Distribution of the Extracellular Free Ca^{2+} in the Periodontal Ligament and Periosteum during Experimental Tooth Movement Using Fluorescent Free Ca^{2+} Indicator "Fluo-3"

Yoshiki Nakamura, Kouji Noda, Takashi Tanaka and Yosuke Kuwahara

Department of Orthodontics, School of Dental Medicine, Tsurumi University, Yokohama 230

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Distribution of extracellular free Ca^{2+} was examined in the rat periodontal ligament and periosteum of alveolar bone during tooth movement, using fluo-3. To stimulate bone formation and osteoclastic bone resorption, upper molars of adult rats were moved lingually for 7 days. After careful removal of the chestal ribs, fluo-3 was injected into the left ventricle of throbbing hearts, immediately after clamping the descending aortae with forceps. 90 sec after the injection, the upper jaw was excised out rapidly and immersed in liquid nitrogen. 5 μm unfixed undecalcified frozen sections from the frozen tissues were prepared for fluorescent microscopy and also electron microprobe analysis (EPMA). Green fluorescence was emitted from the whole periodontal space and pulp cavity. The pattern of bone forma-

tion in the periodontal ligament was quite different from that in the periosteum. A considerable amount of osteoid formed in the buccal periodontal ligament (tension side) and also in the periosteum of lingual alveolar bone (compensative bone apposition). The fluorescence emitted from the osteoid was more intense than that from the periodontal ligament or subperiosteal tissue. EPMA data also showed a higher peak of Ca in the osteoid than in the periodontal ligament or subperiosteal tissues. In addition, a narrow linear green fluorescence was emitted from the interface between the osteoclasts and the bone in the lingual periodontal ligament (pressure side). These results suggest that osteoid contains more free Ca^{2+} than periodontal ligament and subperiosteal tissue.

Key words: Fluo-3, Extracellular free Ca^{2+} , Osteoid, Periodontal ligament, Tooth movement

I. Introduction

Examination of the localization of calcium (Ca) in the calcification front have been essential for understanding of mechanism of calcification. Advances in techniques for detecting Ca in histochemistry and cytochemistry have made it possible to observe intracellular Ca [4, 13]. However, it has been very difficult to detect intracellular free Ca^{2+} , though intracellular free Ca^{2+} [6] as well as cyclic adenosine monophosphate [2] are considered to be second messengers of cells. Recently, the development of fluorescent indicators for intracellular free Ca^{2+} , such as fluo-3 and fura-2, have enabled us to detect free Ca^{2+} in many cell types [5, 15].

On the other hand, with regard to distribution of the extracellular Ca in the interfaces between bone and bone cells, such as osteoblasts and osteoclasts, most of the evidence have been limited to the electron dense grains of calcium phosphate in the matrix vesicles [2] and also in the ruffled border areas [13]. It is quite important to detect free Ca^{2+} in the interfaces for bone biology. However, no efforts have been made to observe the extracellular free Ca^{2+} , due to technical difficulties.

We report here experiments using a fluorescent free Ca^{2+} indicator fluo-3 in order to detect the free Ca^{2+} in the interfaces between bone and bone cells in the alveolar bone of the upper first molars of adult rats.

II. Materials and Methods

Orthodontic appliances with springs were fixed to upper incisors of adult male wistar rats (6 rats) (weighing

Correspondence to: Dr. Yoshiki Nakamura, Department of Orthodontics, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230, Japan.

220–240 g) and the upper first molars were moved lingually for 7 days by the springs [10] (force magnitude: 10–15 g) in order to stimulate bone formation in the periodontal ligament on the buccal side and in the periosteum of the alveolar bone on the lingual side and also osteoclastic bone resorption in the periodontal ligament on lingual side [7, 11, 14].

After 7 days of tooth movement, under anaesthesia with nembutal (0.1 ml/100 g bw, Sankyo Co, Tokyo, Japan) the ribs were removed carefully, and immediately after clamping the descending aorta with forceps, 0.06 ml of 10 mM fluo-3/100 g body weight (Dojindo, Kumamoto, Japan) diluted with 10 mM NaOH was injected into the left ventricle of the throbbing heart. During throbbing, the upper jaw was excised out 90 sec after the injection, and immersed rapidly in liquid nitrogen. The frozen tissues were trimmed into smaller blocks in a frozen condition, embedded in pre-cooled OCT-compound (Miles Inc, Elkhart, IN, U.S.A.), put into liquid nitrogen and left until the OCT-compound was completely frozen. The frozen blocks were trimmed (6 × 6 × 8 mm) and mounted on pre-cooled stubs in the cryostat (−25°C, Reitz, Wetzlar, Germany) with the same compound, and sectioned using a superhard knife (Meiwa shoji Ltd., Tokyo, Japan). 5 µm serial sections were collected one by one with powerful adhesive tapes, which were freeze-dried for 1 hr in the cryostat [12].

One of the sections was fixed with 10% buffered formalin for 60 sec, washed briefly with DW and stained with toluidine blue for histological observation. Two rats without tooth movement were also prepared in the same way for fluorescence microscopy.

Fluorescence images were obtained from the unfixed, undecalcified frozen sections by using fluorescence microscope (Olympas Co. Ltd., Tokyo, Japan). A 100 W mercury burner served as the source for excitation. A blue beam over 455 nm was used for the excitation of fluo-3. An emission filter (G 520) which passes only a green beam between 520 and 530 nm in wavelength was used for observation.

On the other hand, some sections were transferred to carbon specimen stubs for electron probe microanalysis (EPMA) and coated with a layer of carbon in the vacuum evaporator. Secondary electron images and back scattered electron images were obtained and then EPMA was performed using a scanning electron microscope (JSM 840, JEOL, Akishima-city, Japan) with an energy dispersive system (Link system, Bucks, England). Accelerating voltage was 20 kV. The data were collected for 4 mm, using a beam current of 1×10^{-9} µA and a beam size of 1×1 µm.

III. Results

Fig. 1a shows a cross-sectional view of the toluidine blue-stained section of the specimen without tooth movement. The periodontal ligament sandwiched between the

alveolar bone and the root and pulp were clearly observed, indicating good structural preservation. Green fluorescence emitted from fluo-3 combined with Ca^{2+} was seen in the whole periodontal space and pulp cavity. It was noticed in the osteocytic lacunae in the bone, though the calcified matrix didn't show any fluorescence. A circular band of fluorescence was also emitted from the areas corresponding to the predentine in the pulp cavity (Fig. 1b).

When upper molars were moved lingually for 7 days, the periodontal fibers were stretched in the widespread periodontal space on tension side, and new bones protruded from the bone surface, showing a wave like configuration. A considerable amount of osteoid with metachromasia in toluidine blue stain was also formed on the new bone. (Fig. 2a). Green fluorescence was seen in the widened periodontal space (Fig. 2b). It seemed that the fluorescence from the osteoid was somewhat more intense than that from the other periodontal ligament. A secondary electron image of the bone surface areas in similar sections prepared for elemental analysis showed that the osteoid consisted of thick collagen fiber bundles (Sharpey's fibers) and the matrix between the bundles (Fig. 3a). The back scattered electron image of the same bone area clearly revealed the border between bone and osteoid (Fig. 3b). Thus, it was possible to detect the osteoid with EPMA. EPMA of the collagen fiber bundles within the osteoid (Fig. 3a) indicated high peaks of Ca, P and peaks of S, Cl and a low peak of K. But the analysis of collagen fiber bundles a long distance away from the bone surface did not show such high peaks of Ca and P. Both peaks of Ca and P were lower than S. The peak of Ca in the osteoid was higher than that in the other areas of periodontal ligament (Fig. 3b).

Moreover, on the periosteal side of lingual alveolar bone where the compensative bone formation [14] has been taking place, a thick layer of osteoid was formed on the bone by seven days tooth movement and many osteoblasts were lined up on it (Fig. 4a). Aspects of new bone formation on the periosteal side were quite different from that on the periodontal side. A wide strip of intense fluorescence was emitted from the area corresponding to the osteoid. The osteoblast layer showed a weaker fluorescence than that of the connective tissues (Fig. 4b). A secondary electron image of the bone surface areas in periosteum in similar sections prepared for EPMA (Fig. 5a) was different from that in the periodontal ligament (Fig. 3a). No thick collagen fiber bundles were seen in this osteoid. It rather had flat appearance. The bone was easily distinguished from the other tissue, since the border between bone and osteoid was relatively linear. The back scattered electron image of the same area made the border clearer (Fig. 5b). EPMA of the osteoid indicated that high peaks of Ca, P and peaks of S, Cl and a low peak of K, which were very similar to the analyzed data of osteoid in the periodontal ligament. But in the analysis of the subperiosteal tissue (connective tissue), the peaks of Ca

Fig. 1. Cross-sectional view of the periodontal ligament without tooth movement. **a:** Toluidine blue. $\times 100$. **b:** The green fluorescence was seen in the whole periodontal space and pulp cavity. $\times 100$.

Fig. 2. Labial periodontal ligament (tension side). **a:** The periodontal fibers were stretched and the osteoid was formed on the calcified bone (toluidine blue). $\times 200$. **b:** The fluorescence from the osteoid was more intense than that from the widened periodontal ligament. $\times 200$.

Fig. 4. Periosteal side of the lingual alveolar bone. **a:** A considerable layer of osteoid was formed on the bone surface. $\times 400$. **b:** A wide strip of intense fluorescence was emitted from the osteoid. $\times 400$.

Fig. 6. Lingual periodontal ligament (pressure side). **a:** many osteoclasts were seen on the bone surface. $\times 400$. **b:** A narrow linear green fluorescence was observed along the Howship's lacunae. $\times 400$.

Fig. 3. a: Secondary (a) and back scattered (b) electron images of the periodontal ligament on tension side. B=bone. Arrow 1 shows the site of EPMA in the osteoid and arrow 2 shows the site in the periodontal ligament. $\times 5,000$. c: EPMA data of arrows 1 and 2. Horizontal scale: X-ray energy from 1 to 10 keV, Vertical scale: X-ray counts (full scale=500).

Fig. 5. Secondary (a) and back scattered (b) electron images of the periosteum of lingual alveolar bone. B=bone. Arrow 1 shows the site of EPMA in the osteoid and arrow 2 shows the site of EPMA in the subperiosteal tissue (connective tissue). $\times 7,000$. **b:** EPMA data of arrows 1 and 2. Horizontal scale: X-ray energy from 1 to 10 keV, Vertical scale: X-ray counts (full scale=500).

and P were low as compared with that of osteoid (Fig. 5c).

In the lingual periodontal ligament (pressure side), a number of osteoclasts appeared on the bone surface near the degenerating tissue, suggesting that the active bone resorption was occurring (Fig. 6a). The green fluorescence was noticed in the periodontal ligament except for the area corresponding to cellular elements such as osteoclasts. On the bone surface area, a narrow linear fluorescence was noticed along the Howship's lacunae (Fig. 6b).

IV. Discussion

Fluo-3 is one of the members of new fluorescent Ca^{2+} indicators excitable at wavelength in the visible [8]. It combines specifically with Ca^{2+} to form the chelate compound, which emits green fluorescence (530 nm), if it is excited by a beam of wavelength between 480 and 500 nm (blue light) [8]. This chelate compound remains stable and does not combine with Ca-compounds such as Ca-binding protein and calcium phosphate [9]. Unlike fluo-3AM, fluo-3 does not pass through cell membranes [9]. Therefore, the green fluorescence observed in this study indicates the extracellular location of the free Ca^{2+} combined with fluo-3 in the periodontal ligament.

However, there are two possibilities in regard to the combination of fluo-3 with Ca^{2+} ; one is that after combining with Ca^{2+} in the serum within the circulatory system, the chelate compound effused into the periodontal ligament and periosteum. The other is that after effusion from the capillary into the tissues without combining with Ca^{2+} , fluo-3 combined with the extracellular Ca^{2+} . It was difficult to discriminate between them precisely. But most of fluo-3 is considered to be bound with Ca^{2+} in the tissues, because there were differences in the intensity of fluorescence of the osteoid and the other tissues (Figs. 2b, 4b). If fluo-3 combined with Ca^{2+} in the serum, the chelate compound would be spread evenly in all of the periodontal ligament, periosteum and the other tissues since the chelate compound does not have any affinity for the osteoid [9].

After 7 days tooth movement, the pattern of bone formation in the periodontal ligament was quite different from that in the periosteum. In the periodontal ligament the bone surface showed a wavy configuration (Fig. 2a), while in the periosteum it showed a linear configuration (Fig. 4a). This is probably related to differences in the architecture of both tissues, especially in the arrangement of collagen fiber bundles (Figs. 3a, 5a).

However, in the osteoid formation there is no difference between the periodontal ligament and periosteum. A layer of osteoid with metachromasia was always observed on the newly formed bone in both tissues by tooth movement (Figs. 2a, 4a). The fluorescence emitted from the area corresponding to the osteoid tissues was somewhat more intense than that from the other areas (Figs. 2b, 4b). Because one molecule of fluo-3 combines with

one molecule of Ca^{2+} [9], the difference in the intensity suggests that the amount of Ca^{2+} in the osteoid tissues is more than that in other tissues. These phenomena might be supported by the EPMA data that peaks of Ca in the osteoid of both periodontal ligament and periosteum were higher than those in the other area (Figs. 3b, 5b).

On the pressure side, interestingly the interface between osteoclasts and bone emitted the fluorescence (Fig. 6a, b). These facts indicate that fluo-3 or fluo-3 combined with Ca^{2+} is able to pass through the gaps between bone and clear (sealing) zones which isolate the ruffled border area from the other extracellular space to maintain its pH at low level and to make it possible to decalcify bone [1, 3]. Therefore, this would suggest that the gaps have a function of selective permeability that some kinds of molecules are able to pass through except for proton.

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VI. References

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