The effect of anodized implants coated with combined rhBMP-2 and recombinant human vascular endothelial growth factors on vertical bone regeneration in the marginal portion of the peri-implant

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Objectives. The aim of this study was to evaluate the effect of anodized implants coated with combined rhBMP-2 and recombinant human vascular endothelial growth factors (rhVEGFs) on vertical bone regeneration in the marginal portion of the peri-implant.
Study Design. Supra-alveolar defects were created in 3 male beagle dogs. Each animal received 8 implants that were either coated with a single growth factor (rhBMP-2) or combined growth factors (rhBMP-2 + rhVEGF), or an anodized implant (the control group). The amount of the vertical bone regeneration, the bone-implant contact, and the intrathread bone density were investigated using histomorphometric analysis at 8 weeks.
Results. The bone morphogenetic protein (BMP) group and the BMP-VEGF group showed vertical alveolar bone regeneration and enhanced bone-implant contact in the microthread compared with the control group ($P < .05$).
Conclusions. Anodized implants coated with rhBMP-2 and rhBMP - 2 + rhVEGF can induce vertical alveolar bone regeneration, but the combined effect of rhBMP-2 and rhVEGF was not verified. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:e24-e31)

Starting in the late 1960s, studies on the integration of implants and bones have investigated the surface morphology and features of implants and bones, and have attempted to promote their osseointegration via more microscopic physical and chemical surface treatment, and even to biomimetically treat the surface.[1,2] Studies on surface treatment techniques are continuously being performed to reduce the healing time, improve the osseointegration, and enhance the augmentation of the surrounding bones.[3,4] Currently, dental implants are widely used and known to be reliable and safe, although they have such limitations as a low success rate for patients with poor bone quality and quantity, and for patients whose healing and regeneration capability are low.[5] In addition, the most important concern of both patients and dentists is still how to reduce the healing time from the placement of the implant fixture to the placement of the crown. Studies on the application of such growth factors as bone morphogenetic protein (BMP) to dental implants are under way. BMP enhances bone formation by promoting the differentiation of osteoblasts from the mesenchymal stem cell and by helping in the biosynthesis of the bone matrix through control of essential factors in the osteoinduction for the regeneration of osseous tissue.[6,7] BMP can be classified into 16 subgroups. BMP-2, one of the subgroups, has been proven by preclinical and clinical studies for use for various medical treatments.[8] Particularly, a study reported that when the surface of an anodized dental implant was coated with recombinant human BMP-2 (rhBMP-2), which is derived using the genetic recombination technique, the anodized implant can serve as an effective carrier.[9] Several studies reported, however, that the use of rhBMP-2 did not have a significant bone formation effect.[10,11] Such result was suggested to be

This work was supported by Pusan National University Research Grant 2011. The implants and BMP-2 were kindly supplied by Cowellmedi Co. (Pusan, Korea).
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Received for publication Jul 27, 2011; returned for revision Sep 30, 2011; accepted for publication Oct 27, 2011.

2212-4403/$ - see front matter
doi:10.1016/j.oooo.2011.10.040

attributable to the early release of a large amount of rhBMP-2, the lack of ascertainment of the optimum concentration, and the fact that only one type of growth factor, rhBMP-2, was used, unlike in the natural regeneration process of the human body wherein multiple growth factors are involved.[12-15] On the other hand, additional growth factors have been proposed to use in order to improve osseointegration. The vascular endothelial growth factor (VEGF) is a growth factor that helps in angiogenesis by activating endothelial cells.[16] VEGF is a key and strong modulator of vascular formation.[17] Recent studies have shown that in addition to the role of VEGF in angiogenesis in osseous tissue, it performs various other functions, such as enhancement of bone growth by transporting precursor mesenchymal cells to the mineralized region via newly formed vessels, differentiation of cartilage cells, differentiation of osteoblasts, and introduction of osteoclastic cells.[18-20] Deckers et al.[21] reported that endothelial cells that can be found in newly formed vessels as well as both osteoblasts and preosteoblasts are always found in regions where new bones are formed. Numerous studies have shown, however, that the use of VEGF alone could not sufficiently help enhance bone formation,[22,23] and that VEGF could prolong the survival time of cells and help in osteogenesis through interactions with BMP,[22,24] could induce proliferation and differentiation of osteoblasts,[25] and could help in the migration of osteoblasts.[26] Recent studies assessed the osseointegration capability of dental implants coated with rhBMP-2 and rhVEGF, and presented the applicability to the patients.[27,28] However, these 2 studies were limited to osseointegration. We wanted to verify the ability of bone regeneration in a vertical defect around the implants using the implants coated with rhBMP-2 and rhVEGF. There was no previous research on this. This study was conducted to compare the vertical bone regeneration effects of the alveolar bone among the experimental group wherein an anodized implant coated with both rhBMP-2 and rhVEGF was used, the experimental group wherein an anodized implant coated with only rhBMP-2 was used, and the control group after the implants were placed in the healed alveolar bone to form a vertical defect, and then to assess the osseointegration capability of the bone-implant interface.

MATERIALS AND METHODS

Experimental animals

The rearing, management, and surgical procedures for the experimental animals in this study were approved by the Animal Ethics Committee of Chonnam National University (approval no. CNU IACUC-YB-2010-10). Three beagle dogs aged 2 to 3 years, each of which weighed 15 kg, were used. Each beagle was reared in an individual cage wherein the temperature was maintained at 20 to 25°C, and the relative humidity, at 30% to 50%. A soft diet (Science Diet, Hill's Co., Topeka, KS) was provided with water before and after the surgery.

Table I. Characteristics of the groups and numbers of implants

	Control group	*BMP group**	*BMP-VEGF group†*
Test item	Anodized implant	Anodized implant coated with rhBMP-2	Anodized implant coated with rhBMP-2 and rhVEGF
No. of implants	8	8	8

The total dose of the coated growth factors was 10 μg in each experimental group.
*rhBMP-2 (Cowellmedi Co., Pusan, Korea)—0.75 mg/mL.
†rhVEGF (Woongbee Meditech Co., Seoul, Korea)—0.075 mg/mL.

Preparation of the experimental implant

A total of 24 implants (length: 8 mm, diameter: 4 mm; Cowellmedi Co., Pusan, Korea) were prepared. All the implants were screw-type and made of pure titanium. The top 3-mm portion had a microthread, and the bottom 5-mm portion had a macrothread.

The experimental implants were divided into the following 3 groups (with 8 implants per group):

1. the group of anodized implants without coating (control);
2. the group of anodized implants coated with rhBMP-2 (0.75 mg/mL concentration); and
3. the group of anodized implants coated with both rhBMP-2 (0.75 mg/mL concentration) and rhVEGF (0.075 mg/mL concentration).

The surface of the implants was properly anodized (0.1 mol/L H2SO4, 0.1 mol/L H2PO4, 3Å, 180 V: Cowellmedi Co., Pusan, Korea) and coated with such growth factors as rhBMP-2 (Cowellmedi Co., Pusan, Korea) and rhBMP-2 and rhVEGF (Woongbee Meditech Co., Seoul, Korea) (Table I). The BMP group was coated with 0.75 mg/mL of rhBMP-2, and the BMP-VEGF group was coated with 0.75 mg/mL of rhBMP-2 and 0.075 mg/mL of rhVEGF. These coating concentrations of the growth factors were based on previous experiments in which 0.75 mg/mL of rhBMP-2 safely improved the bone formation without particular adverse events.[29,30] In addition, a lower concentration of rhVEGF than of rhBMP-2 was used, as described in a report by Young et al.,[31] who reported that a higher concentration of rhBMP-2 than of rhVEGF helped improve bone formation.

For coating with growth factors, the implants were immersed 3 times in a protein solution and freeze-dried at −40°C. The total coating amount of the BMP group and the BMP-VEGF group was 10 μg each.

Primary surgery

Food was withheld the night before surgery. Surgery was performed under general anesthesia. The surgical area was injected with lidocaine (Yu-Han Co., Gunpo, Korea) that contained 1:100,000 epinephrine for the local anesthesia. All the mandibular premolars and first molar were extracted. The furcation region of the first molar was cut using a fissure bur and extracted carefully to avoid damaging the extraction socket. After verifying that no dental root remained, a suture was made with 4-0 silk (Mersilk, Ethicon Co., Livingston, UK). After the extraction, meloxicam (Metacam, 0.1 mg/kg orally; Boehringer Ingelheim Co., Ridgefield, CT) was administered to the animals for pain relief, and amoxicillin (20 mg/kg orally; Choongwae Co., Seoul, Korea) was administered to them every 12 hours for 6 days.

Secondary surgery

After checking that the alveolar bone healed well 2 months after the extraction surgery, implant placement was performed. The general and local anesthesia procedures were the same as that of the primary surgery. Four implants were placed on each side of the mandible. The position of the implants was decided according to a computer-generated random number. An incision was made at the midcrestal of the alveolar bone. After the full-thickness flap was dissected, the cortical bone was leveled. Then, the implant site was prepared using a 3.2-mm-diameter drill as the final drill. As such, a total of 8 implants were placed on both sides. Implants of the same group were on one side (split-mouth design), as the implants coated with growth factors might influence the surrounding environment. The implantation was performed in such a way that the implant would be exposed 2.5 mm from the uppermost alveolar bone. The alveolar ridge and the buccal cortical bone were perforated using a 1-mm round bur to expose the cancellous bone and the blood. For tension-free suture of the flap, a releasing incision was made to ease the tension of the periosteum. The suture was performed using 5-0 Gore-Tex (W. L. Gore and Associates, Flagstaff, AZ) (Figures 1 and 2).

Postoperative treatment

Penicillin G procaine and penicillin G benzathine (1 mL/5 kg) were intramuscularly injected in the experimental animals immediately after the surgery and 48 hours after the surgery, as was 2% chlorhexidine twice a day in the oral cavity (Figure 3). A soft diet was allowed.

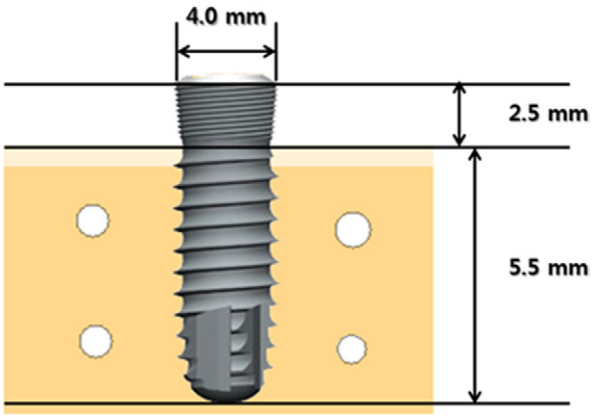

Fig. 1. Schematic diagram of the implant position. In each group, 8 implants were inserted, and the position of the implant placement per group was randomized based on the split-mouth design. While maintaining the cortical bone, the implants were placed so that 2.5 mm of the upper part was exposed out of the alveolar bone, with the microthread as the guide. To reduce bleeding, the cancellous bone between the implants was exposed by punching out the cortical bone (buccal, crestal, and lingual aspects) using a 1-mm round bar.

The experimental animals were killed 8 weeks after the implant surgery. They were first sedated using azaperone and midazolam (1 mg/kg, intramuscularly) before they were killed via intravenous injection of 20% pentobarbital solution (Dermocal AG, Buenos Aires, Argentina).

Preparation of the specimen

The specimen that included the implant was prepared after the experimental animals were killed. The specimen was fixed for 2 weeks in a neutral buffered formalin solution (Sigma Aldrich, St. Louis, MO), and then dehydrated by increasing the ethanol concentration to 70% to 100%. The dehydrated specimen was embedded in a Technovit 7200 resin (Heraeus KULZER, South Bend, IN). A block of the polymerized specimen was cut in the direction of the long axis of the implant at the center of the implant using an EXAKT diamond cutter (KULZER EXAKT 300, EXAKT, Norderstedt, Germany). A slide with a final thickness of 30 μm was made from the 400-μm-thick slide using an EXAKT grinding machine (KULZER EXAKT 400CS, EXAKT). The tissue was stained using Goldner's trichrome.

Histometric analysis

An observer who was blind to the experimental conditions analyzed the histologic findings. The measurement was performed 3 times to reduce the possibility of

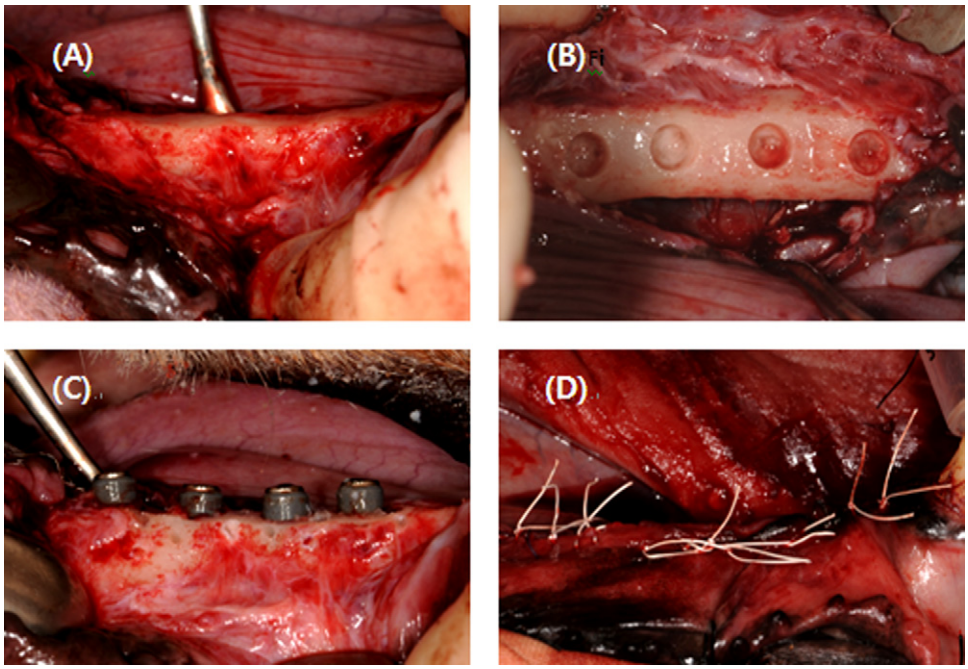

Fig. 2. Clinical photographs of implant surgery. **A,** Alveolar bone flattening to decide a base bone level. **B,** Drilling. **C,** Implant placement. **D,** Suture.

error. The images were obtained using an optic microscope (Olympus BX, Tokyo, Japan) linked to a computer, with a charge-coupled device camera (Polaroid DMC2 Digital Microscope Camera, Polaroid Co., Cambridge, MA) attached to the microscope. The obtained images were analyzed using an image analysis computer program (Image-Pro Plus, Media Cybernetic, Silver Spring, MD). The center of the specimen that was cut in the direction of the buccolingual side was used for the histologic analysis. All the images of the specimens were taken under ×2.5 magnification; ×40 magnified images were used for the histologic analysis, and ×100 magnified images for accurate assessment of the bone-to-implant contact (BIC). The following factors were analyzed.

1. Bone growth height: The length of the bone growth that increased upward along the implant from the reference point of the implant site on the alveolar ridge was measured.
2. BIC in the microthread: The BIC ratio in the area where the bone grew along the implant from the reference point of the implantation on the alveolar ridge was measured.
3. BIC in the macrothread: The BIC ratio in the existing bone where the implant was implanted was measured.
4. Intrathread bone density in the macrothread: The intrathread bone density in the existing bone where the implant was placed was measured.

Statistical analysis

The mean and standard deviation of the BIC, the intrathread bone density (ITBD), and the bone growth height were measured. A Shapiro-Wilk test was performed to test the normal distribution, and a 1-way analysis of variance was performed to compare the difference in the BICs, ITBDs, and bone growth of the groups. A Bonferroni test was performed for the post hoc test with a significance level of 95%. SPSS ver. 18.0 (SPSS, Chicago, IL) was used for the statistical analysis.

RESULTS

Clinical findings

The healing process progressed well without particular problems. No implantation failure occurred. No implant had seroma around the implantation site. There were no cases in which an implant was exposed owing to the opening of the flap. No findings suggestive of inflammation or a particular change in the mucosa around the implant were observed.

Histologic findings

The histologic analysis of the specimen that was collected at the time the experimental animals were killed at 8 weeks showed that the bone growth was 0.1, 1.13, and 1.03 mm in the control, rhBMP-2, and rhBMP-2 + rhVEGF groups, respectively. The bone regeneration in the rhBMP-2 and rhBMP-2 + rhVEGF groups was

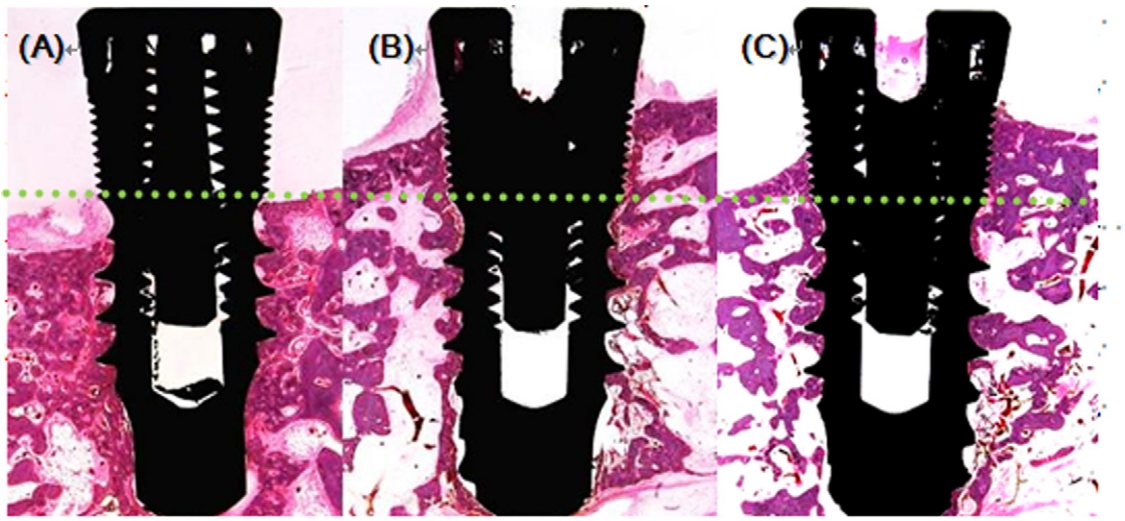

Fig. 3. Light microphotographs. **A,** Control group. **B,** BMP group. **C,** BMP-VEGF group. The dotted line indicates the placement level with the resident alveolar bone. The BMP group and the BMP-VEGF group showed alveolar ridge augmentation and enhanced bone-implant contact in the microthread. This enhancement was significant compared with that in the control group ($P < .05$).

significantly better than that in the control group, although no significant difference was observed between the rhBMP-2 and rhBMP-2 + rhVEGF groups ($P > .05$). The BICs at the site of the microthread where the bone growth occurred were 1.57%, 12.72%, and 19.41% in the control, rhBMP-2, and rhBMP-2 + rhVEGF groups, respectively. The BICs of the experimental groups significantly differed from that of the control group. The BICs of the rhBMP-2 and rhBMP-2 + rhVEGF groups did not significantly differ, however ($P > .05$).

The BICs at the site of the macrothread that was implanted in the existing bone were 22.46%, 48.79%, and 41.92% in the control, rhBMP-2, and rhBMP-2 + rhVEGF groups, respectively. The BICs at the sites of the macrothread were not significantly different between the 3 groups ($P > .05$).

The intrathread bone densities at the sites of the macrothreads that were implanted in the existing bones were 65.00%, 68.89%, and 52.13% in the control, rhBMP-2, and rhBMP-2 + rhVEGF groups, respectively, but did not significantly differ between the 2 experimental groups and between the 2 experimental groups and the control group (Table II) ($P > .05$).

DISCUSSION

The entire surface area of an implant that is being covered by a sufficient amount of alveolar bone is very important to the long-term survival and functionality of the implant.[32] When the bone resorption process is excessive, however, owing to periodontitis after tooth extraction, it may sometimes be impossible to achieve the bone height required for implantation. In this case, a bone graft is required to restore the vertical bone height in the alveolar ridge of the edentulous jaw where such excessive bone resorption occurred. Because of the advances in the surgical procedure for bone regeneration, surgical procedures that can restore the alveolar ridge vertically have become very diverse. Despite these advances, recent studies have reported that the result of the vertical ridge augmentation procedure is still highly unpredictable, and involves frequent adverse events.[33,34] Although currently the autogenous bone graft is considered the first-line treatment,[35] it

Table II. Results of the histomorphometric analysis at 8 weeks (mean ± SD)

	Bone augmentation, mm	*Bone-to-implant contact of the microthread, %*	*Bone-to-implant contact of the macrothread, %*	*Intrathread bone density of the macrothread, %*
Control group	0.11 ± 0.33	1.57 ± 1.53	22.46 ± 11.5	65.00 ± 12.18
rhBMP-2 group	1.13 ± 0.73*	12.72 ± 5.14*	48.79 ± 9.64*	68.89 ± 16.92
rhBMP-2+rhVEGF group	1.06 ± 0.81*	19.41 ± 12.71*	41.92 ± 16.47	52.13 ± 16.84

rhBMP, recombinant human bone morphogenetic protein; *rhVEGF*, recombinant human vascular endothelial growth factor.
*Compared with the control group, 1-way analysis of variance test, $P < .05$.

involves donor site pain and dysfunction, and has a limitation in the collectable amount of bone.[36] With advancements in tissue engineering, bone graft procedures have been reported with many successful results for the alveolar ridge where bone resorption is excessive. Vertical ridge augmentation still has to overcome the challenges related to the alveolar ridge of the edentulous jaw, however, where bone resorption is particularly excessive.

Numerous studies have been conducted on ways to enhance vertical bone regeneration without using a bone graft by placing an implant fixture with its surface coated with a bone growth factor. It was reported that among such growth factors, rhBMP-2 could effectively play the role of carrier when used to coat the surface of an anodized implant.[9,11]

Particularly in animal studies, wherein an implant coated with rhBMP-2 was exposed above the alveolar bone without additional bone graft materials, the rhBMP-2 resulted in significant regeneration of the bone wherein a new bone was formed along the surface of the rhBMP-2–coated implant.[29,30] Limitations were also reported, however, such as a low density around the newly formed bone, and a low bone-to-implant contact.[29]

In this context, this study was performed to assess the synergic effect of rhBMP-2 when combined with other growth factors, by comparing the vertical bone regeneration of the alveolar bone in the experimental group where the anodized implant was coated only with rhBMP-2, with that of the experimental group wherein the anodized implant was coated with both rhBMP-2 and rhVEGF, and with that of the control group, and by assessing the osseointegration in the bone-implant interface after such implants were placed high above the healed alveolar bone.

In this study, the osteoinduction effect of rhBMP-2 was observed, and the osseointegration effect of the experimental group significantly differed from that of the control group, except for the ITBD. In a study by Wikesjö et al.,[29] the BIC was low in the rhBMP-2–coated implant owing to pinpoint-type osseointegration, but high in the control group owing to thin bone formation along the surface of the implant. Consistent with this, the present study showed that BIC was lower in the control group than in the experimental group, which was deemed to have been possibly because of differences in the materials and methods. Further studies are required on these differences.

No synergic effect was observed when rhVEGF and rhBMP-2 were used in combination. The bone formation effect was significantly greater when rhVEGF and rhBMP-2 were used in combination than in the control group, although it did not significantly differ between the rhVEGF + rhBMP-2 group and the rhBMP-2 group. A recent study on the effect of the combination coating of an implant with rhBMP-2 + rhVEGF on osseointegration showed that although the bone density was higher in the combination-coating group than in the group coated with either rhBMP-2 or rhVEGF, the BICs of the combination-coating group and of the group coated with either rhBMP-2 or rhVEGF did not significantly differ.[27] Most other studies showed that the BICs were similar or were even lower with the coated surface.[37,38]

In contrast, some studies on bone regeneration using rhBMP-2 and rhVEGF had positive outcomes. One of these studies that used periosteum-derived cells showed that the bone formation significantly increased when rhBMP-2 and rhVEGF were combined.[39] In addition, a study wherein a scaffold that was made by combining rhVEGF and rhBMP-2 with bone-marrow–derived multipotent stem cells (BMSCs) was implanted onto the ectopic site,[40] and a study wherein the implantation was performed using combined rhVEGF, rhBMP-2, and BMSC, also showed good outcomes.[41] Another study showed that an implant for which a combination of rhVEGF and rhBMP-2 was used had significantly higher bone formation than when either one of them was used in a rat with a critical defect.[23] Simultaneous expression of rhVEGF and BMP4 with high concentrations resulted in a high level of endochondral bone formation in the ectopic site.[42]

Several studies have presented various opinions on such limitations in in vivo experiments wherein an implant was coated with 2 types of growth factors. Such limitations include limitations in effect owing to the rapid degeneration of the growth factors, and nonstandardization of the optimum concentration of the growth factors[14,23]; and the possible deformation of the functional unit and structural deformation on the surface of the implant owing to the coating of the surface of the implant with rhBMP-2 and rhVEGF, which could decrease the affinity with the bone morphogenic cells that are attached to the surface of the implant via mediation by integrin.[27] Unlike the exposed upper portion of the implant, the portion that is implanted in the existing bone may affect the osseointegration because of problems, such as difficulty in standardizing insertion stability.[43,44]

In a study that assessed vertical-bone regeneration with an anodized implant that had been coated with rhBMP-2 via the dip and dry process, the coated surface showed a significantly better outcome than the uncoated surface did.[30] Thus, in the experiment in the present study, the anodized implants were coated with a combination of rhBMP-2 and rhVEGF via the dip and dry process. The dip and dry process can be easily

applied to clinical practice because it is very simple, consisting only of drying after 3-time immersion. In the experiment in the present study, however, the application of a combination of 2 other growth factors did not result in a significantly good outcome.

If the emergence timing and degeneration rate of the 2 growth factors that were used to coat the implant can be controlled, a more favorable outcome can be expected. The control should imitate the serial emergence and degeneration of growth factors that naturally occur within the human body. In addition, much evidence was accumulated from many previous studies on the timing of the involvement of VEGF in bone formation. VEGF temporarily exhibits strong expression, and particularly 5 to 7 days after the implementation of distraction osteogenesis, its expression is highest.[45] Other studies also showed that angiogenesis occurred in the early stage before bone formation in a fracture model.[45,46] Thus, if the serial-release of growth factors based on the slow-release system will be used such that VEGF will be released earlier than BMP to promote angiogenesis, a more favorable outcome can be achieved. This is likely to be responsible for the fact that in this study, the results obtained by the group that used an implant coated only with rhBMP-2 and those obtained by the group that used an implant coated with a combination of rhVEGF and rhBMP-2 did not show a great difference.

If how long such growth factor should be released when the implant is coated with a bioactive material can be ascertained by further studies, the potential of implants coated with a bioactive material is believed to be great.

CONCLUSION

This study showed that an anodized implant coated with rhBMP-2 could achieve vertical bone regeneration in the defect site around the implant without an additional bone graft, and could enhance the BIC area. Further studies are required, as this study could not show if combined application of rhBMP-2 and rhVEGF to the implant could have a further synergistic effect compared with the single use of rhBMP-2.

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