Comparative Observations of Bone Formation in Peri-implant Tissue using Soft X-ray, Micro-computed Tomography and Confocal Laser Scanning Microscopy

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SYNOPSIS

Objectives: To establish a method for nondestructive observation of bone in peri-implant tissue, we compare radiographs with soft X-ray, microcomputed tomography (micro-CT) and confocal laser scanning microscopy (CLSM).

Materials and Methods: After extraction of the 3rd incisors in the maxillary bone of beagle dogs (n=3), six titanium implants were placed in the bone. Two fluorescent dyes, calcein and alizarin red, were administered to the animals at different weeks. At 4, 8 and 12 weeks after implantation, the animals were sacrificed and temporal patterns of new bone formation at the interface between implant and host bone were observed in undecalcified materials using soft X-ray, micro-CT and CLSM.

Results: Bone density was analyzed with dental X-ray and soft X-ray. Micro-CT enables three-dimensional analysis of the bone in peri-implant tissue. CLSM had the advantage of enabling observation of the process of calcification at high magnification. In addition, the analysis of CLSM showed that a little newly formed bone appeared close to the host bone at 4 weeks after implantation, after which porous bone formation tended to increase with time up to 12 weeks, and filled the screw grooves of the implant. It was concluded that these three techniques are useful in assessing the relation of bone formation to implant design and surgical methods.

Key words: Implant; Soft X-ray, Microcomputed tomography, Confocal laser scanning microscopy, Bone

INTRODUCTION

Bone formation in peri-implant tissue is a key factor in maintaining implant stability, and is influenced by implant design, surgical factors, and post operative infection\(^1,2\). Biological analysis of newly formed bone in peri-implant tissue have been reported using scanning electron microscope (SEM)\(^3\), radiography\(^4\), microcomputed tomography (micro-CT)\(^5\), confocal laser scanning microscopy (CLSM)\(^6\), and tissue sections examined by routine light microscopy\(^7\). Radiography with soft X-ray is a nondestructive
procedure that enables observation of both bone formation and bone density\(^4\). Micro-CT enables three-dimensional analysis of the peri-implant tissue morphology during bone formation\(^5\). CLSM has been used to study undecalcified specimens of bone labeled with fluorescent dyes, in order to elucidate the process of calcification of newly formed bone around implants\(^9\).

In the present study, bone defects were created in the maxillae of beagle dogs, and implants were positioned together with autologous bone fragments containing platelet-rich plasma (PRP) to promote bone formation\(^10\). Calcein and alizarin red were administered to facilitate observation of newly formed bone by CLSM. The aim of the present study was to establish a nondestructive method to compare these three detection techniques. At the same time, evidence obtained with the use of these methods will provide a clearer picture of the process of new bone formation.

MATERIALS AND METHODS

1. Animals

The protocol 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 Osaka Dental University.

Three female beagle dogs weighing 10–12 kg were used in the experiment. With the animals under general anesthesia, the 3rd right and left maxillary incisors were extracted and the surrounding alveolar bone was ablated with a round bur to create bone defects measuring 4 mm in diameter and 12 mm in depth. Into these defects were then placed titanium implants (TiUnite Mark III, Nobel Biocare, Sweden; 3.75 mm x 10 mm) together with autologous PRP using SmartPreP\(^\text{TM}\) (Harvest, USA) and crushed chin region bone for filler material. Wounds were closed by suturing.

Calcein (Wako Pure Chemical Industries, Osaka, Japan; 20 mg/kg body weight) and alizarin red (Wako Pure Chemical Industries, Osaka, Japan; 40 mg/kg body weight) in physiological saline solution were administered to the animals by intraperitoneal injection. Calcein was administered at seven days before the collection of specimens and alizarin red at three days before. Animals were sacrificed by overdose with sodium pentobarbital, at 4, 8, and 12 weeks postoperatively.

2. Specimen preparation

Maxillary bone blocks containing the implants were dissected free of the animals, fixed in 10% phosphate buffered formalin, and dehydrated using graded ethanol alcohols. Specimens were then embedded in polymethyl methacrylate resin, and cut longitudinally along the center of the long axis, using a micro cutting machine (Exakt 30/784, EXAKT Vertriebs GmbH, Nordstedt, Germany). Thus, sections representing the mesial-distal aspects of each block were prepared.

3. X-ray

Dental X-ray (MAX-DC70, J. MORITA MFG. CORP., Japan) was used to examine the specimens at a tube voltage of 70 kV and 3 mA for 0.17 sec., and soft X-ray irradiator (Softex, CMB-2, Japan) using a tube voltage of 40 kV and 2 mA for 20 sec. The developed films were scanned with a scanner (CanoScan 9950F, Canon, Japan) at 1200 dpi and 12 bit gray scale and stored in jpeg format.

4. Microcomputed tomography (micro-CT)

A series of images of the maxillary bone including the implant were obtained using a micro-focus X-ray system (SMX-130-SV3, Shimadzu, Kyoto, Japan). The exposure conditions were
**Fig 1.** Radiographic images with dental X-ray. 1a; at 4 weeks after implantation, 1b; at 8 weeks, 1c; at 12 weeks.

**Fig 2.** Radiographic images with soft X-ray. 1a; at 4 weeks after implantation, 1b; at 8 weeks, 1c; at 12 weeks.

**Fig. 3.** Slice images with micro-CT at 4 weeks after implantation. 3a; cross sectional image at 1 mm distance from the bottom implant, 3b; cross sectional image at 2 mm, 3c; cross sectional image at 3 mm, 3d; cross sectional image at 4 mm, 3e; slice image of vertical section, with appearance of an artifact (arrow). 3f; three-dimensional image.
56 kV and 45 μA at 12.8 μm intervals for 13.2 μm thick slices. The information from all slices was saved and the images were reconstructed with a computer (Macintosh G3 266; Apple Computer, Cupertino, CA). After reconstruction, microstructural data of the bone block including the implants were obtained, and slice images at the center of the long axis, cross sectional images at distances of 1 mm, 2 mm, 3 mm, and 4 mm, from the bottom of the implant, and three-dimensional images were produced.

5. Confocal laser scanning microscopy (CLSM)

Samples were examined using an inverted CLSM (LSM-GB200, Olympus, Tokyo, Japan; excitatory argon laser wavelength 488 nm). Both a 535 nm pass filter (CH1) and a 590 nm barrier filter (CH2) were used. Calcein was detected as a greenish color (CH1) and alizarin red appeared as a reddish color (CH2). To obtain the implant image, both the 535 nm and 590 nm filters were removed, and reflected light only, which was collected through CH2, was recorded as a bluish color. Next, a composite achieved by superimposition of the images was obtained from detection of both calcein and alizarin red, and the implant image.

RESULTS

1. Radiograph

At 4 weeks after the implantation, radiopacity was seen at the deep part of the implant (Figs. 1a, 2a). At 8 weeks postoperatively, increased bone formation and density were seen around the implant (Figs. 1b, 2b), and at 12 weeks postoperatively, further increased bone formation and density were seen in the peri-implant tissue (Figs. 1c, 2c). Radiographic images with soft X-ray (Figs. 2a, b, c) were finer than those with dental X-ray (Figs. 1a, b, c)

2. Micro-CT

Slice images at 4 weeks after the implantation showed almost no bone formation in peri-implant tissue in the space between the bone and the implant (Figs. 3a, b, c, d, e). In addition, an artifact of reflective X-ray from metallic implant was seen around the implant (Fig. 3e). A wide gap was evident around the implant on a cross sectional image (Figs. 3a, b, c, d). On a three-dimensional image, there was little bone formation around the implant (Fig. 3f). Slice images at 8 weeks postoperatively showed the bone formation in the peri-implant tissue (Figs. 4a, b, e). However, some areas had no bone formation (Figs. 4c, d). On a three-dimensional image, bone formations, with many spheroidal cavities of various sizes were seen (Fig. 4f). Slice images at 12 weeks postoperatively showed a lot of bone formation in the peri-implant tissue (Figs. 5a, b, c, d, e). On cross sectional images, increased bone mass was seen in direct contact with the implant surface (Figs 5a, c). However, there were also some areas without bone formation (Figs. 5b, d). On the three-dimensional image, there was increased porous bone mass, which wrapped the deep portion of the implant (Fig. 5f).

3. CLSM

At 4 weeks after the implantation, a little newly formed bone was seen close to the host bone, with the direction of bone formation indicated by sequential calcine and alizarin red labeling (Fig. 6a). At 8 weeks postoperatively, fine porous bones were seen extending through to the implant (Fig. 6b). At 12 weeks postoperatively, the porous bone formation spread from the host bone to the implant surface, and filled the screw grooves of the implant (Fig. 6c, d). In addition, non-calcified tissue of at least 10 μm was seen between new bone and
Fig. 4. Slice images with micro-CT at 8 weeks after implantation. 4a; cross sectional image at 1 mm distance from the bottom implant, 4b; cross sectional image at 2 mm, 4c; cross sectional image at 3 mm, 4d; cross sectional image at 4 mm, 4e; slice image of vertical section, 4f; three-dimensional image.

Fig. 5. Slice images with micro-CT at 12 weeks after implantation. 5a; cross sectional image at 1 mm distance from the bottom implant, 5b; cross sectional image at 2 mm, a narrow gap was seen. 5c; cross sectional image at 3 mm, 5d; cross sectional image at 4 mm, a narrow gap was seen. 5e; slice image of vertical section, 5f; three-dimensional image.
Fig. 6. Histological images with CLSM. 6a; at 4 weeks after implantation (I: implant, B: newly formed bone), 6b; at 8 weeks (I: implant, B: newly formed bone), 6c and d; at 12 weeks (I: implant, B: newly formed bone), a narrow space in the peri-implant tissue (arrow), 6e; high magnification image at 12 weeks (B; newly formed bone, O; osteoblast).
the implant surface (Fig. 6d). High magnification images revealed many osteoblasts labeled with alizarin red near the newly formed bone (Fig. 6e).

**DISCUSSION**

An exact understanding of the features of bone formation in peri-implant tissue is important in clinical practice optimum implant design and surgical technique\(^{11}\). It is well known that thin paraffin sections are impossible to prepare from specimens containing implants, and that observation with light microscopy is difficult. There is loss of organic substances as a result of chemical solution under decalcification, and also physical damage occurs in peri-implant tissue as a result of implant removal. In the present study, we have compared the respective advantages of X-ray, micro-CT, and CSLM in non-invasive observation of peri-implant bone tissues.

With X-ray, specimens are observed while still embedded in resin, so there is no chemical or physical damage to the specimen. If specimen thickness is uniform, bone density can be estimated. Moreover, dental X-ray images can be used for X-ray diagnosis, and they make it possible to observe the bone while the animal is alive.

With soft X-ray the energy is weaker than with normal dental X-ray and so images can be taken for a longer time, and fine information can be obtained\(^{12}\). In the present experiment, bone formation in the peri-implant tissue and bone density increased with time up until 12 weeks after the implant was placed.

With micro-CT, X-ray images are taken from different angles around the specimen, and cross-sectional images on any plane and three-dimensional images can be obtained using a computer\(^{8,13}\). The bone formation seen with X-ray and CSLM in the present study was not expressed in some areas around the implant on micro-CT images. It was also found that the opposite was true in some cases. This indicates that bone formation in the peri-implant tissue was non-uniform, and observation with the use of two-dimensional images was insufficient. Thus, tomographic images are necessary, but reflected X-rays from the metallic implant appear as an artifact.

With CLSM, an aspect of the specimen is observed while the specimen is still embedded in resin. Since the same image is observed as with a specimen having a thickness of 0.1 μm, a high-magnification image finding can be obtained\(^{14}\). CLSM requires that the tissue to be observed emits fluorescence, for which it is necessary to administer a fluorescent dye such as tetracycline\(^{15}\), calcein, or alizarin red that is involved in calcium dynamics\(^6\). With these dye reagents it is difficult to distinguish between host bone and new bone in normal tissue specimens, but with CLSM only newly formed bone is observed. In addition, by administering two different kinds of fluorescent dyes, the progress and direction of bone growth can be assessed\(^{14}\). With CLSM any depth from the bone surface can be observed, and cross-sectional and three-dimensional images can be obtained at the histological level\(^6\). However, in the specimens containing implants, the implant does not allow penetration of laser light. As a result, observation of bone formation in deep portions is virtually impossible unless measures such as changing the direction of the laser irradiation are taken.

The results obtained from the non-destructive observations revealed processes of new bone formation in the peri-implant tissue. First, a little bone formation appeared close to the host bone at 4 weeks after the implantation. At 8 weeks postoperatively, newly formed bone had increased through to
the implant. At 12 weeks, the newly formed bone with porous appearance had spread to the implant surface and filled the screw grooves of the implant. A narrow space of at least 10 μm was seen separating the new bone from the implant surface. At high magnification, many osteoblasts were seen near the newly formed bone.

Non-destructive analysis, soft X-ray, micro-TC, and CLSM have advantages and disadvantages in examining for target tissues and materials, in non-calcified or well calcified bones existing in peri-implant tissue, and also metal implant materials. X-ray images can be used for bone density analysis, and they can be obtained in living subjects. Micro-CT enables three-dimensional analysis. CLSM makes it possible to the morphology in the process of calcification at high magnification. Exact examination of newly formed bone around implants, by taking advantage of the strengths of these individual techniques may be beneficial in achieving the final goal of successful implant technology. It was concluded that three detection techniques are advantageous for assessment of bone formation, and can provide useful information for optimum implant design and surgical methods.

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