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Confocal laser scanning microscopy in study of bone calcification

Tetsunari Nishikawa[a,*], Mayu Kokubu[a], Hirohito Kato[a], Koichi Imai[b], Akio Tanaka[a]

[a] Department of Oral Pathology, Osaka Dental University, Osaka, Japan
[b] Department of Biomaterials, Osaka Dental University, Osaka, Japan

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ABSTRACT

Bone regeneration in mandible and maxillae after extraction of teeth or tumor resection and the use of rough surface implants in bone induction must be investigated to elucidate the mechanism of calcification. The calcified tissues are subjected to chemical decalcification or physical grinding to observe their microscopic features with light microscopy and transmission electron microscopy where the microscopic tissue morphology is significantly altered. We investigated the usefulness of confocal laser scanning microscopy (CLSM) for this purpose. After staggering the time of administration of calcein and alizarin red to experimental rats and dogs, rat alveolar bone and dog femur grafted with coral as scaffold or dental implants were observed with CLSM. In rat alveolar bone, the calcification of newly-formed bone and net-like canaliculi was observed at the mesial bone from the roots progressed at the rate of 15 μm/day. In dog femur grafted with coral, newly-formed bones along the space of coral were observed in an orderly manner. In dog femur with dental implants, after 8 weeks, newly-formed bone proceeded along the rough surface of the implants. CLSM produced high-magnification images of newly-formed bone and thin sections were not needed.

1. Introduction

The bone defects in the jaw bones, following extraction or tumor resection, need to heal in such a way that the regenerated or reconstructed bone ultimately affects the prosthetic rehabilitation and quality of life. Therefore, the scaffold materials that are grafted for the bone formation at sites of bony defects should promote new bone formation both in quantity and quality. Scaffold materials that are porous in structure and with rough surfaces are effective in the process of new bone formation. When missing teeth are rehabilitated by using dental implants, the rough surface implants have better outcomes in the new bone formation on the implant surface/jaw bone interface.

In the study of the process of new bone formation, hard tissues are viewed either with a light microscope or an transmission electron microscope. During this process the calcified sections are decalcified chemically or ground mechanically. Therefore, the histological findings of hard tissues are not as straightforward and are not as easy to apply as in soft tissues [1].

The confocal laser scanning microscope (CLSM) has the advantage of confocal imaging, which enables observation of different image planes at varying depths of the sample, without slicing the specimen. The confocal scanning and sectioning of a plane 0.1 μm in thickness [2,3] allow comparative study of the morphology and further histology could be evaluated using thin tissue sections. This makes it possible to recognize portions not affected by acid or mechanical damage, and tomographic images could also be detected and studied at microscopic levels [4]. In the present study, we evaluated the characteristics of CLSM and histology on newly-formed bone on the surface of materials grafted in bony defects.

2. Materials and methods

2.1. Metabolism with calcification in mandibular bone of rat

Newly-formed bone of male Wistar rats (four-weeks-old) were labeled by intra-peritoneal administration of two fluorescent dyes, calcein (Wako Pure Chemical Industries, Osaka, Japan, 2 mg/100 g rat) and alizarin red (Wako Pure Chemical Industries, Osaka, Japan, 4 mg/100 g rat), on 5 days and 3 days before sacrifice, respectively. After the rats were euthanized and subjected to perfusion fixation, their mandibles were dissected out and embedded in epoxy resin for preparation of undecalcified sections of 500 μm in thickness. The calcification of the bone growth was observed on undecalcified samples using CLSM.

* Corresponding author. Tel.: +81 72 864 3057; fax: +81 72 864 3157.
E-mail address: tetsu-n@cc.osaka-dent.ac.jp (T. Nishikawa).

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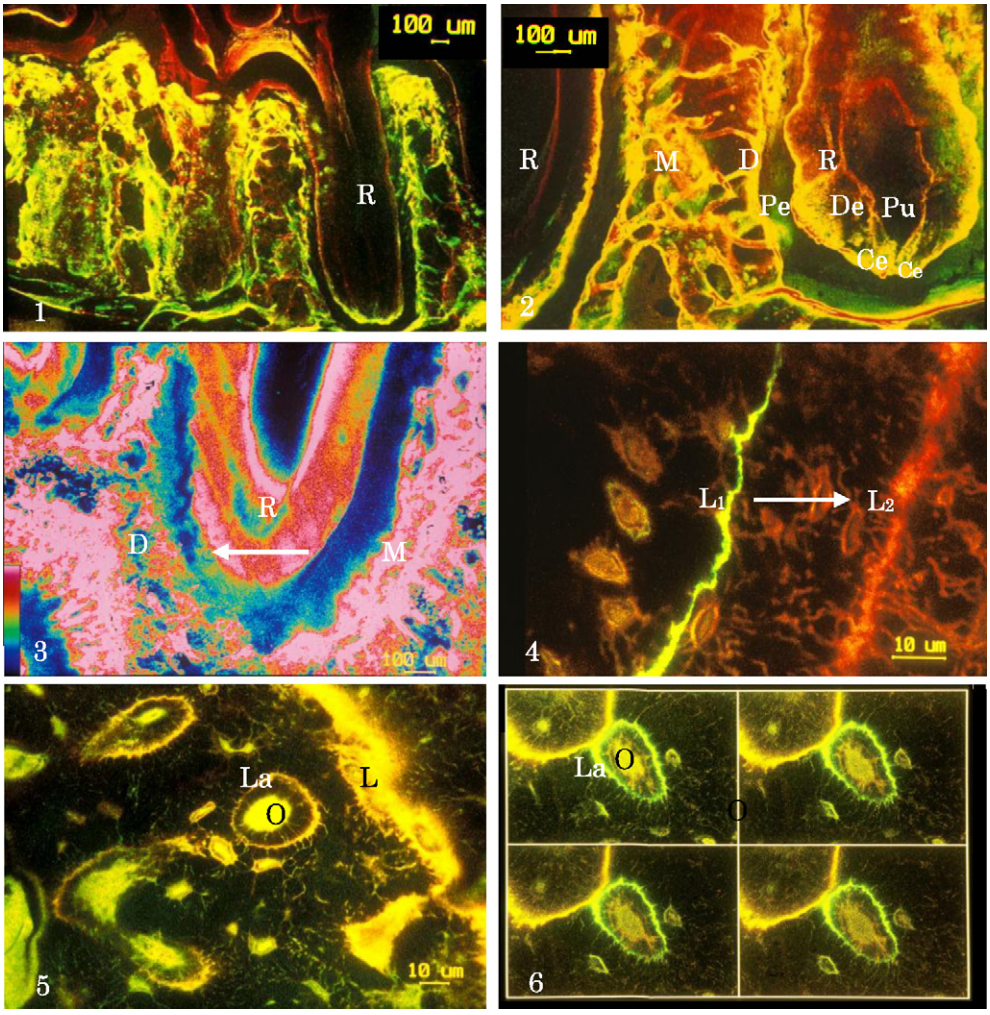

Fig. 1. CLSM image of rat alveolar bone. (1) Calcification stained with calcein as greenish and alizarin red as reddish. (2) High magnification view of alveolar bone around the roots (R). Periodontal ligaments: Pe, cementum: Ce, dentin: De, dental pulp: Pu, mesial bone: M, and distal bone: D. (3) Images of a gradated florescence intension of alizarin red. The calcification at mesial bone (M) of the root (R) was increased compared to that at distal bone (D). The mandible grew toward distal side (arrow). (4) High magnification view of rat mandible by CLSM. A greenish bone lamellae (L_1) with calcein and a reddish bone lamellae (L_2) with alizarin red were observed. The bone grew about 30 μm per 2 days toward the outside (arrow). (5) High magnification view of rat mandibular by CLSM. Lacunae: La, osteocytes: O, and lamellae: L. (6) Tomographical view of rat mandible. Parallel images at 2 μm slices from the surface of the specimen were shown. Lacunae: La and osteocytes: O. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.2. Bone regeneration at defects of dog bone grafted scaffold with surface roughness

Staghorn coral (*Acropora palifera*) deproteinized with NaOH was used as a bone scaffold. The surfaces of the corals were observed with scanning electron microscopy (SEM, S-4000, Hitachi, Tokyo, Japan). Experimental holes (4.3 mm in diameter) were made in the femur of beagle dogs, and were grafted with coral. After 4 weeks, calcein (20 mg/kg body weight) was injected in the experimental dogs. One week later, the dogs were euthanized and their femurs collected. Undecalcified sections including the holes were observed with CLSM. The bony defects without coral implantation were observed as controls.

2.3. Newly-formed bone of dog grafted dental implant with surface roughness

The third left maxillary incisors of beagle dogs were extracted, and the surrounding alveolar bones were removed to create bone defects (4.0 mm × 4.0 mm in diameter and 12 mm in depth). Crushed bone from the chin region of the same dogs was filled in around dental implants (3.75 mm in diameter and 10 mm in length, TiUnite Mart III, Nobel Biocare, Gothenberg, Sweden). Calcein (20 mg/kg body weight) was administered to the dogs by intraperitoneal injection 7 days before the sacrifice. Three days before sacrifice of dogs, alizarin red (40 mg/kg body weight) was also administered. The dogs were sacrificed at 4, 8, and 12 weeks postoperatively. Maxillary bones of dogs containing dental implants were then collected, embedded in resin, and undecalcified 500 μm thick sections were observed with CLSM.

The protocol of this study was approved by the Animal Research Committee of Osaka Dental University, and the study was conducted in accordance with the Guidelines for Animal Research at the same university. Animals were under intubation anesthesia combined with local anesthetic injection, and were administered orally following surgery to provide pain relief and prevent infection.

2.4. CLSM observation

The specimens were observed by CLSM (LSM-GB200, Olympus, Tokyo), followed by image processing *via* a computer (Vectra 386/25, Hewlett Packard, USA) to obtain tomographic images taken