcein labels were examined to compare the TRAP activity and PAS reactivity in osteocytes from ECB and MCB.

Neither TRAP activity nor PAS-positive material was detected in osteocytes between the periosteal surface and label #1 in ECB (Fig. 3A, B) and MCB (Fig. 4A, B). Osteocytes observed in this area, especially in ECB, had a plump appearance. Between calcein labels #1 and #2, many osteocytes had PAS-positive material in their

Fig. 1. Schematic drawing of a cross section at the mesial root of the first molar in rat mandible. The two sites used in this study, the enamel organ covering bone (ECB) and the medial cortical bone of the mandible (MCB), are indicated by rectangles.

Fig. 2. Calcein labeling and tartrate-resistant acid phosphatase (TRAP) activity in ECB (A and B) and MCB (C and D) of the mandible. In both ECB and MCB, two calcein labels, label #1 (labeled at 1 week before sacrifice) and #2 (labeled at 2 weeks before sacrifice) are shown in bone matrix (A and C). TRAP-positive large cells are prominent on the enamel organ (EO) facing surface in ECB (B). Only faint activity of TRAP is detected on the endosteal surface of MCB (D). Bar = 100 μ m.

cytoplasm, while TRAP activity was not detected in osteocytes in ECB (Fig. 3C, D) or MCB (Fig. 4C, D). Many osteocytes showed TRAP activity between label #2 and the EO facing surface of ECB, but they had less PAS-positive material (Fig. 3E, F). On the EO facing surface, osteoclasts with intense TRAP activity were prominent. In MCB, osteocytes located deeper than label #2 showed similar characteristics to osteocytes between label #1 and #2. Those osteocytes had PAS-positive material, but TRAP activity was not detected in their cytoplasm (Fig. 4E, F).

Ultrastructurally, osteocytes between the periosteal surface and label #1 were of plump appearance and showed well developed cell organelles such as rER and Golgi complex (data not shown). The osteocytes between label #1 and #2 had PA-TCH-SP-positive granule accumulations (Fig. 5A). ACP activity was detected in some of the osteocytes in this area by electron microscopy (Fig. 5B). PA-TCH-SP-positive granules were few; however, intense ACP activity was detected in lysosomes of osteocytes found between label #2 and the EO facing surface of ECB (Fig. 5C, D).

IV. Discussion

Mature osteocytes referred to by Baud [1, 2] have been characterized by their possession of PAS-positive reticulum, using light microscopy. They are also characterized by glycogen granules in the cytoplasm, seen with electron microscopy, as well as by less rER and a characteristic quantity of lysosomes. Because there are no descriptions by other investigators regarding glycogen granules in resorptive osteocytes [10, 12–14], we focused on the morphological and histochemical characteristics of mature osteocytes.

TRAP activity was detected only in osteocytes between calcein label #2 and the EO facing surface in ECB. In MCB, osteocytes located deeper than label #2 lacked TRAP activity and showed similar characteristics to osteocytes between label #1 and #2, even the osteocytes that had been enclosed in bone matrix as long as the osteocytes that did show TRAP activity in ECB. This contrast strikingly indicates that TRAP activity in osteocytes is closely related to osteoclast activity. TRAP activity detected in the osteocytes displayed a granular appearance. Moreover, ACP activity in lysosomes of the osteocytes was also demonstrated, suggesting that the TRAP activity occurred within the lysosomes. We cannot completely rule out the possibility that the TRAP originates from osteoclasts and is taken up by the osteocytes after traveling through bone canaliculi to bone lacunae. However, ACP [24] and TRAP [4] activity in osteocytes as well as osteoblasts lying adjacent to osteoclasts have been reported. Yamamoto and Nagai [25] demonstrated TRAP activity ultrastructurally in lysosomes and in the Golgi complex of osteoblasts and also pointed out that TRAP activity of osteoblasts was detected only at areas of bone resorption or bone remodeling [26]. In this context, our present findings suggest that the osteocytes adjacent to osteoclastic bone resorption express TRAP. TRAP in osteocytes may play a role in bone resorption, such as modifying the bone surface to facilitate osteoclastic bone resorption or breaking down of the bone matrix by the osteocytes themselves (osteocytic osteolysis [3]).

The appearance of PAS-positive material in osteocytes corresponds with accumulation of PA-TSC-SP-positive granules. In osteocytes the granules appear to be glycogen. Scott and Glimcher [20] demonstrated changes in the quantity of glycogen granules during cytodifferenti-

Fig. 3. PAS reaction (A, C, E) and TRAP activity (B, D, F) in osteocytes in three distinct areas of ECB. In the area near the periosteal surface, neither PAS-positive material (A) nor TRAP (B) activity is detected in osteocytes. In the area between the two calcein labels in Fig. 2A, many osteocytes show PAS-positive material (C, arrow heads), but TRAP activity is not detected in the osteocytes (D). In the area near the EO facing surface, PAS-positive material are not seen in osteocytes (E), but TRAP activity (arrows) is detected in the osteocytes (F). Osteoclasts (OC) on the EO facing surface show intense TRAP activity. OB, osteoblast. Bar=20 μ m.

ation of fetal osteoblasts, suggesting the phasic synthesis and regeneration of glycogen and lipid in the cell. It is probable that the osteocytes subjected to anaerobic conditions produce glycogen for their energy source. It is also

noteworthy that the PAS-positive material in osteocytes was absent in the area between label #2 and the EO facing surface of ECB. The cancellation of anaerobic conditions by closer proximity to the bone surface may alter the

Fig. 4. PAS reaction (A, C, E) and TRAP (B, D, F) activity of osteocytes in MCB. In the area near the periosteal surface, neither PAS-positive material (A) nor TRAP activity (B) is detected in osteocytes. In the area between the two calcein labels in Fig. 2C, many osteocytes show PAS-positive material (C, arrow heads), but TRAP activity is not detected in the osteocytes (D). In the area deeper than the calcein label #2 in Fig. 2C, osteocytes show PAS-positive material (E), but TRAP activity is not detectable (F). The osteocytes are similar to the osteocytes in the area between the two calcein labels. Bar = 20 μ m.

metabolism of osteocytes so as not to produce glycogen. Baud [1] described the possibility of involvement of lactic acid derived from glycolysis in dissolving mineral substance. Glycolytic activity may be stimulated by

osteoclasts to produce lactic acid. Ohshima *et al.* [17] suggested that the disappearance of glycogen pools in the cells during tooth development was related to the high level of signaling activity of the mesenchymal cells in the

Fig. 5. Electron micrographs of osteocytes in ECB after detecting neutral glycoconjugates by the periodic acid-thiocarbohydrazide-silver protein method (A and C) and acid phosphatase (ACP) activity (B and D). Glycogen pools (A, arrow heads), and faint activity of ACP at the Golgi region (B, arrows) are detected in osteocytes at the region between the two calcein labels. In osteocytes near the EO facing surface of alveolar bone, lysosomes (double arrows) are evident but glycogen granules are few (C). ACP activity is evident in lysosomes (arrows) of osteocytes near the EO facing surface (D). Bar=2 μ m.

dental follicle. Thus, disappearance of glycogen in osteocytes adjacent to bone resorption may also be related to the acquisition of TRAP activity in osteocytes or bone resorption activity of osteocytes themselves or of osteoclasts.

Since neither disappearance of glycogen nor the acquisition of TRAP in osteocytes was observed in MCB, we emphasize that these changes in osteocytes are likely to be closely related to osteoclastic bone resorption.

The osteocytes characterized by glycogen accumulation must be in the phase between formative and resorptive osteocytes, in which osteocytes are transferring their activity from formation to resorption. Mature osteo-

cytes, referred to by Baud [1], include both transferring osteocytes and resorptive osteocytes. Osteocytes with glycogen granules are also found between formative and degenerative osteocytes, as demonstrated in MCB. Thus the osteocytes characterized by glycogen accumulation must be in the phase after formative osteocytes. Osteocytes in this phase show neither formation or resorption activity.

V. References

1. Baud, C. A.: Submicroscopic structure and functional aspects

- of the osteocyte. *Clin. Orthop.* 56; 227–236, 1968.
2. Baud, C.: Morphologie et structure inframicroscopique des osteocytes. *Acta Anat.* 51; 209–205, 1962.
 3. Baylink, D. and Wergedal, J.: Bone formation and resorption by osteocytes. In "Cellular Mechanisms for Calcium Transfer and Homeostasis", ed. by G. J. Nichols and R. Sasserman, Academic Press, New York, 1971, pp. 257–289.
 4. Bianco, P., Ballanti, P. and Bonucci, E.: Tartrate-resistant acid phosphatase activity in rat osteoblasts and osteocytes. *Calcif. Tissue Int.* 43; 167–171, 1988.
 5. Clark, S. A., Ambrose, W. W., Anderson, T. R., Terrell, R. S. and Toverud, S. U.: Ultrastructural localization of tartrate-resistant, purple acid phosphatase in rat osteoclasts by histochemistry and immunocytochemistry. *J. Bone Miner. Res.* 4; 399–405, 1989.
 6. Hammarström, L. E., Hanker, J. S. and Toverud, S. U.: Cellular differences in acid phosphatase isoenzymes in bone and teeth. *Clin. Orthop.* 78; 151–167, 1971.
 7. Hoshi, K., Amizuka, N., Sakou, T., Kurokawa, T. and Ozawa, H.: Fibroblasts of spinal ligaments pathologically differentiate into chondrocytes induced by recombinant human bone morphogenetic protein-2: Morphological examinations for ossification of spinal ligaments. *Bone* 21; 155–162, 1997.
 8. Irie, K. and Ozawa, H.: Relationships between tooth eruption, occlusion and alveolar bone resorption: cytological and cytochemical studies of bone resorption on rat incisor alveolar bone facing the enamel. *Arch Histol. Cytol.* 53; 497–509, 1990.
 9. Izumi, N., Amizuka, N., Oda, K., Misumi, Y., Ikehara, Y. and Ozawa, H.: Ultrastructural alteration of Osteoclasts treated with brefeldin A and wortmanin. *Acta Histochem. Cytochem.* 32; 393–405, 1999.
 10. Jande, S. S.: Fine structural study of osteocytes and their surrounding bone matrix with respect to their age in young chicks. *J. Ultrastruct. Res.* 37; 279–300, 1971.
 11. Jande, S. S. and Bélanger, L. F.: Electron microscopy of osteocytes and the pericellular matrix in rat trabecular bone. *Calcif. Tissue Res.* 6; 280–289, 1971.
 12. Jande, S. S. and Bélanger, L. F.: The life cycle of the osteocyte. *Clin. Orthop.* 94; 281–305, 1973.
 13. Luk, S. C., Nopajaroonsri, C. and Simon, G. T.: The ultrastructure of cortical bone in young adult rabbits. *J. Ultrastruct. Res.* 46; 184–205, 1974.
 14. Luk, S. C., Nopajaroonsri, C. and Simon, G. T.: The ultrastructure of endosteum: a topographic study in young adult rabbits. *J. Ultrastruct. Res.* 46; 165–183, 1974.
 15. Minkin, C.: Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif. Tissue Int.* 34; 285–290, 1982.
 16. Nijweide, P., Burger, E., Klein Nulend, J. and Van der Plas, A.: The osteocyte. In "Principles of Bone Biology", ed. by J. Belezikian, L. Raisz and G. Rodan, Academic Press, Inc., San Diego, 1996, pp. 115–126.
 17. Ohshima, H., Wartiovaara, J. and Thesleff, I.: Developmental regulation and ultrastructure of glycogen deposits during murine tooth morphogenesis. *Cell Tissue Res.* 297; 271–281, 1999.
 18. Owen, M.: Cell population kinetics of an osteogenic tissue I. *J. Cell Biol.* 19; 19–32, 1963.
 19. Sano, Y.: Histological Technics. Theoretical and Applied, 6th ed., Nanzando, Tokyo, 1981.
 20. Scott, B. L. and Glimcher, M. J.: Distribution of glycogen in osteoblasts of the fetal rat. *J. Ultrastruct. Res.* 36; 565–586, 1971.
 21. Smith, C. E.: Ultrastructural localization of nicotinamide adenine dinucleotide phosphatase (NADPase) activity to the intermediate saccules of the Golgi apparatus in rat incisor ameloblasts. *J. Histochem. Cytochem.* 28; 16–26, 1980.
 22. Takagi, M., Parmley, R. T., Toda, Y. and Denys, F. R.: Ultrastructural cytochemistry of complex carbohydrates in osteoblasts, osteoid, and bone matrix. *Calcif. Tissue Int.* 35; 309–319, 1983.
 23. Van de Wijngaert, F. P. and Burger, E. H.: Demonstration of tartrate-resistant acid phosphatase in un-decalcified, glycol-methacrylate-embedded mouse bone: a possible marker for (pre)osteoclast identification. *J. Histochem. Cytochem.* 34; 1317–1323, 1986.
 24. Wergedal, J. E. and Baylink, D. J.: Distribution of acid and alkaline phosphatase activity in undemineralized sections of the rat tibial diaphysis. *J. Histochem. Cytochem.* 17; 799–806, 1969.
 25. Yamamoto, T. and Nagai, H.: Ultrastructural localization of tartrate-resistant acid phosphatase activity in rat osteoblasts. *J. Electron. Microsc.* (Tokyo) 47; 659–663, 1998.
 26. Yamamoto, T., Yamagata, A. and Nagai, H.: A histochemical study of tartrate-resistant acid phosphatase activity in rat osteoblasts. *Acta Histochem. Cytochem.* 29; 221–225, 1996.