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CONFOCAL LASER SCANNING MICROSCOPIC STUDIES ON ALVEOLAR BONE REMODELING WITH ORTHODONTIC TOOTH MOVEMENT AND RETENTION

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Abstract

Alveolar bone reconstruction in growing dog during the retention period following orthodontic tooth movement was studied. Three beagle dogs (8-10 kg body weight, about one-year-old) were used and two of the animals were subjected to histological observation. The upper 2nd and lower 3rd premolars on both sides were extracted prior to the orthodontic treatments. After a healing period of one month, the upper 3rd premolar and the lower 4th premolar on the right side were moved mesially with a conventional orthodontic force for 8 weeks, and then retained in their new position for 4 weeks. The contralateral corresponding premolars were used as control. The alveolar bone was double--labeled with tetracycline (TC) during the movement and calcein (Cal) during the retention period. Alveolar bone structure and labeling patterns were examined by contact microradiography, conventional fluorescence microscopy, and confocal laser scanning microscopy (CLSM). Optimizing the separation of TC and Cal labelings in the alveolar bone was attained by the simultaneous use of ultraviolet (364 nm) and argon (488 nm) laser sources for excitation of TC and Cal, respectively. Cal labeling, indicative of new bone deposition showed two distinct patterns: lamination at the periodontal surface and rings circumscribing the vascular canal. The cementum surface also exhibited active deposition during the experimental period. Bone formation was affected by slight changes in magnitude and direction of orthodontic or occlusal forces. CLSM is valuable in deciphering the process of alveolar bone remodeling.

Key Words: Confocal microscopy, alveolar bone remodeling, orthodontic tooth movement and retention.

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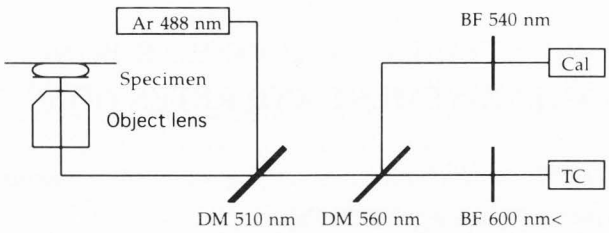
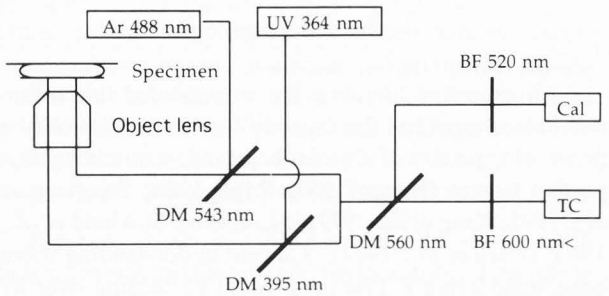
Introduction

An extensive literature has documented that orthodontic treatment has the capacity to alter dramatically a preexisting pattern of alveolar bone and surrounding supportive tissues (Reitan 1967; Rygh 1985; Steigman *et al.*, 1991; King *et al.*, 1991a; Lindskog-Stokland *et al.*, 1993; Dolce *et al.*, 1994). Current understanding about bone remodeling is that bone could be turning over by site-specific alterations in a remodeling sequence comprising the characteristic phases of activation, resorption, reversal, formation, and quiescence (Parfitt, 1984; King *et al.*, 1991b). In connection with orthodontic movement of a tooth and its retention in a new position, Roberts *et al.* (1992) stated that following active orthodontic therapy, dental corrections should be retained for at least 6-8 months to allow for mineral maturation of the newly formed bone. However, a paucity of information remains as to the length of time that retainers should be worn and the details of bone modeling and remodeling taking place with retention of the moved teeth.

Since alveolar bone structures and their changes caused by orthodontic treatment are known to be similar in man and dog (Reitan, 1959), we have been investigating the temporal and spatial patterns of alveolar bone remodeling of beagle dogs. In a previous report (Yagishita, 1994), the alveolar bone turnover taking place during orthodontic tooth movement (2 through 8 weeks) and subsequent retention (2 through 52 weeks) was monitored by a double labeling technique using tetracycline (during the active movement) and calcein (during the retention period). However, the combination of conventional microradiography and fluorescence microscopy did not allow us to view separately the localization of tissue markers in remodeling bone. It was also reported that the spatial resolution of double labeling patterns reduces significantly with an increase in section thickness (Birkenhager-Frenkel and Birkenhager, 1987). Thus, we designed a new series of the animal experiments in conjunction with the use of confocal laser scanning microscopy (CLSM). The specific aim of the current work was to demonstrate that CLSM has possibilities as an improved means for obtaining a discrete view



System 1



System 2

Figure 1. Schematic diagrams of the CLSM systems used. System 1 comprised an argon laser (Ar 488 nm) and one dichroic beam splitter (DM 560 nm). System 2 allowed us to simultaneously use two laser sources: an ultraviolet laser (UV 364 nm) for TC excitation and an argon laser for Cal excitation. Various combinations of barrier filters (BF: for instance, > 600 nm for TC detection, and BF 540 or 520 nm for Cal) were tested to attain optimal separation of TC and Cal labelings.

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of localization of tetracycline and calcein in alveolar bone tissue. Our hypothesis was that active bone reconstruction occurs continuously during the retention period after orthodontic tooth movement.

Materials and Methods

Experimental protocol

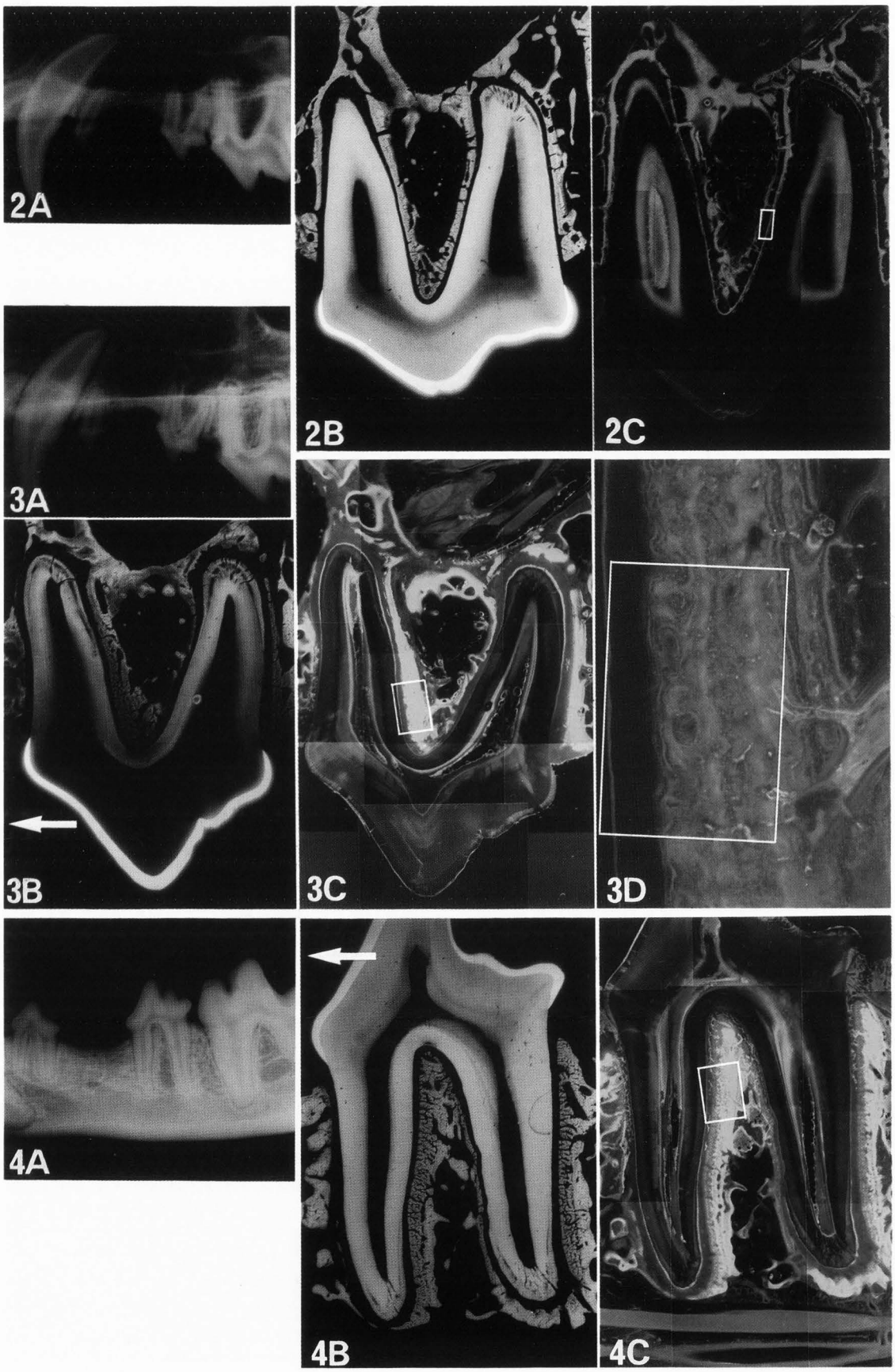
Three beagle dogs (approximately 1 year old), weighing 8-10 kg at the beginning of the experiment, were housed with special care to maintain their good health condition. Clinical and radiographic examination showed that the animals had gingival inflammation (redness and swelling) but did not exibit bleeding or periodontal tissue breakdown in the premolar-molar tooth regions. From the day of initial examination and throughout the course of the study, mechanical plaque control measures were performed once every 2-3 days under general anesthesia with a minimum dose of sodium pentobarbital (Pitman Moor Co., Mundelein IL).

Figure 2. (A) Radiogram of a maxillary bone segment dissected from the control (left) side. The second premolar (P2) was extracted prior to the orthodontic experiment but no orthodontic appliances were applied over the experimental period. (B) Contact microradiogram of a sagittal ground section (100 μm thick) of the P3 and surrounding alveolar bone, showing that the medial and distal roots of the tooth were surrounded entirely by cortical bone relatively uniform in thickness. (C) Non-confocal image of the corresponding section. Note the presence of a thin layer of tetracycline (TC, yellow) labeling covering the periodontal side of the alveolar bone. Although TC labeling with spots of Cal (green) were discerned on endosteal surfaces, the originally existed cortical bone in the core region remained unlabelled. TC and Cal lines corresponding to the numbers of their injections were discerned in dentin formed during the experimental period.

Figure 3. (A) Radiogram of the maxilla in the experimental side. The target tooth (P3) had been bodily moved in the medial direction for 8 weeks and subsequently was retained for 4 weeks. Orthodontic movement and retention of the tooth were achieved with only minimal vertical displacement of the teeth. (B) Contact microradiogram of the P3 and surrounding alveolar bone. Arrow indicates the direction of tooth movement by the orthodontic appliance. (C) Non-confocal images of the corresponding section. (D) Enlarged image of the area shown in box of Figure 3C. Note the apposition of Cal labeled (green) bone on the periodontal side, as well as its appearance around a large capillary wall. A TC labeled thin band on the left side corresponds to the cementum surface.

Figure 4. (A) Radiogram of a mandibular segment including the target tooth (P4), which was dissected from the same animal shown in Figure 3. The third premolar was extracted prior to the orthodontic experiment. The extraction socket was filled with bone trabeculae. (B) Contact microradiogram of the medially moved P4 and surrounding alveolar bone. The arrow indicates the direction of tooth movement by the orthodontic appliance. (C) Non-confocal image of the corresponding section. Note that the Cal labeled bone replaced the outer (periodontal side) half of the TC labeled cortical bone in the original tension sides.

The current experimental protocol was designed on the basis of our previous findings. After arrival of the animals, we kept the animals for 1 week to confirm whether they could be used in the required experiment. The upper 2nd premolars and the lower 3rd premolars in both sides were extracted under general anesthesia (0.4 mg/kg body weight, b.w., sodium pentobarbital).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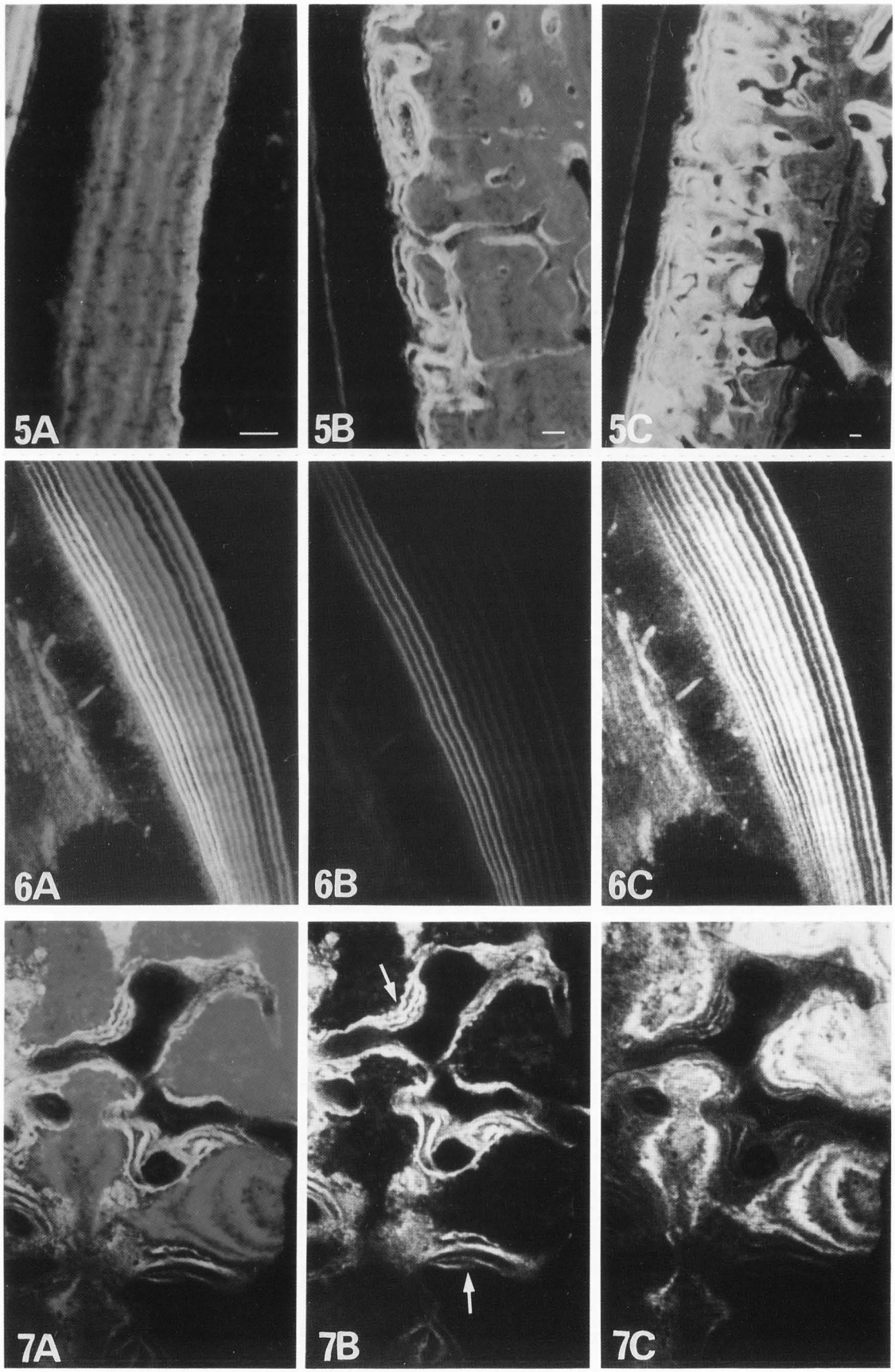
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Figure 5. (A) Confocal images of dog alveolar bone, recorded from a single plane in focus. TC and Cal labelings are displayed using pseudo-colors, red and yellow, respectively. The area observed is shown in the box in Figure 2C. In this control side without orthodontic appliances, TC labeling appeared as parallel lamellae and Cal labeling as a single band on the periodontal side. The cementum, as well as the endosteal side of alveolar bone, was barely labeled with neither fluorochrome. (B) Confocal imaging of the reconstructed alveolar bone in the experimental side of the upper jaw 4 weeks after completion of tooth movement. The area observed is shown in box of Figure 3D. Note the appearance of sharp red line on the cementum surface, yellow lamination on the periodontal side, and yellow rings around the canal walls. (C) Confocal imaging of the alveolar bone in the experimental side of the lower jaw. The area observed is marked in box of Figure 4C. The outer (periodontal side) half of the cortical bone was replaced by the Cal labeled bone during the 4-week-retention period. Bar = 100 μm.

Figure 6. Confocal images of fluorescence localization in dentin of the lower first molar. The picture was recorded using system 1 with a single argon laser illustrated in Figure 1. (A) A simultaneous display of both TC (red) and Cal (yellow) labelings. Corresponding to the sequence of fluorochrome injections during the experimental period, initial 8 sharp lines of TC and subsequent 4 lines of Cal were discerned. (B) The emission signal collected between 540 and 560 nm. Since the system used was optimal for calcein excitation and collection of its emission signal, the 4 lines of Cal were clearly visualized with negligible interference with TC. (C) The emission signal passed through > 600 nm BF. Although the filter condition was selected to collect only the TC signal, the total 12 bands including deceptive Cal interference were still produced.

Figure 7. Confocal images of fluorescence localization in alveolar bone surrounding the orthodontically treated lower premolar. The area observed is indicated in the box in Figure 4. All photographs were recorded with system 2 having both UV and Ar laser sources (see Fig. 1). (A) A superimposed display of the simultaneously recorded TC and Cal images, representing an early stage of replacement of red-colored bone by concentric yellow bands arising from the Haversian canal walls. (B) Cal signals, which were generated with excitation at 488 nm and collected between 520 and 560 nm. Note the separation of 4 Cal lines (arrow) corresponding to the number of Cal injections administered at 1 week intervals. (C) The emission signal passed through > 600 nm BF. A much stronger TC signal was produced by UV excitation, yielding better discrimination between TC and Cal.

After a healing period of one month, the upper 3rd premolar (P3) and the lower 4th premolar (P4) in right side were moved medially (toward the healed extraction socket) by means of an open coil spring (Sentalloy coil spring, 0.25 mm x 0.90 mm, Tomy International Inc., Tokyo, Japan). The movement force (around 0.3-0.5 N) was applied for 8 weeks. The canines and the distal anchor teeth (upper 4th premolar and lower 1st molar) on the test side were fixed to individually fabricated archwires (stainless steel wire, 0.8 mm, Ormco, Tokyo, Japan). An open coil spring was attached to the main archwires distal to the target teeth. The contralateral corresponding premolars, which served as control, were not subjected to orthodontic tooth movement. After elimination of the orthodontic force, each of the moved teeth was ligated individually to the main archwire and then retained in a new position during the rest of experimental period. Placement of the appliance and its subsequent adjustment were performed under general anesthesia with a minimum dose of sodium pentobarbital.

Bone double-labelling with tetracycline and calcein

Tetracycline (TC, 20 mg/kg b.w., Meiji Co., Tokyo, Japan) and calcein (Cal, 8 mg/kg b.w., Wako Chem. Co., Osaka, Japan) were used as bone markers, respectively, during the tooth-movement and retention periods. The markers were injected subcutaneously with an interval of 1 week in the course of the specified experimental phase. All animals remained healthy through the experimental course without any apparent symptoms related to the above regimen. At the end of the retention period, the animals were killed with an overdose of sodium pentobarbital. Both jaw bones were dissected into halves and fixed in 10% neutral formalin for 24 hours at 4°C. Each hemijaw was further excised using a hard tissue cutter (EXAKT, model BS3000N, Meiwa Co., Tokyo, Japan) to prepare specimen segments comprising the target (moved and then retained) tooth, the mesial extraction socket, and the distal supporting tooth. In order to examine the tooth movement in the anterior-posterior direction along the incisor-molar vector, radiograms of the tissue segments were recorded using a soft X-ray apparatus (Model M-60, Softex Co., Tokyo, Japan) at 30 kV and 3 mA.

Preparation of ground sections and their examination by confocal laser scanning microscopy

The fixed tissue blocks were dehydrated in an ascending series of alcohols and then embedded in a polyester resin (Rigolac, Ouken Co. Inc., Tokyo, Japan). Sagittal ground sections (100 μm thick) were prepared by hand on grindstones. Specimen thicknesses were measured using a caliper. These sections were first examined by contact microradiography (CMR) and conventional fluorescence microscopy. Microradiograms

were produced on a Softex CMR at 7 kV and 5 mA. Next, confocal images of the same sections were obtained using two commercially available systems as illustrated in Figure 1. One system (model Sarastro 2000, Molecular Dynamics Inc., Sunnyvale, California) was equipped with a single argon laser source (Ar at 488 nm), while the other (model LSM 410, Carl Zeiss, Jena, Germany) allowed us to use simultaneously two laser sources: Ar at 488 nm and ultraviolet (UV) laser at 364 nm: the former was used for detection of calcein (its excitation occurs at 495 nm and its maximum emission around 540 nm), and the latter for TC labeling (excitation at 390 nm and maximum emission around 560 nm). In each system, the optimal dichroic mirror (DM) and barrier filter (BF) were empirically selected to minimize interference between the two fluorochromes. Photographs were taken directly from the monitor after computer processing of the data and generation of pseudocolors, namely, TC by red and Cal by green.

Results and Discussion

The initial signs of gingival inflammation decreased during the course of the study at both test and control sites as a result of the plaque control regimen executed. None of the sites exhibited bleeding on probing at times of periodical examination and at the termination of the experiment. Observations of radiograms and ground sections revealed that the target teeth in one animal were tipped markedly after the treatments. Thus, those teeth were excluded from further analysis by CLSM.

Figure 2A shows a radiogram of a maxillary segment dissected from the control side. The alveolar bone crest exhibited a concave defect but the extraction socket was almost filled by bony trabeculae. No visible movement or tipping of the P3 occurred over the experimental period. The cortical bone surrounding both medial and distal roots of the tooth remained uniform with respect to thickness and degree of mineralization (Fig. 2B). Observation by conventional fluorescent microscopy (Fig. 2C) revealed that a thin, continuous layer of TC labeling (yellow) covered entirely the periodontal side of the alveolar bone, while labelings by TC (as meandering yellow lines) and Cal (spotty in green) were discerned on the endosteal side of the interradicular septum within the root bifurcation. It is to be noted that the core region of the cortical bone remained unlabelled, indicating that most of the original bone has not been remodeled during the experimental period without the orthodontic appliances.

On the experimental side of the upper jaw of the same animal, the target tooth (P3) moved mesially toward the extraction socket (Fig. 3A). Examination of a ground section by microradiography and fluorescent microscopy (Figs. 3B and 3C) showed that: (a) the cortical bone in the original tension sides (i.e., the distal region of interdental space of a distal root and the medial surface of the interradicular septum within the root bifurcation) became thickened with the apposition of TC labeled bone, which exhibited lower degrees of X-ray opacity as compared to the unlabeled (original) bone in the endosteal sides; (b) in contrast, the cortical bone in the compression sides (e.g., the distal surface of the interradicular septum) was extremely thin and most of the bone tissues were labeled by TC and Cal; and (c) a continuous yellow band was visible on the cementum surface without indication of root resorption. All observations evidenced the bodily tooth movement. The marked TC labeling of the cortical bone represents the occurrence of active bone formation with orthodontic tooth movement. Additionally, careful examination of the apposed bone at high magnification disclosed Cal labeling (green) on the periodontal side and around a large blood canal (Fig. 3D), indicative of the active bone turnover during the retention period. However, the details of TC and Cal localization remained obscure mostly due to overlapped images stemming from many planes in the thick ground section.

Figures 4A, 4B and 4C depict alveolar bone reorganization around the moved mandibular premolar (P4) of the same animal. The target lower tooth was slightly tipped, as evidenced by the non-uniform periodontal space, particularly wider in the medial and apical region of the medial root. In this case, root resorption took place in the medial and middle region of the distal root as reported recently by Brudvick and Rygh (1995). Other findings were essentially similar to those found for the maxillary alveolar bone surrounding the moved tooth, except for the observation that one third or half of the cortical bone on the original tension sides was replaced by Cal labeled bone. This outcome associated with tooth tipping suggests that modulation of the magnitude or vectorial direction of occlusal force may accelerate the kinetics of alveolar bone turnover during the retention period.

Despite the general picture on alveolar bone reorganization obtained by conventional microscopy, further distinction of TC and Cal localization in the formed alveolar bone was hindered mainly by a superposition of fluorescence at many depths in thick sections. As shown in Figures 5A, 5B and 5C, confocal imaging of the same sections reduced substantially background fluorescence, allowing us to scrutinize the TC and Cal distribution in a single plane of the alveolar bone. In those views, TC labeled areas were pseudo-colored by red, while Cal labeled areas by yellow or yellowish green. Confocal imaging of the cortical bone in the control side (Fig. 5A) unveiled parallel lamination patterns by TC and Cal

corresponding to the order of their periodic injections. This proves that even without the orthodontic appliance, alveolar bone structure changed steadily but at modest rates to accommodate to new environment after extraction of the adjacent tooth. Careful examination of those patterns indicated that the initial administration of TC produced wider bands as compared with those caused by the later administrated TC and Cal. These subtle but appreciable differences in labeling patterns appear to have reflected the rapid adjustment and attenuation of alveolar bone reconstruction after extraction of the adjacent tooth. Confocal images of the alveolar bone in the experimental sides (Fig. 5B) were characterized by homogeneous TC distribution, indicating that more extensive replacement of the original alveolar bone occurred with the tooth movement. Moreover, on the basis of Cal labeling patterns, it was interpreted that active bone formation during the subsequent tooth retention progressed in two manners: (a) advancement in lamination form from the periodontal surface toward the interior, and (b) propagation in a form of concentric waves stemming from Haversian canal walls. These observations and interpretation are in good agreement with the results reported by others (Ohya *et al.*, 1992; Roberts *et al.*, 1992). Of interest, parts of the TC labeled bone were demarcated by yellow Cal lines, giving rise to red-colored islands. With the advancement of bone deposition as observed in the experimental side of the lower jaw (Fig. 5C), the apposition of Cal laminae merged progressively with Cal rings stemming from the canal walls, leaving red islands in a row at an inward front of bone turnover. Notably, active tissue reorganization also occurred on the cementum surface in the same manner, leaving small red areas within a narrow yellowish green (Cal labeled) belt. The cementum formation in both control and experimental sides may be due in part to growth changes with tooth eruption.

The results described above indicate that both bone and cementum were actively reconstructed during the experimental period, although the magnitude of the tissue-turnover was much greater in bone. Prior to acceptance of the results based on the apparent TC and Cal localization, however, caution should be exercised due to possible interference between TC and Cal because (a) whereas Cal emission exhibits a relatively sharp spectral profile, the corresponding spectrum of TC is broad, and (b) the maxima of TC and Cal emission are close, around 560 nm and 540 nm, respectively. As demonstrated in Figure 6A, confocal imaging of dentin formed during the experimental period clearly exhibited 8 red bands (TC) and 4 yellow bands (Cal); this picture was obtained using system 1 (see Fig. 1) having a single argon laser (488 nm) and the following filter conditions: 540-560 nm for Cal detection and > 600 nm for TC. Since the laser used was optimal for excitation of Cal (495 nm), as expected, emission signals collected between 520 and 560 nm produced four sharp and intense bands corresponding to the number of Cal injections, with only marginal interference of TC emission (Fig. 6B). This discrete separation of Cal labeling supports the general features and progress of alveolar bone replacement interpreted above on the basis of Cal labeling patterns. On the other hand, the interference effect became more serious in attempts to localize TC labeling. When only the emission signals collected > 600 nm were displayed, the total 12 bands were still discernible, and almost equal in their intensity (Fig. 6C). This undesirable interference remained distinct even after selection of various filter conditions. Alternatively, the dual excitation system illustrated in Figure 2 substantially improved the differential observation of TC and Cal labelings (Fig. 7). Obviously, the TC labeling increased in intensity (see Fig. 7C), making it possible to discern the undulated interface between TC and Cal labeled areas.

In recent years, CLSM has been proven to be useful in a wide range of bone studies (Boyde and Reid, 1986; Kazama *et al.*, 1993; Piattelli *et al.*, 1993, 1994). To the best of our knowledge, the present report is the first attempt using CLSM to monitor alveolar bone formation associated with orthodontic tooth movement and retention. The results obtained so far support the view that alveolar bone and adjacent supportive tissues must be allowed to be reorganized around the newly positioned teeth over the long term. At present, it is not certain how long it takes to stabilize the moved tooth with a full strength of the supportive tissue. In this aspect, our preliminary observations showed that a complete replacement between TC and Cal labeled bone is only achieved after extended retention up to 52 weeks (unpublished data). The details of such long-term remodeling processes are currently under investigation using CLSM. Finally, it should be pointed out that orthodontically-treated teeth surrounded by newly mineralized alveolar bone are not immune to relapse, and numerous factors other than bone remodeling (e.g., occlusion, growth, jaw reflexes) are known to effect orthodontic relapse.

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Discussion with Reviewers

G.J. King: The study does not compare confocal scanning microscopy with the thinner types of sections possible today on mineralized samples. Certainly, fluorochrome resolution using conventional microscopy can be much improved over that demonstrated on the 100 μm sections shown in this paper. Did you take any thinner sections from your blocks?

Authors: We used 100 μm thick sections, simply to avoid an accidental loss of experimental material during specimen preparation. It is practically difficult to prepare thinner sections which covered a wide tissue region of dog jaw bones including two teeth and an extraction socket.

G.J. King: Radiograms were taken to examine "tooth movement in the anterior-posterior direction along the incisor-molar vector", but no measurements were taken from radiograms and they add little to the paper except some orientation to the anatomy in question.

Authors: Radiograms are included to show the relative position of the target tooth. The dog model (with its practical limitations) may not be the best to control the distance of tooth movement with orthodontic force and the stability of tooth location during the retention period. In this regard, the rat model you have used is certainly advantageous.

J. Appleton: How was the force applied to the teeth assessed?

Authors: The force was not determined *in situ*. The values were obtained using a replica model in the laboratory.

J. Appleton: How were the ground sections kept plano-parallel and how was the thickness measured?

Authors: Ground sections were prepared by a sandwich method using a pair of flat grindstones. After polishing, the thickness was measured at multiple points of a section by a caliper.