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Assessment of the Bone Regenerative Process from Fibular Periosteum by *in vivo* Micro Computed Tomography

Takayuki Mashimo^{1,2)}, Tadahito Saito^{1,2)}, Hiroshi Shiratsuchi^{1,2)}, Jun Iwata^{1,2)}, Takeshi Uryu^{1,2)},
Takaaki Tamagawa^{1,2)}, Shunsuke Namaki²⁾, Kunihito Matsumoto³⁾, Shouji Kawashima³⁾,
Yoshiyuki Mori⁴⁾, Yoshinori Arai⁵⁾, Kazuya Honda^{3,6)} and Yoshiyuki Yonehara^{2,7)}

¹⁾ Division of Oral Structural and Functional Biology, Nihon University Graduate School of Dentistry, Tokyo, Japan

²⁾ Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, Tokyo, Japan

³⁾ Department of Oral and Maxillofacial Radiology, Nihon University School of Dentistry, Tokyo, Japan

⁴⁾ Department of Oral and Maxillofacial Surgery, The University of Tokyo Hospital, Tokyo, Japan

⁵⁾ Nihon University School of Dentistry, Tokyo, Japan

⁶⁾ Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

⁷⁾ Division of Systemic Biology and Oncology, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

(Accepted for publication, June 20, 2013)

Abstract: The aim of this study was to examine the bone regenerative process from fibular periosteum in rats. Twenty male Wistar rats were divided into two groups : a periosteum preservation (PP) group (n=15) and a periosteum removal (PR) group. In the PP group, the fibula was totally removed, but the periosteum and blood supply were preserved. In the PR group (n=5), the fibula was totally removed, including the periosteum. Radiological and histological findings were evaluated after operation. In the PP group, the increase in regenerative bone volume was highest at 1 week. At 2 weeks, the bone volume decreased transiently, but then continued to increase gradually until 4 weeks. There was little change after 4 weeks. The regenerative bone mineral density continued to increase gradually from 5 days until 8 weeks. In the PR group, there was no evidence of regenerative bone. These results suggest that the periosteum has osteogenic capacity and the peak of bone regeneration from the periosteum occurs around 4 to 6 weeks.

Key words: Fibula, Periosteum, Regenerative bone, Micro CT

Introduction

Maxillofacial bone defects resulting from tooth extraction, trauma, or tumor resection have been repaired with autogenous bone grafts or artificial materials. Autogenous bone grafting is generally an effective technique, but has disadvantages such as donor-site morbidity. Artificial materials have been developed to overcome the disadvantages of autogenous bone grafts, but can cause adverse effects such as foreign-body reactions. Since both autogenous bone grafts and artificial materials have inherent advantages and disadvantages, it is necessary to develop new reconstruction materials. We have focused on the use of periosteum as a new material with low morbidity and no foreign-body reactions. The use of bone regenerated from periosteal grafts is considered clinically significant.

The periosteum is a connective tissue that covers the surface

of bone. This tissue consists of two layers, a thick outer fibrous layer and an inner osteogenic layer (cambium layer)¹⁾. The cambium layer plays an important role in osteogenesis. The osteogenic capacity of periosteum was first reported by Duhamel et al.²⁾ in 1739. However, stable results with free periosteum grafts were not obtained in experimental studies, and the osteogenic potential of periosteum remained controversial³⁻⁵⁾. Clinically, Skoog^{6,7)} introduced the use of maxillary periosteal flaps for primary repair of alveolar clefts in the 1960s. Ritisila et al.⁸⁾ used free periosteal grafts from the tibia for primary repair of the palate. These reports restimulated interest in the osteogenic capacity of periosteum. Several studies reported that vascularized periosteum has good osteogenic properties, suggesting that vascularity has an important role in periosteum-related osteogenesis⁹⁻¹²⁾. In addition, Acland and van den Wildenberg et al.^{13,14)} reported that mechanical stress influences the osteogenic capacity of periosteum. Takato et al.^{15,16)} found that the osteogenic capacity of periosteum depends not only on weight and stress, but also on the volume of periosteum and the blood circulation. Uddströmer et al.^{17,18)}

Correspondence to: Dr. Takayuki Mashimo, Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan; Tel: +81-3-3219-8102; Fax: +81-3-3219-8355; E-mail: deta10027@g.nihon-u.ac.jp

Figure 1. Intraoperative images.

A: The epiphysis of the fibula was held by surgical clips. (scale bar: 5 mm); B: The fibula was pulled out in the peripheral direction with the surgical clips. (scale bar: 5 mm); C: The removed fibula with the periosteum preserved in the PP model. (18 mm in length); D: The removed fibula with the periosteum removed in the PR model. (18 mm in length)

Figure 2. Measurement of regenerative bone.

A: The line indicates the longest length of regenerative bone in the longitudinal plane images. (scale bar: 5 mm); B: The line indicates the longest diameter of regenerative bone in the cross-sectional plane images. (scale bar: 5 mm); C: The square indicates the measuring range of regenerative bone volume and mineral density. (scale bar: 5 mm)

demonstrated that periosteum is intimately involved in fracture healing. Oni et al.^{19,20)} found that removing the periosteum prevents fracture healing. Li et al.²¹⁾ showed that cartilaginous and osteoid bone derived from periosteum participate in fracture healing. Thus, the periosteum has been reported to have osteogenic potential, but the bone regenerative process from periosteum, including the time course of osteogenesis, regenerative bone volume and bone mineral density, remains poorly understood.

Vascularized fibula grafting has been shown to be an effective technique for reconstruction of the maxillofacial region^{22,23)}. However, the disadvantages of this technique are restricted availability of suitable grafts and high invasion associated with acquiring bone from donor sites. If regenerative bone from fibular periosteum could be used in place of direct bone grafts, it would be possible to reconstruct jaw bones without directly using the fibula.

A better understanding of the timing and properties of regenerative bone from periosteum may provide important clues to the optimal timing of periosteal grafting. Recently, most studies of periosteum examined the repair process in segmental bone defect models and bone fracture models²⁴⁻²⁷⁾. Few studies have focused exclusively on fibular periosteum. Studies examining the details of bone regeneration from periosteum in the same subjects are extremely rare.

A recently developed micro-computed tomography (micro-CT) system, R_mCT® (Rigaku Co., Tokyo, Japan), has made it

possible to obtain images of anesthetized experimental animals. Clear hard tissue images of small animals can be obtained with short exposure time and low dose. A lot of studies have proven the value of micro-CT for the assessment of changes over time in the animal experiments^{28,29)}. Saito et al.³⁰⁾ have concluded that micro-CT is suitable for evaluating bone regenerative process after mandibular condylectomy in rat model and periosteum plays a critical role in bone regeneration of mandibular condyle. However, the detail of regenerative process of long bones such as fibula is unclear. Thus, we conducted the assessment of the detailed process of bone regeneration from fibular periosteum of experimental animals over time using micro CT.

Materials and Methods

Animals

Twenty 6-week-old male Wistar rats with a mean body weight of 130 g (Sankyo Laboratory, Tokyo, Japan) were used in the study. The rats were divided into two groups : a periosteum preservation (PP) group (n=15) and a periosteum removal (PR) group (n=5). The animals were housed in an experimental animal room (22 °C, 55% relative humidity, and a 12-h light/dark cycle) and fed a standard laboratory diet. Water was provided ad libitum. The Animal Experimentation Committee of the Nihon University School of Dentistry approved this study.

Surgical Procedure

All surgical procedures were performed with the animals under intraperitoneal anesthesia with sodium pentobarbital (50 mg/kg body weight; Somnopentyl, Schering-Plough, Munich, Germany). The surgical site was shaved, and the skin was washed with 70 % ethanol. The site was then locally anesthetized with an intramuscular injection of 1 ml 2 % lidocaine (Xylocaine, Astra-Zeneca, Osaka, Japan). A lateral skin incision about 20 mm in length was made in the lower leg, and a smaller incision was made into the gastrocnemius muscle. The muscle was retracted to expose the connections of the tibia and fibula. An osteotomy was performed at the epiphysis of the fibula.

In the PP group, the gastrocnemius muscle and periosteum were detached from the fibular diaphysis, and the fibula was totally

Figure 3. CT images in the PP model.

A: Longitudinal plane images are shown. The yellow arrow indicates the regenerative bone; B: Cross-sectional plane images are shown. The indicates the regenerative bone; C: Three-dimensional (3D) images are shown. The arrow indicates the regenerative bone, and the yellow arrowhead indicates the articular cartilage.

Figure 4. CT images of the PR model.

A: Longitudinal plane images are shown. No regenerative bone was observed; B: Cross-sectional plane images are shown. No regenerative bone was observed; C: Three-dimensional (3D) images are shown. No regenerative bone was observed, but the articular cartilage was evident (arrowheads).

removed by pulling peripherally with the use of surgical clips; the blood supply to the periosteum was maintained (Fig. 1-A, B, C). In the PR group, the same procedure was performed; however, the periosteum was not detached and removed with the fibula (Fig. 1-D). After the fibula was removed, the muscle and skin were closed with 5-0 nylon sutures (Bear Medic Co., Tokyo, Japan). Rats were sacrificed 5 at days and 1, 2, 4, 6, and 8 weeks after operation.

Radiographic analysis

Before operation, immediately after operation, and 3 days, 5 days, and 1, 2, 4, 6, and 8 weeks after operation, the bone-defect region underwent radiographic analysis by *in vivo* micro computed tomography system (R_mCT; Rigaku Co., Tokyo, Japan). Longitudinal, cross-sectional, and 3-dimensional images (3D

images) were examined. During exposure, the rats were anesthetized with isoflurane (DS Pharma Animal Health Co. Ltd., Osaka, Japan). The exposure conditions were 17 seconds at 90 kv/100 mA. The image volume was a cylinder 2.4 cm in diameter and 2.4 cm high with a voxel matrix size set to $481 \times 481 \times 483$. The longest lengths and diameters of regenerative bone were measured in millimeters (mm) with the use of image reconstruction software (i-VIEW-R; Morita Co., Kyoto, Japan) (Fig. 2-A, B).

Quantitative analysis

The bone volume and bone mineral density of the whole regenerative bone were measured in cubic millimeters (mm^3) and milligrams per cubic centimeter (mg/cm^3) with the use of quantitative analysis software (3-by-4 viewer 2011, Kitasenju Radist Dent, Tokyo, Japan) (Fig. 2-C).

Figure 5. Time course of the length of the regenerative bone. The length tended to increase gradually until 8 weeks. Each bar indicates the mean $\pm$ SD of five independent experiments.

Figure 6. Time course of the diameter of the regenerative bone. The diameter of the cross-sectional image was thickest at 1 week, but then gradually decreased. Each bar indicates the mean $\pm$ SD of five independent experiments.

Figure 7. Time course of regenerative bone volume. Bone volume increased remarkably at 1 week ($p < 0.05$), but decreased at 2 weeks. After 2 weeks, bone volume increased slightly up to 4 weeks. Little change was seen from 4 weeks to 8 weeks. Each bar indicates the mean $\pm$ SD of five independent experiments.

Figure 8. Time course of regenerative bone mineral density. Bone mineral density continued to increase gradually up to 6 weeks. There was little change between 6 and 8 weeks. Each bar indicates the mean $\pm$ SD of five independent experiments.

Table 1. Mean of Each Measurements in the Regenerative Bone

	3 days	5 days	1 week	2 weeks	4 weeks	6 weeks	8 weeks
Length of bone (mm)	0	10.29 (0.73)	11.07 (0.31)	12.43 (0.64)	14.90 (0.29)	15.60 (0.59)	16.06 (0.54)
Diameter of bone (mm)	0	1.36 (0.24)	1.45 (0.18)	0.99 (0.06)	0.87 (0.17)	0.81 (0.11)	0.76 (0.05)
Bone volume (mm³)	0	2.97 (0.39)	5.01 (0.15)	2.53 (0.38)	2.89 (0.51)	2.87 (0.59)	2.97 (0.52)
Bone mineral density (mg/cm²)	0	396.75 (0.39)	465.63 (0.15)	483.63 (0.38)	508.5 (0.52)	555.38 (0.59)	559.25 (0.52)

The mean $\pm$ SD of five independent experiments ($\pm$ SD)

Statistical analysis

These data expressed as the mean $\pm$ SD for each group. Statistical difference were analyzed using Scheffe's test. Values of $P < 0.05$ were considered statistically significant.

Histological analysis

Five days and 1, 2, 4, 6, and 8 weeks after surgery, regenerative bone was extirpated and fixed in 4 % paraformaldehyde phosphate buffer solution. The resected specimens were decalcified in 10% EDTA (Dojindo, Kumamoto, Japan) for 10 days at 4 °C. The

specimens were then dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned transversely at a thickness of 8 μ m. The sections were stained with hematoxylin and eosin. Chondrocyte were identified by alcian bule staining, and osteoclasts were identified by tartrate-resistant acidic phosphate (TRAP) staining using a TRAP/ALP stain kit (Wako, Tokyo, Japan). Histologic examination was performed under a light microscope equipped with a morphometric system connected to a personal computer.

Figure 9. The section at the center of regenerative bone in the PP model (hematoxylin-eosin staining, 200-fold magnification, cross sections, scale bar: 100 μ m)

A: 5 days after operation; B: 1 week after operation; C: 2 weeks after operation; D: 4 weeks after operation; E: 6 weeks after operation; F: 8 weeks after operation; (Black arrow: periosteum. Yellow asterisk: woven bone. Green asterisk: lamellar bone.)

Figure 10. The section at the epiphysis of regenerative bone in the PP model (5 days)

A: hematoxylin-eosin staining, 200-fold magnification, cross sections, scale bar: 100 μ m (black asterisk: cartilage-like tissue); B: alcian blue staining, 200-fold magnification, cross sections, scale bar: 100 μ m (black asterisk: cartilage-like tissue)

Fig. 11. The section at the center of regenerative bone in the PP model (Tartrate-resistant acidic phosphate (TRAP) staining, 200-fold magnification, cross sections, scale bar: 100 μ m).

A: 5 days after operation; B: 1 week after operation; C: 2 weeks after operation; D: 4 weeks after operation; E: 6 weeks after operation; F: 8 weeks after operation; (Black arrowhead: TRAP⁺ osteoclast)

Results

Radiographic analysis

Regenerative bone on the fibula was examined using micro computed tomography and was quantified with reconstruction software (i-VIEW) in cross-sectional and longitudinal planes. Immediately after operation, both groups showed that the fibula had been totally removed (Fig. 3, 4). The CT images of the PP groups showed initial signs of bone formation on the fibula 5 days after operation (Fig. 3-A, B, C; yellow arrow). Bone regeneration began at the center of the diaphysis and gradually progressed to the epiphysis (Fig. 3-A, C). Three-dimensional images of regenerative bone showed that the bone surface was rough at 5 days and 1 week, but then gradually became smooth up to 8 weeks (Fig. 3-C). Morphometric analysis of CT image slices showed that the length of regenerative bone on longitudinal images continued to increase gradually until 8 weeks (Fig. 5, Table 1). The diameter of regenerative bone on cross-sectional images was thickest at 1 week, but then gradually decreased until 8 weeks (Fig. 6, Table 1). At the knee joint, epiphyseal cartilage had increased (Fig. 3-C, yellow arrowhead), but this tissue was not studied because it was not bone regenerated from fibular periosteum, but tissue regenerated from the stump of cartilage. In the PR group, the stump of the fibula at the knee joint increased (Fig. 4-C, yellow arrowhead), but there was not bone regeneration at the center of the diaphysis up to 8 weeks (Fig. 4-C).

Quantitative analysis

Quantitative analysis of bone regeneration on the fibula was performed with analysis software (3-by-4 viewer 2011) to estimate regenerative bone volume and bone mineral density (Fig. 2-C). In the PP group, regenerative bone volume increased remarkably at 1 week, but decreased at 2 weeks. After 2 weeks the bone volume increased slightly up to 4 weeks, but there was little change thereafter (Fig. 7, Table 1). In contrast, regenerative bone mineral density continued to increase gradually up to 6 weeks. There was little change after 6 weeks (Fig. 7, Table 1). In the PR group, there was no evidence of regenerative bone during the 8 weeks of observation.

Histological findings

In the PP group, woven bone surrounded by periosteum (Fig. 9-A, black arrow) emerged at the center of regenerative bone at 5 days (Fig. 9-A, yellow asterisks) and was present until 2 weeks (Fig. 9-B, C, yellow asterisks). During 2 to 4 weeks, the woven bone changed to lamellar bone (Fig. 9-D, green asterisks). After 4 weeks, there were no significant changes in the histological findings (Fig. 9-D, E, F). At the epiphysis of regenerative bone, cartilage-like tissue was observed at 5 days (Fig. 10-A, black asterisk). The cartilage-like tissue was stained with alcian blue. In addition, at the diaphysis of that, TRAP⁺ osteoclasts were

observed inside the regenerative bone at 5 days and 1 week (Fig. 11-A, B). At 2 weeks and 4 weeks, TRAP⁺ osteoclasts were observed not only inside the bone, but also around the bone (Fig. 11-C, D). At 6 weeks and 8 weeks, TRAP⁺ osteoclasts were observed only around the bone (Fig. 11-E, F).

Discussion

The present study showed that when the fibula was totally removed, and the periosteum and its blood supply were preserved (PP group), bone formation was evident. However, when the fibula was removed including the periosteum (PR group), there was no bone formation at all. These results suggest that the periosteum has osteogenic capacity. As mentioned above, many investigators have reported the osteogenic capacity of the periosteum¹⁻¹⁸. Bone regeneration from the periosteum was found to depend on mechanical stress and blood supply^{16,17}. However, the most important events in bone formation occur at the cellular level. Recent studies have demonstrated that progenitor cells, such as mesenchymal stem cells (MSCs), exist inside the periosteum, especially in the inner cambium layer^{31,32}. Such MSCs can form bone *in vitro* and *in vivo*^{33,34}. MSCs derived from periosteum have a greater osteogenic potential *in vitro* than those derived from other local tissues, such as adipose tissue or synovium³⁵. The PP group showed obvious bone formation, whereas the PR group showed no bone formation. The reason for the difference between the groups was attributed to the presence of MSCs. However, we could not identify the existence of MSCs in periosteum, because we did not investigate marker proteins expressed by MSCs, such as CD29, CD90, and CD105³⁶. Further research is needed on the detection of MSCs by immunohistochemical staining.

In the present study, the time course of bone regeneration was followed in the same subjects. The regenerative bone at defects was examined in detail by means of *in vivo* micro CT. Micro CT showed that bone formation began at the center of the diaphysis and gradually progressed to the epiphysis in the PP group. Morphometric analysis revealed that the regenerative bone tended to change into long and narrow bone. These changes in the bone appeared to be due to the fibular periosteum promoting extension and growth of the tibia and gastrocnemius muscle. With the use of analysis software, it was possible to quantitatively assess regenerative bone. In the PP group, bone volume clearly increased until 1 week, but then decreased at 2 weeks. After 2 weeks, the bone volume gradually tended to increase again until 8 weeks. However, there was no obvious difference between 4 and 8 weeks. The bone mineral density continued to increase until 6 weeks, although there was no apparent difference between 6 and 8 weeks. Three-dimensional images of regenerative bone showed that the bone surface was rough at 5 days and 1 week, but then gradually became smooth up to 8 weeks. In addition, HE staining of the

sections showed that regenerative bone from periosteum was initially observed as woven bone at the epiphysis at 5 days. The woven bone gradually changed to lamellar bone up to 8 weeks. There were no significant histological changes from 4 to 8 weeks. These findings showed that immature bone was replaced by mature bone throughout bone remodeling. Remodeling is responsible for changes in bone shape and mass to renew bone, associated with the presence of osteoblasts and osteoclasts³⁷⁾. In this study, we used TRAP staining to detect the localization of osteoclasts. Localization of TRAP⁺ osteoclasts changed with time. TRAP⁺ osteoclasts were found only inside woven bone at 5 days to 1 week, inside and around bone at 2 to 4 weeks, and only around lamellar bone at 6 to 8 weeks. These results suggest that remodeling occurs within regenerative bone at an early stage, and the site of the remodeling shifts to the bone surface with bone mature at the final stage. Remodeling of mature bone is generally thought to occur at the cortical bone surface³⁷⁾. Therefore, our study demonstrated the mature process of regenerative bone during 8 weeks. Our findings suggested that the development of mature regenerative bone from periosteum was completed at 4 to 6 weeks. Most previous studies using fibular periosteum examined the repair of segmental bone defects as a model of fracture healing¹⁹⁻²¹⁾. These studies showed that bone repair involves ossification in the fracture gap and subperiosteal direct bone formation over cortical bone^{24, 25)}. These previous investigations examined the mechanism of bone fracture healing and did not focus only on the function of the periosteum. Another purpose of these studies of fibular defects was to observe bone regeneration using particulate artificial bone, cells, and biomaterials^{26, 27)}. The results focused on conditions associated with the formation of more regenerative bone. To our knowledge, no previous study used a model lacking fibula in which the blood supply to the periosteum was maintained to directly investigate the periosteal function. The PP model we used specifically allowed assessment of the functions of fibular periosteum for the first time. However, the process of bone regeneration in our model was similar to that previously reported in segmental bone defects.²⁴⁻²⁷⁾ These regenerative bone in previous reports were thick in the early stage, but gradually became thinner during processes such as fracture healing³⁸⁾. In our previous study, a similar process was observed in a temporomandibular joint defect model in which the periosteum was maintained³⁵⁾. It is interesting to note that the process of bone regeneration from only periosteum was associated with a transient decrease in bone volume. This phenomenon can be explained by the mechanism of bone-fracture healing. After fracture, a hematoma forms beneath the periosteum, and the hematoma is replaced by callus bone, which has a woven structure. The bone undergoes repeated resorption and formation by remodeling and changes to lamellar bone. Consequently, repaired bone becomes thin and functional³⁸⁾. These processes underlie bone regeneration in the PP model.

In addition, nearly all previous studies reported that rat fibular defects were repaired in 4 to 8 weeks^{24, 26, 27)}. We believe that the regenerative process described in past reports followed a similar course as bone regenerated from only periosteum.

Another similarity with previous studies was the ossification pattern of bone regenerated from fibular periosteum. Periosteum of long bones such as the tibia and fibula has osteogenic capacity due to endochondral ossification, while that of membrane bones such as the calvaria has osteogenic capacity due to membranous ossification³⁹⁾. We expected that regenerative bone in this study would be formed by endochondral ossification because our model used fibular periosteum. Endochondral ossification is a process in which bone replaces cartilage. In this type of ossification, formed cartilage is replaced by bone from the central part, and replacement of cartilage by bone then proceeds longitudinally³⁹⁾. Radio-opacity images at 5 days indicated where bone replaced cartilage, but could not identify cartilage tissue. However, histological examination at 5 days showed the presence of woven bone at the center of regenerative bone, but cartilage tissue at the epiphysis. This cartilage tissue indicated the top of the epiphysis during endochondral ossification. We thus demonstrated that bone regenerated from fibular periosteum was formed by endochondral ossification.

Clinically, there appear to be two methods to promote the formation of regenerative bone from periosteum. The first method is to induce the formation of regenerative bone by securing a space between bone and periosteum at the donor site. The second method is to form bone at the recipient site by grafting vascularized periosteum. Thus, if bone regenerated from fibular periosteum can be used for bone grafts, it is possible to reconstruct the jawbone without using the fibula itself. We believe this is clinically significant. Our results are promising because they showed that the properties of regenerative bone depend on the observation period. The volume of bone derived from periosteum was greatest at 1 week, but the bone was immature. At 4-8 weeks, the bone was more mature than that at 1 week with respect to bone mineral density and histological characteristics. This study shows that it is necessary to determine the optimal timing of grafting. Future studies are required to investigate whether bone regenerated from fibular periosteum can be used for bone grafts and to define the optimal timing for grafting and the conditions that most efficiently promote the formation of bone regenerated from periosteum.

In conclusion, we successfully followed the process of bone regeneration from fibular periosteum in detail, including the time course of bone regeneration, bone volume, and bone mineral density by means of micro CT. In addition, we demonstrated bone regeneration from only fibular periosteum, unaffected by regeneration from a bone stump. Our results suggested that the peak of bone regeneration from rat fibular periosteum occurred around 4 to 6 weeks.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (24593063) from the Japan Society for the Promotion of Science (JSPS), Grants from the Dental Research Center, Sato Funds, Uemura Fund of Nihon University School of Dentistry, as well as special research grants for the development of distinctive education for private schools of Japan.

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