Existing Bone-Derived Bone Morphogenetic Protein-2 Initiates Contact Osteogenesis on the Surface of a Titanium Implant

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Research Article

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Abstract

The dental implant relies on osseointegration and the response of bone to the implant surface. This process comprises bidirectional bone formation, including bone deposition on the implant surface toward the existing bone (contact osteogenesis) and vice versa (distance osteogenesis). It is unclear whether these processes are independent or whether contact osteogenesis is initiated by other factors. Therefore, this study aimed to identify the initiator of contact osteogenesis. We hypothesized that contact osteogenesis does not occur when it is physically isolated from distance osteogenesis, which would imply that some factors from the wounded bone normally promote contact osteogenesis. Using a rabbit tibial implant model, we tested the effects of human recombinant bone morphogenetic protein-2 (BMP-2) and plasma-rich plasma, which are possible initiators from bone and blood, respectively. Titanium implants with BMP-2 showed a better bone-to-implant contact (BIC) ratio. We concluded that BMP-2 initiated contact osteogenesis on the surface of titanium implants.

Introduction

The structural and functional connection between living bone and metal implants, known as osseointegration, was first described by Per-Ingvar Brånemark in the 1960s¹.

The insertion of a dental implant, typically made of titanium (Ti), is the primary option for replacing a missing tooth². When a dental implant is inserted into the jawbone, healing of the bone and direct contact of the bone with the implant surface are accomplished by two mechanisms: contact osteogenesis and distance osteogenesis³. In contact osteogenesis, bone formation begins on the implant surface and extends toward the implant, whereas distance osteogenesis involves the formation of new bone on the surface of the existing bone and its subsequent progression toward the implant surface (Supplementary Fig. 1). While some authors have described a certain affinity of bone to Ti, others have interpreted osseointegration as an immunologic response to the metal surface^{4,5}. Either way, the nature of the contact between the bone and the Ti implant surface remains unknown.

A previous *in vivo* study showed that contact osteogenesis was largely eliminated when dental implants were inserted into rabbit tibiae within a Ti tube⁶, suggesting that contact osteogenesis is not independent of distance osteogenesis. Various modifications of Ti implant surfaces can accelerate the osseointegration process^{7–11}. However, in a previous *in vivo* study using fluorochrome labeling, distance osteogenesis was unaffected by modifications to the surface properties of dental implants, whereas there were significant differences in bone-to-implant contact (BIC) when the modified surfaces were used, suggesting that the quality and efficiency of osseointegration depend largely on contact osteogenesis¹². In view of these findings, it has been suggested that contact osteogenesis is initiated by the release of a certain substance from the existing bone⁶. A previous immunohistochemical analysis of bone samples surrounding metal implants revealed high levels of bone morphogenetic protein-2 (BMP-2)⁶, a protein that activates bone healing^{13–16}, suggesting that it may be an initiator of contact osteogenesis.

Osteogenesis around implants is similar to the bone healing process after injury or extraction. In implant surgery, bone injury and hemorrhage occur. Blood clots formed after hemorrhage have a major role in recruiting osteogenic stem cells around dental implants and promoting the differentiation of osteogenic stem cells into bone cells through several factors, such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF- β)¹⁷. Additionally, factors are released from the injured bone itself, such as bone morphogenetic proteins (BMPs). Bone morphogenetic proteins (BMPs) are proteins that are responsible for osteogenesis and bone regeneration and remodeling and were first discovered by Urist in 1965¹⁸. They are members of the transforming growth factor beta (TGF- β) superfamily, and approximately 20 BMPs have been discovered. Among them, BMP-2 and BMP-7 have strong osteoinductivity. For pharmaceutical use for osteoinductivity, recombinant human BMPs (rhBMPs) were developed for bone graft substitutes and were approved by the United States Food and Drug Administration (FDA). In 2002, rhBMP-2 was first approved by the FDA, and in 2007, it was approved in bone grafts for sinus and localized alveolar ridge augmentation. In 2004, rhBMP-7 received limited approval from the FDA under a Humanitarian Device Exemption, but in 2009, it failed to gain FDA Premarket Approval. As a result, only a rhBMP-2 product on the market is approved for clinical use today^{19–21}.

In the bone healing process, factors from both bone and blood participate. Therefore, we assumed that the factor from bone injury is BMP-2, the only BMP approved for human use, and that plasma-rich plasma from blood has many factors that can promote clotting in the bone healing process.

The aim of our current study was to identify an initiator of bone formation on a Ti implant surface. To this end, we used a rabbit tibial implant model for its advantages of easy handling and sustaining, the short life span and easier access to the surgery site²². The effects of BMP-2 and plasma-originating factors on the bone healing capacity were examined by blocking the influx of endogenous substances from the existing bone by using a Ti tube. A histomorphometric analysis was done to examine the interface between the bone and the implant surface. The results of this study provide insight into the mechanism of the osseointegration of a Ti surface into a hard tissue system.

Results

Surface characteristics of the titanium implants and tubes

After the screw-shaped Ti implants were prepared, the surfaces were modified by sandblasting and hydrochloric acid etching (SLA) of all implants. Ti tubes were also constructed to physically block the flow of substances from the existing bone in vivo. FE-SEM analyses of the SLA-modified surfaces of the Ti implants revealed honeycomb-shaped irregularities (Fig. 1a, top row). The inner and outer surfaces of the Ti tubes displayed a grooved pattern, which is a characteristic feature of the computer numerical control milling process used to manufacture the tubes (Fig. 1a, middle and bottom rows). Analysis of the confocal laser scanning microscopy images also showed that the SLA-modified surfaces of the implants were rougher than those of the unmodified Ti tubes (Fig. 1b, Supplementary Fig. 2, and Supplementary

Table 1). Subsequent X-ray photoelectron spectroscopy analysis of the surfaces of the tubes and the SLA-modified surfaces of the implants showed that they were mainly composed of Ti and oxygen; the average Ti and oxygen contents of the implants were $75.70 \pm 0.35\%$ and $24.30 \pm 0.35\%$, respectively, whereas those of the tubes were $83.64 \pm 1.30\%$ and $16.36 \pm 1.30\%$, respectively (Fig. 1c). The altered oxygen content of the Ti tubes may have been due to the oxide layer on the tube surface.

In vivo experiments and histomorphometric analyses

Three experiments were performed in which Ti implants and tubes were inserted into rabbit tibiae. The first in vivo experiment was performed to compare the response to bone-containing implants surrounded by Ti tubes with that to bone-containing Ti tubes only. The second in vivo experiment was performed to compare the response to bone-containing implants with vs. without Ti tubes. The BIC ratio in the Ti tube-only group was not measured, as there was no implant in this group, but the bone healing pattern was monitored. Bone healing in the Ti tube-only group started in the distal (or bottom) region of the rabbit tibia, and very little bone healing occurred in the proximal region (Fig. 2a, top row).

The mean BIC ratio in the implant-only group was $47.32 \pm 12.09\%$, which was significantly higher than that in the implant + tube group ($P = 0.030$). The mean BIC ratio in the implant + tube group was comparable between the first ($19.13 \pm 6.45\%$) and second ($21.41 \pm 13.81\%$) experiments.

Finally, we evaluated the effects of substances originating from bone and blood on bone healing. We included an implant-only group (positive control), an implant + tube group (negative control), an implant + tube + rhBMP-2 group, and an implant + tube + PRP group. In the last two groups, rhBMP-2 or PRP was applied to both the implant and the wounded bone after implantation. The average BIC ratios were as follows: implant only, $53.33 \pm 6.09\%$; implant + tube, $33.22 \pm 9.83\%$; implant + tube + PRP, $16.35 \pm 15.98\%$; and implant + tube + rhBMP-2, $66.53 \pm 14.06\%$ (Fig. 2b and Table 1). The differences among the four groups were significant ($P = 0.004$, one-way ANOVA), and Duncan's post hoc test revealed that the BIC ratio in the implant + tube + rhBMP-2 group was significantly higher than those in the implant + tube and implant + tube + PRP groups ($P < 0.05$). Platelet-rich plasma, our source of blood-originating factors, had little effect on bone healing around the implants (with reference to the negative control group), while the bone morphogenetic protein, one of the factors from the existing bone matrix, did have an effect on bone formation, inducing significantly more bone formation around the implant.

Table 1
Bone-to-implant contact (BIC) ratios in the third experiment (n = 4).

Treatment	Implant only	Implant + tube	Implant + tube + PRP	Implant + tube + rhBMP-2
	54.87	24.74	0	51.37
BIC (%)	46.62	44.00	17.11	79.12
	58.50	30.91	31.94	69.09
Mean	53.33	33.22	16.35	66.53

Discussion

Bone regeneration around an implant comprises two processes: contact osteogenesis and distance osteogenesis. To examine whether contact osteogenesis is independent of distance osteogenesis or is driven by a bone-derived or blood-derived factor, we used a Ti tube to physically isolate Ti implants inserted into rabbit tibiae. We concluded that contact osteogenesis on the Ti implant surface is dependent on distance osteogenesis. The Ti tubes used in our experiments not only blocked the effects of the existing bone (distance osteogenesis) but also limited the blood supply to the implant by allowing blood flow from the bottom of the cortical bone only. The first experiment was designed to compare the response of bone to the insertion of implants within Ti tubes vs. Ti tubes only. The second experiment was designed to compare the response of bone to the insertion of implants with vs. without Ti tubes. Bone healing in the Ti-tube-only group occurred primarily in the lower region of the tube, likely due to the release of initiating substances from the existing bone in that region, while bone regeneration around the implant placed within the Ti tube was lower than that around the implant placed alone. Overall, these results indicate that physically blocking the effects of the existing bone using a Ti tube reduces bone regeneration around the implant. Similar results were also reported in a previous study⁶.

Based on the results of that previous study⁶, we hypothesized that BMP-2 derived from the existing bone was an initiator of contact osteogenesis. To test this hypothesis, we examined the effect of applying rhBMP-2 to the implant surface and the bone defect on the BIC ratio. To determine whether a blood-derived factor could initiate contact osteogenesis, we examined the effect of applying PRP to the implant and existing bone on the BIC ratio. New bone formation in the implant + tube + rhBMP-2 group was similar to that in the positive control (implant only) group and was substantially higher than that in the negative control (implant + tube) group. These results suggest that BMP-2 released from the existing bone might act as an initiator of contact osteogenesis on the implant surface (Supplementary Fig. 3). Supporting this potential role of BMP-2, previous studies have shown that BMP-2 plays an important role in bone development and can stimulate the production of new bone. In a recent study, Alhussaini²³ examined the effects of platelet-rich fibrin (PRF) and BMP-2 on the implant stability quotient. At the time of implant insertion, there were no significant differences among the implant-only (control), implant + PRF, or

implant + rhBMP-2 groups. However, 6 weeks and 12 weeks after surgery, the implant stability was significantly higher in the rhBMP-2 group than in the control or PRF group. Similarly, in our experiments, bone regeneration around the implant inside the Ti tube was increased significantly when BMP-2 was applied.

For comparison to BMP-2, we added PRP to the bone-implant interface. PRP contains a number of growth factors, such as VEGF, PDGF, TGF- β , EGF, IGF-1, and HGF, that stimulate bone formation^{24–26}. Consequently, PRP is used in various treatments for tissue regeneration, including bone regeneration. Marx et al.²⁷ described an enhancing effect of PRP on the density of bone grafts in mandibular continuity defects: specifically, the trabecular bone density of bone grafted with PRP was significantly higher than that of bone grafted without PRP. Another study concluded that PRP may improve the outcome of grafting in sinus floor elevation and alveolar ridge preservation, as well as postoperative pain and swelling²⁶. Despite these findings, in our experiments, the BIC ratio was clearly higher in the implant + tube + rhBMP-2 group than in the implant + tube + PRP group. In addition, PRP was unable to promote bone regeneration around the implants that had Ti tubes blocking the effects of existing bone. Blood-originating factors may be unable to initiate bone healing in the absence of bone-originating factors, although they may promote bone formation after initiation of the healing process.

In conclusion, our findings suggest that bone-derived BMP-2 is an initiator of contact osteogenesis. Further research is needed to identify additional biological and molecular mechanisms involved in osteogenesis and to find ways to improve the clinical prognosis of dental implants.

Methods

Sample preparation and implant surface modification

Thirty-nine implants with a diameter of 3.0 mm and a length of 12.0 mm were prepared by Deep Implant System, Inc. (Seongnam, Korea) using a computer numerical control system (CINCOM L20; Citizen Machinery Co., Ltd., Kitasaku-gun, Japan). The implants were screw-shaped and made of grade 4 commercially pure Ti, and their surfaces were modified by sandblasting with alumina particles and etching with hydrochloric acid. To physically block the implants from exposure to substances from the existing bone in the in vivo experiments, 24 Ti tubes were constructed using the CINCOM L20 system. The tubes were made of commercially pure grade 4 Ti and had the following dimensions: outer diameter, 4.0 mm; inner diameter, 3.6 mm; thickness, 0.2 mm; and length, 6.0 mm. Unlike the implant specimens, the tubes had no surface modifications.

Surface characteristics of the implants and Ti tubes

Six implants and six Ti tubes underwent surface analysis: field emission scanning electron microscopy (FE-SEM; S-4700; Hitachi, Tokyo, Japan) was used to generate overall surface images, while X-ray photoelectron spectroscopy (Sigma Probe; Thermo Scientific, Waltham, MA, USA) was used to analyze the components and contents of the surfaces. Three implants and three tubes were used to analyze the

topographical features of the surfaces. Confocal laser scanning microscopy (LSM 5-Pascal; Carl Zeiss AG, Oberkochen, Germany) was performed, and the Sq, Sa, and Sdr parameters were measured (Supplementary Fig. 4).

In vivo surgery

The animal experiments performed in this study were approved by Institutional Animal Care and Use Committees (IACUC, #201805001) of CRONEX Co., Ltd. (Hwaseong, South Korea). After the approval, the animal experiments were conducted under the guidelines of the IACUC of CRONEX Co., Ltd. and were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for in vivo animal experiments²⁸. Eight male New Zealand white rabbits, aged 1–2 years and weighing 2.6–3.0 kg, were used.

All rabbits were anaesthetized via an intravenous injection of tiletamine/zolazepam (15 mg/kg; Zoletil® 50; Virbac Korea Co., Ltd., Seoul, Korea) and xylazine (5 mg/kg; Rompun™; Bayer Korea, Ltd., Seoul, Korea). Then, the skin over the proximal tibia was shaved and washed with betadine, after which the antibiotic cephalosporin (cefazolin; Yuhan Co., Seoul, Korea) was administered intramuscularly. Subsequently, 0.9 mL of 2% lidocaine with 1:100,000 epinephrine (2% lidocaine HCl injection; Huons Co., Ltd., Seongnam, Korea) was injected locally into each surgical site of the tibia. The skin was then incised, and the tibia was exposed by muscle dissection and periosteal elevation. Drills and profuse sterile saline irrigation were used to prepare the implant sites on the flat tibial surface. Drilling was performed bicortically, with a final diameter of 4 mm in the upper cortical bone (used to compare the experimental and control groups) and 2.8 mm in the lower cortical bone (the anchorage site for implant placement) (Supplementary Figs. 5 and 6).

In the first experiment, the response to bone-containing implants surrounded by Ti tubes was compared with the response to bone-containing Ti tubes only. In the Ti tube + implant group, the Ti tubes were inserted into the prepared holes and contacted the upper cortex only; subsequently, the implants were inserted and anchored to the lower cortex. In the Ti-tube-only group, the Ti tubes were inserted into the holes, and no implant was placed. Two rabbits were used for the experiment, and each tibia contained two surgical sites. A two-by-two Latin square arrangement was applied (Supplementary Fig. 7a) to ensure complete randomization of the arrangement of the groups.

The second experiment was performed to compare the response of bone-containing implants with vs. without Ti tubes. The Ti tube + implant group was established as described in the first experiment. In the implant-only group, the implants were inserted through the upper holes and anchored to the lower cortex, but no tubes were inserted. Two rabbits were used, and each tibia contained two surgical sites. As in the first experiment, the arrangement of the groups was randomized completely using a two-by-two Latin square arrangement (Supplementary Fig. 7b).

The third experiment was performed to evaluate the effects of substances from bone and blood on bone healing. Four rabbits were used in this experiment, and as in the other in vivo experiments, each tibia

contained two surgical sites. There were four groups: the implant-only, Ti tube + implant, Ti tube + implant + rhBMP-2, and Ti tube + implant + PRP groups. The Ti tube + implant group served as a negative control, and the implant-only group served as a positive control. The first of the two experimental groups received an implant with a Ti tube, as well as the application of recombinant human BMP-2 (rhBMP-2; CowellMedi, Busan, South Korea). In this group, rhBMP-2 solution (total 0.1 mL, 0.25 mg/mL) was applied to the implant surface before implantation and around the bone defect after implantation. The second experimental group involved the placement of an implant with a Ti tube, as well as the application of platelet-rich plasma (PRP), which was prepared using the protocol described by Pazzini et al.²⁹. Briefly, rabbit blood was collected in a tube containing sodium citrate (anti-coagulant) and centrifuged at $401 \times g$ (relative centrifugal force, or RCF) for 10 minutes. After discarding the red blood cells, the sample was centrifuged at $626 \times g$ (RCF) for an additional 10 minutes. Subsequently, PRP was collected and applied to the implant and bone defect after implantation. The methods used for insertion of the implants and tubes were as described for the first and second experiments. The treatments were arranged in the rabbit tibiae according to the split-plot design (Supplementary Fig. 7c).

After surgery, the muscle and fascia were sutured with resorbable 4–0 Vicryl sutures, and the outer dermis was closed with nylon sutures. Each rabbit was housed separately after surgery, and an antibiotic was administered intramuscularly for 3 days. After a 4-week period of bone healing, all of the experimental animals were anaesthetized and sacrificed via an intravenous overdose of potassium chloride. The tibiae were exposed, and the implants were surgically removed en bloc with an adjacent bone collar. The bone blocks containing the implants were immediately fixed in 10% neutral formaldehyde.

Histomorphometry

Histomorphometry specimens were prepared using the method described by Choi et al.⁶ and Donath and Breuner³⁰. Briefly, the implants and bone samples were embedded in light-curing resin (Technovit 7200 VLC; Kultzer, Wehrheim, Germany), and undecalcified specimens were ground to a thickness of 50 μm , stained with hematoxylin and eosin, and subjected to a general histological evaluation under a light microscope (Olympus BX; Olympus, Tokyo, Japan). The image analysis software that was associated with the light microscope (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to calculate the BIC ratio for each implant. Specifically, the three best consecutive threads of each implant were examined to determine the proportion of each thread that was in contact with bone. The mean BIC ratio for each implant was then calculated.

Statistical analysis

Statistical analyses were performed using R software (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as the mean and standard deviation. For the BIC data, one-way analysis of variance (ANOVA) was performed to identify significant differences among the groups. When ANOVA indicated a significant difference, pairwise comparisons were conducted using Duncan's post hoc test. $P < 0.05$ indicated statistical significance.

Declarations

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request

Competing interests

The authors declare no completing interests.

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Figures

Figure 1

Figure 1

Physical properties of the titanium implants and tubes used in the study. (a) Scanning electron microscopy images of the titanium implant and tube at magnifications of 30 $\times$, 1000 $\times$, 2000 $\times$, and 5000 $\times$ (left to right). (b) Surface roughness analysis of the titanium implant and tube under confocal laser scanning microscopy, showing the Sa, Sq, and Sdr parameters. Error bars: standard deviation. (c) Surface

chemical analysis of the titanium implant and tube under electron spectroscopy. The atomic ratios of the implant and tube surfaces are shown. Error bars: standard deviation.

Figure 2

Figure 2

Representative two-dimensional histomorphometric analysis of each group. (a) Hematoxylin and eosin staining in the experiment to see the effect of a titanium tube that blocked the effect of existing bone on osteogenesis (specimens from the first and second experiments). Specimens containing only titanium

tubes showed little bone formation. Specimens containing implants and tubes showed less bone formation between the threads of the implant than specimens containing only implants. (b) Hematoxylin and eosin staining in the experiment to find the initiator of contact osteogenesis (specimens from the third experiment). Specimens treated with rhBMP-2 showed more bone formation than the other specimens. The images were obtained at magnifications of 12.5×, 40×, and 100× (left to right).

Supplementary Files

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