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Histomorphometric Analysis of the Alveolar Bone for Two Weeks after Bone Morphogenetic Protein Transfer

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Abstract

Alveolar bone regeneration therapy is critical to retain the teeth and proper occlusion. Currently, alveolar bone loss is treated surgically using bone grafts or artificial bone, both of which carry the risk of complications, such as post-operative infection. A new, non-surgical therapy would help to improve patient safety levels and treatment success. Alveolar bone is always proceeding remodelling, and this makes it difficult for the clinicians and researchers to evaluate alveolar bone tissues after some regenerative treatments. In our previous studies, we developed a system for bone regeneration using non-viral bone morphogenetic protein (*BMP*) gene-expression plasmid vectors and *in vivo* electroporation for the ectopic bone formation in rat skeletal muscles. Here, we used bone morphometric analyses using calcein and tetracycline labelling in rats to evaluate changes in alveolar bone with our *BMP* gene-transfer system. We concluded that *BMP-2/7* gene transfer to the periodontal tissues was an optimal therapy for the alveolar bone regeneration.

Keywords: Alveolar bone; Histomorphometric analysis; Bone labelling; Gene therapy

Introduction

Alveolar bone is pivotal for the maintenance of teeth [1]. However, when lost, alveolar bone has limited potential for spontaneous regeneration [2]. Therefore, numerous studies have investigated ways to successfully engineer new bone that is efficient and safe for clinical therapy [3]. However, because alveolar bone is in a constant state of remodeling-more so than most other bony regions-it can be difficult to evaluate whether a method has been successful in regenerating bone at the intended site [4-6]. Moreover, bone and teeth are generally more difficult to handle than other types of tissues, requiring lengthy decalcification procedures before the specimens can be assessed [7].

Previously, we developed a gene-transfer system for bone regeneration therapy by combination a non-viral *BMP* gene expression plasmid vector and *in vivo* electroporation [8,9]. Our final goal is to apply our constructed method clinically for alveolar bone regeneration [10] and to limit new bone formation to the appropriate site. Our previous study revealed that the observation for one week after BMP gene transfer to the periodontal tissues was not enough to evaluate the regenerated alveolar bone [11]. To resolve this problem, we require a suitable and reliable evaluation method to detect regenerated alveolar bone after gene therapy for continuous period of time. Here, we used a histomorphometric analysis to value alveolar bone regeneration for two weeks after *BMP-2/7* gene transfer.

Aim

Aim of this study is to reveal the availability for histomorphometric analyses on the alveolar bone for two weeks after BMP gene transfer.

Materials and Methods

Gene transfer

Nine-week-old male Wistar rats (*n*=3) were anesthetized via an intraperitoneal injection of pentobarbital sodium (5.0 mg/100 g body weight). The *BMP-2/7* gene expression plasmid vector detailed in our previous study (9) was diluted to 0.5 μg/μL in phosphate-buffered saline and 50 μL was injected into the palatal region of the periodontal tissues of the first molar in the right maxilla using a syringe with a 31-gauge needle. *In vivo* electroporation was performed immediately in the condition of 50 V, 50 ms and 32 pulses [12]. All animal experimental procedures were approved by the Animal Care and Use Committee, Okayama University (Approval number: oku-2012137) and Animal Research Committee of Osaka Dental University (Approval number: 16-1009).

Double-staining of bone

Nine-week-old male Wistar rats (n=3) were intraperitoneally injected with calcein (10 mg/kg) on the day of gene transfer. Three days later, tetracycline hydrochloride (30 mg/kg) was intraperitoneally injected. Rats were again injected with calcein on days 6 and 12, and tetracycline on day 9, and then sacrificed with an overdose of pentobarbital sodium on day 14 (Figure 1). The maxillary regions of rats were dissected and fixed with 70% ethanol for 8 days, stained with Villanueva osteochrome bone stain for 10days, dehydrated with increasing concentrations of ethanol, and embedded in methyl methacrylate without decalcification [13].

After polymerization, 10-μm frontal sections were obtained from the mesiolingual center of the upper first and second molars the region of alveolar bone surrounding the second molar was used as an untreated control for the experiment. In addition, a site of alveolar bone around the first molar away from the site of injection was also used as a control, as was the same site in the second molar. Sections were observed by fluorescence microscopy under UV irradiation for tetracycline (364 nm) and calcein (477 nm) labeling. The distances between the calcein and tetracycline labels were measured vertically at 10 points within the

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region in which gene transfer had been performed, using a Histometry RT Camera (System Supply, Tokyo, Japan). Statistical analyses was performed by analysis of variance (ANOVA), following by Fisher's comparison test.

Results

Villanueva bone staining

Villanueva osteochrome bone staining was used to measure alveolar bone changes following gene transfer into the region of the first molar, comparing against the region around the second molar as an untreated control. With this staining, osteoid is transparent green to jade green or homogeneous red low-density bone is red, the nuclei of osteoblasts or osteocytes are greenish-blue to dark purple, and the cellular cytoplasm green or light green represented the cytoplasm. After *BMP-2/7* gene transfer, we found no significant differences in osteoid or low-density bone formation between the alveolar bones of the first molars and second molars (Figure 2A and 2B). However, in comparing the morphology of the osteoblasts, numerous osteoblasts in the alveolar bone around the first molars were cuboidal in shape (Figure 2A arrow), typical of active osteoblasts. In contrast, osteoblasts in the alveolar bone of the second molar were squamous-like, reminiscent of lining cells (Figure 2B, arrow).

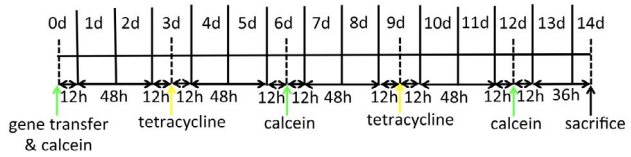

Figure 1: Schematic of the double bone staining protocol used in our study. Calcein and tetracycline were intraperitoneally injected alternatively every 3 days and the distances between the stains were measured and used to determine the mineral apposition rate.

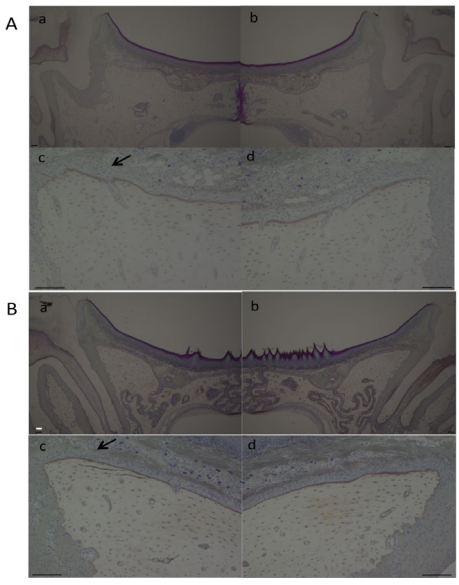

Figure 2: Villanueva bone staining. Alveolar bone surrounding the (A) first and (B) second (control) molars. Rats received the BMP-2/7 gene transfer injection at "a". Position "c" marks the same site in the second molar; "b" marks a control side of the first molar where the injection was not given; and "d" marks the same site in the second molar. Scale, 100 μm.

Bone labeling and MAR

We found five labels in the alveolar bones of the first molars and second molars (Figures 3A and 3B). We measured the distances between each label (Mineral Apposition Rate: MAR) and compared them between the alveolar bones of the first molars and second molars. The MAR values for the first molar with BMP-2/7 from 0–3 days, 3–6 days, 6–9 days and 9–12 days after gene transfer were significantly different to those for the second molar (Figure 4A). In comparison, the MAR for the uninjected control sites of both the first and second molars was not significantly different (Figure 4B).

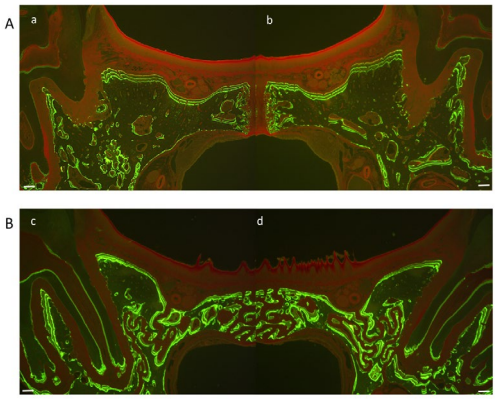

Figure 3: Double bone staining. (A) Representative images of calcein and tetracycline double bone staining of the alveolar bone in the first molar. "a" marks the site of injection for BMP-2/7 gene transfer and "b" shows the control site (area not injected) of the alveolar bone in the first molar. (B) Representative images of double bone staining of alveolar bone of the second molar. "c" marks the same position as the site of injection in "a"; "d" is the control site, as in "b". Transparent green to jade green or homogeneous red is osteoid, red is low-density bone, greenish-blue to dark purple marks the nuclei of osteoblasts or osteocytes, and green or light green marks the cytoplasm. Scale, 200 μm.

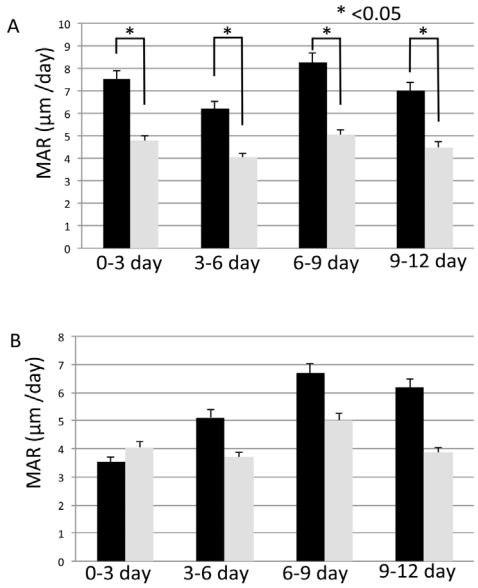

Figure 4: Mineral apposition rate (MAR). (A) MAR in the alveolar bone of the first molars following *BMP-2/7* gene transfer (black columns), and alveolar bone at the same position in the second molars (gray columns). (B) MAR of the control sites in the alveolar bones of the first and second molars.

Discussion

We transferred a *BMP-2/7* non-viral vector into the periodontal tissues of the first molars of rats with electroporation, and found that *BMP-2/7* gene transfer can increase the MAR of alveolar bone. Moreover, the influence of *BMP-2/7* gene transfer was limited to the targeted region of alveolar bone, without affecting adjacent alveolar bone of the second molar. In the past, gene therapy has been used to target a general change rather than a local change [14-16]. However, in alveolar tissues, it is very important that *BMP-2/7* gene transfer targets only the intended periodontal tissues and not the surrounding regions, which lie adjacent to the site of interest.

Preparing bone samples for histological analysis can require an extensive processing time because of the need to decalcify the samples [17]. Moreover, histological staining of sections with hematoxylin and eosin demonstrates only a snapshot or the fragmental changes in bone tissue growth. Histomorphometric analyses with the use of dyes can reveal the time-dependent changes in bone formation [18]. This is an important distinction, as alveolar bone is always remodeling and changing [19]. Therefore, histomorphometric analyses offer a suitable way to evaluate potential alveolar bone regeneration [20].

In our previous study, although we found the inflammatory cells until three days in the target site, new bone like tissues were formed on day five after *BMP-2/7* gene transfer [11]. Our final goal is to apply our alveolar bone regeneration system for the patients are under the control of the periodontitis.

Conclusion

Our histomorphometric analyses revealed that *BMP-2/7* gene transfer by *in vivo* electroporation could increase the potential for alveolar bone regeneration at specific periodontal tissues sites. This method may represent a new clinical therapy for alveolar bone regeneration.

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