# Osseointegration of Anodized Titanium Implants Coated with Fibroblast Growth Factor–Fibronectin (FGF-FN) Fusion Protein

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***Purpose:*** *The synergistic effect of fibroblast growth factor (FGF) and human fibronectin fragment ($hFNIII_{9\text{-}10}$) on osteoblast cell adhesion has been demonstrated in vitro. The purpose of this study was to evaluate the bone response around anodized titanium implants treated with FGF-FN fusion protein using the histomorphometric analysis and the removal torque test.* ***Materials and Methods:*** *Threaded implants were manufactured by machining a commercially pure titanium (grade 4). Two different groups of samples were prepared: Group 1 samples were anodized under a constant voltage of 300 V, and group 2 samples were anodized under a constant voltage of 300 V and then soaked in a solution containing fusion protein (65 μg/mL) for 24 hours. Ten implants from each group were placed in rabbit tibiae (1 implant per group per rabbit; each rabbit served as its own control). After a 3-month healing period, the animals were sacrificed. Removal torque testing and histomorphometric analysis was then carried out.* ***Results:*** *The mean removal torque value of group 2 (44.8 Ncm) was greater than that of group 1 (37.6 Ncm). The percentages of bone-implant contact of the best 3 consecutive threads were 76.37% for group 1 and 88.02% for group 2. The percentage of bone-implant contact for the total length of the implant was higher for group 2 (36.91%) than for group 1 (29.47%). However, the percentage of the area inside the threads that consisted of bone did not differ significantly for the 2 groups.* ***Discussion and Conclusion:*** *This study demonstrated that the FGF-FN fusion protein coating on anodized implants may enhance osseointegration. However, the influence of fibronectin- and FGF-treated rough surfaces on long-term prognosis and the propagation of inflammation are subjects for further study.* Int J Oral Maxillofac Implants 2006;21:859–866

**Key words:** *anodic oxidation, fibroblast growth factors, fibronectin, fusion proteins, histomorphometric analysis, removal torque*

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Poor bone quality and insufficient quantity are major challenges in implant treatment. Implant characteristics such as surface chemistry, charge, texture, and porosity can be used to influence bone response in vivo. Other approaches involve treating implants with biologically active substances, such as growth factors.[1] Growth factors are believed to act as autocrine and paracrine effectors of bone formation by increasing osteoblastic proliferation and bone matrix biosynthetic activity.

Osborn and Newesley[2] described the phenomena of contact and distance osteogenesis, through which bone becomes juxtaposed to an implant surface. In the case of poor bone quality, optimizing contact osteogenesis by implant surface design (such as the use of a rough surface) to ensure early stability is important.[3]

Cells do not bind directly to the implant but rather to extracellular glycoproteins that are adsorbed to its surface. Numerous reports have

demonstrated that there is an amorphous layer of proteoglycans and unmineralized collagen, varying in thickness from 40 to 400 nm, between the bone and the implant surface.[4–6]

Growth-factor–induced proliferation, differentiation, and migration have been shown to require the adhesion of cells to the extracellular matrix (ECM). Fibronectin (FN) is a large glycoprotein composed of 2 large subunits joined by a pair of disulfide bonds, which acts as a major ECM component. FN is used as a substrate for cell attachment. Each subunit is folded into a series of functionally distinct rodlike domains, and the domains in turn consist of smaller modules.[7] The Arg-Gly-Asp (RGD) sequence within the tenth type III fibronectin (FNIII10) repeat (the main type of module) is part of the major cell-binding site.[8] Fibronectin binds to the $\alpha_5\beta_1$ integrin through the consensus site including the RGD sequence. Cellular binding sites for RGD peptides have been reported to play a major role in mediating cell adhesion through integrin receptors, which transduce information to the nucleus through cytoplasmic signaling pathways.[9] The Pro-His-Ser-Arg-Asn (PHSRN) sequence within the ninth type III module ($FNIII_9$) has also been identified as a synergistic motif for binding to $\alpha_5\beta_1$ integrin.[10] Mechanical pull-out testing of RGD-coated titanium implants in rat femurs conducted at 4 weeks revealed that the average interfacial shear strength had increased.[1] In the histomorphometric analysis of RGD-coated titanium implants placed in beagle dogs, an increase in bone-implant contact at 3 months was reported.[11]

Fibroblast growth factors (FGFs) are polypeptide growth factors that show potent mitogenic activities for cells of mesodermal and neurodermal origin.[12] They probably enhance bone formation by increasing the number of cells capable of synthesizing bone collagen.[13] FGFs are also angiogenic factors, which are important for neovascularization during bone healing. FGF incorporated into a demineralized bone matrix was implanted intramuscularly in rats; this led to increased new bone formation.[14] Bone graft incorporation was also enhanced, both by treatment of the graft with FGF and by continuous use of a miniosmotic pump.[15]

Jang and colleagues[16] showed that FGF enhances fibronectin-mediated adhesion in human osteoblast-like MG63 cells. The mechanism of the synergistic adhesion was due to the activation of extracellular-regulated kinase(ERK)-type mitogen-activated protein kinase (MAPK) upon interaction of integrin to recombinant human fibronectin peptide ($hFNIII_{9\text{-}10}$) and its downstream activation of signaling pathways.[16]

In this study, FGF-FN fusion protein was prepared. On complementary DNA (cDNA) level, FGF-1 and $FNIII_{9\text{-}10}$ fragments were ligated with the histidine (His) $His_6$-tagges at the COOH terminus to provide convenient purification, and then fusion proteins were expressed and purified. The aim of this study was to examine the effect of the biomimetic coating of anodized titanium implants with FGF-FN fusion protein on the formation of peri-implant bone in rabbit tibia.

## MATERIALS AND METHODS

### Implant Preparation (Anodic Oxidation)

Threaded implants were manufactured by machining a commercially pure titanium (grade 4). The length was 7.0 mm, the outer diameter was 4.1 mm, and the pitch height was 0.4 mm. The anodic oxidation treatment of the implant was performed at 300 V in an aqueous electrolytic solution of 0.02 mol/L calcium glycerophosphate and 0.15 mol/L calcium acetate. All procedures were executed at room temperature, and the total time for anodization of 1 implant was 3 minutes.[17,18] Implants were washed with distilled water and then dried. Implants were sterilized in ethylene oxide (EO) gas before animal surgery.

### Construction of FGF1-$hFNIII_{9–10}$ Fusion Protein (Expression Plasmids and Purification)

FGF-1 was amplified from human cDNAs. Polymerase chain reaction (PCR) primers were designed to recognize FGF-1:

- Forward "F1-BF" primers: 5′-GAAGATCTGCT-GAAGGGGAAATC-3′
- Reverse "F1-KR" primers: 5′-GGGGTACCATCAGAA-GAGACTGG-3′

PCR was performed with 1 minute of pre-denaturation at 94°C, 1 minute of annealing at 58°C, and 1 minute of extension at 72°C. After 35 cycles, amplified cDNAs were digested by BglII and KpnI. After digestion, the PCR products were in-frame ligated into the multiple cloning sites of pBAD-His-$FNIII_{9–10}$ with the C-terminal 6X His tag.[16] The FGF1-$FNIII_{9–10}$ fusion proteins containing the poly-His tag were expressed and purified using a nickel ion ($Ni^{2+}$) affinity column under denaturing conditions according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).[16]

The implant samples were divided into 2 groups:

- Group 1: Anodized under constant 300 V
- Group 2: Anodized under constant 300 V, then soaked in a solution containing fusion protein (65 μg/mL) for 24 hours.

### Surgical Procedures

Ten New Zealand white rabbits, aged 6 to 9 months and weighing 3 to 3.5 kg, were used in this study. The animals were kept in separate cages and fed a standard diet. For surgery, general anesthesia was induced by an intramuscular injection of 10 mg/kg ketamine (Yu-han, Gunpo, South Korea) and 0.15 ml/kg Rompun (Bayer Korea, Ansan, South Korea). Both legs were shaved and washed with iodine solution. Two percent lidocaine (1.0 mL) (Yu-han; 1:100,000) was administered at the tibial area.

Using sterile surgical techniques, an incision was made in the skin to expose the proximal aspect of each tibia, and the muscles were dissected to allow the elevation of the periosteum. The flat surface on the lateral aspect of the proximal tibia was selected for implant placement. The holes were drilled with a low-speed rotary instrument under constant irrigation with sterile saline.

Four implants were placed in each rabbit. Two anodized implants were placed in the right tibia, and 2 implants soaked in a solution containing fusion protein for 24 hours were placed in the left tibia. Thus, each rabbit served as its own control.

The surgical site was closed in layers. Muscle and fascial layers were sutured with Vicryl resorbable sutures (Woori Medical, Namyangju, South Korea), while skin was sutured with black silks for primary closure. All animals received 50 mg/kg Kanamycin (Dong-A, Pochun, South Korea) intramuscularly.

### Removal Torque Test

Animals were sacrificed after 12 weeks of healing. Removal torque values were measured on each implant located in the superior part of the tibia (n = 20). The implants were exposed, and removal torque values were measured with a torque measurement device (Shinsung, Seoul, Korea). Torque measurement was carried out by increasing the amount of weight on the device by 20 g until rupture between bone and implant occurred.

### Preparation of Specimens and Histomorphometric Analysis

The remaining intact implants and surrounding tissue (n = 20) were removed en bloc for the purpose of histomorphometric analysis. The implants and the surrounding bone were fixed in neutral buffered formalin; dehydrated in 70%, 90%, 95%, and 100% alcohols; and embedded in a light curing resin (Technovit 7200 VLC; Kulzer, Wehrheim, Germany). The embedded implants were divided longitudinally by sawing (Exakt cutting and grinding equipment; Exakt Apparatebau, Norderstedt, Germany). The sections were ground to approximately 30 μm thick, as described by Donath and Breuner, and were stained with 1% toluidine blue.[19]

The histomorphometric analysis was performed with the aid of an Olympus BX51 microscope (Olympus, Tokyo, Japan) connected to a computer. The software used was KAPPA Imagebase (KAPPA Opto-electronics, Kleines Feld, Germany). All the measurements were calculated with a 10× magnification objective and with a 10× magnification eyepiece. The percentage of bone-implant contact in the 3 consecutive best threads, the percentage of the total implant length,[20] and the percentage of bone inside the same threads were calculated.[21] A higher magnification objective and zoom were used to help determine whether the bone was in contact with the implant surface.

### Statistical Analysis

SPSS 12.0 for Windows software (SPSS, Chicago, IL) was used for statistical analysis. A *t* test ($P < .05$ considered significant) was used to evaluate the removal torque measurement data and the histomorphometric analysis.

## RESULTS

### Removal Torque Values

The removal torque values, measured after a 12-week healing period, are presented in Table 1. The mean removal torque values were 37.6 ±15.0 Ncm for group 1 and 44.8 ±14.7 Ncm for group 2. A significant difference was demonstrated between the 2 groups (*t* test; $P < .05$; Fig 1). The peak force value fell quickly after rupture, and until that moment, no macroscopical movement of the implant was evident.[21]

### Histomorphometric Analysis

After 12 weeks, histomorphometric analysis was performed with a microscope (Figs 2 to 5). The best 3 consecutive threads were analyzed for the percentage of bone-implant contact (Table 2). Group 2 had significantly greater bone-implant contact than group 1 ($P < .05$, Fig 6). The mean values were 76.37% ± 5.61% for group 1 and 88.02% ± 4.42% for group 2.

The percentage of bone-to-implant contact for the total implant length was calculated as well (Table 3). The mean percentage of the total length was 29.47% ± 8.42% for group 1 and 36.91% ± 10.57% for group 2. The *t* test demonstrated a significant difference between group 2 and group 1 ($P < .05$; Fig 7).

In contrast, there was no statistically significant difference in the values for the percentage of the area inside the threads that was filled with bone (Fig 8). The percentage of the area within the threads that was

**Table 1 Removal Torque Values (Ncm) After 12 Weeks of Healing**

| Rabbit no. | Group 1 | Group 2 |
|---|---|---|
| 1 | 38.8 | 55.1 |
| 2 | 33.7 | 53.1 |
| 3 | 18.4 | 25.5 |
| 4 | 33.7 | 40.8 |
| 5 | 74.0 | 70.4 |
| 6 | 33.7 | 38.8 |
| 7 | 26.5 | 25.5 |
| 8 | 48.0 | 57.1 |
| 9 | 38.8 | 49.0 |
| 10 | 30.6 | 32.7 |
| Mean | 37.6 | 44.8 |
| SD | 15.0 | 14.7 |

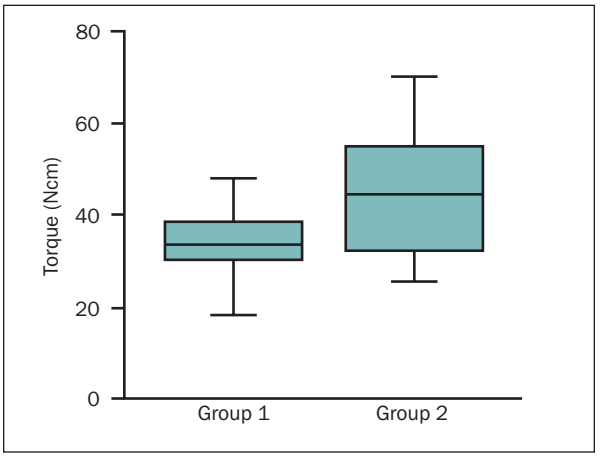


**Fig 1** Mean removal torque values (Ncm). A statistically significant difference between the 2 groups was demonstrated ($P < .05$).

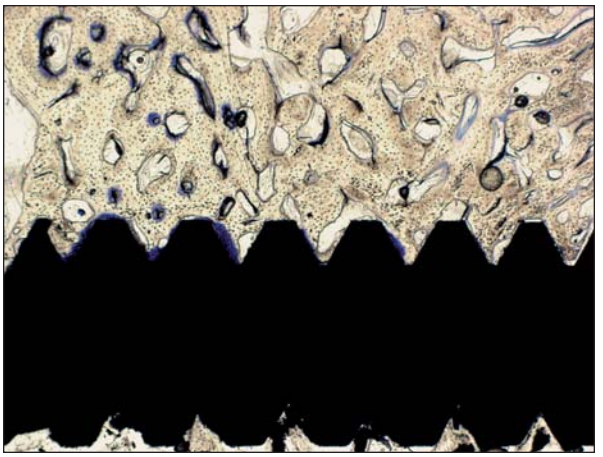

**Fig 2** Light microscopic view of group 1 (toluidine blue; original magnification ×40).

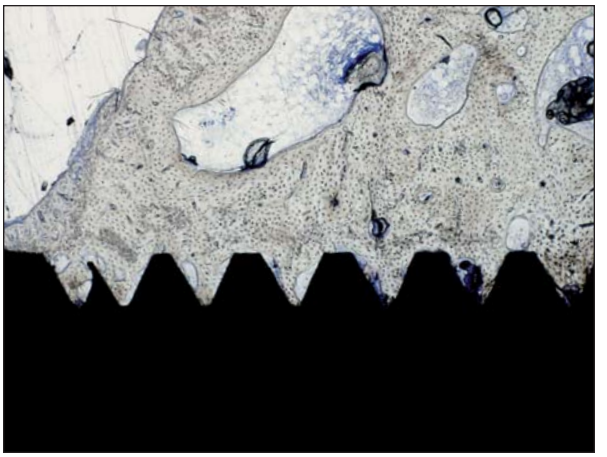

**Fig 3** Light microscopic view of group 2 (toluidine blue; original magnification ×40).

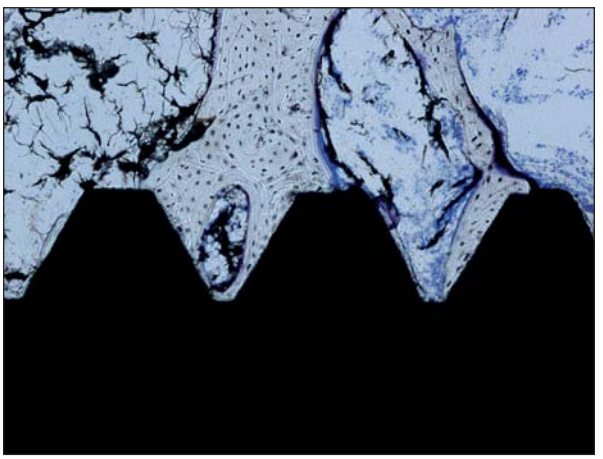

**Fig 4** Light microscopic view of group 1 (toluidine blue; original magnification ×100).

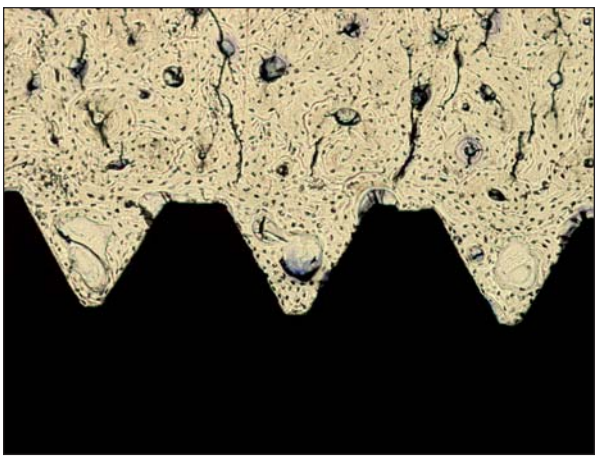

**Fig 5** Light microscopic view of group 2 (toluidine blue; original magnification ×100).

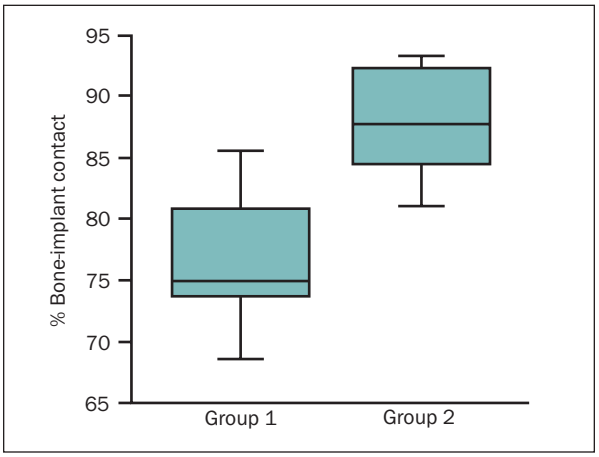


**Fig 6** Mean percentage of bone-implant contact in the best 3 consecutive threads. A statistically significant difference between the 2 groups was demonstrated ($P < .05$).

**Table 2 Percentage of Bone-Implant Contact in the Best 3 Consecutive Threads**

| Implant no. | Group 1 | Group 2 |
|---|---|---|
| 1 | 74.59 | 86.20 |
| 2 | 77.63 | 80.90 |
| 3 | 75.26 | 92.35 |
| 4 | 73.96 | 83.64 |
| 5 | 83.90 | 91.70 |
| 6 | 80.76 | 93.25 |
| 7 | 73.67 | 84.52 |
| 8 | 85.57 | 92.94 |
| 9 | 69.86 | 85.66 |
| 10 | 68.53 | 89.07 |
| Mean | 76.37 | 88.02 |
| SD | 5.61 | 4.42 |

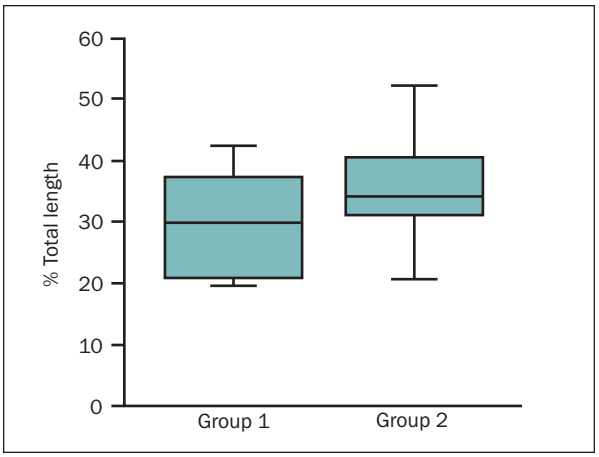


**Fig 7** Mean percentage of bone-implant contact for the total length. A statistically significant difference between the 2 groups was demonstrated ($P < .05$).

**Table 3 Percentage of Bone-Implant Contact for the Total Implant Length**

| Implant no. | Group 1 | Group 2 |
|---|---|---|
| 1 | 31.50 | 32.23 |
| 2 | 37.34 | 40.60 |
| 3 | 31.10 | 29.28 |
| 4 | 20.78 | 20.57 |
| 5 | 19.57 | 32.44 |
| 6 | 39.37 | 52.63 |
| 7 | 28.50 | 36.45 |
| 8 | 42.85 | 55.36 |
| 9 | 22.62 | 31.07 |
| 10 | 21.03 | 38.43 |
| Mean | 29.47 | 36.91 |
| SD | 8.42 | 10.57 |

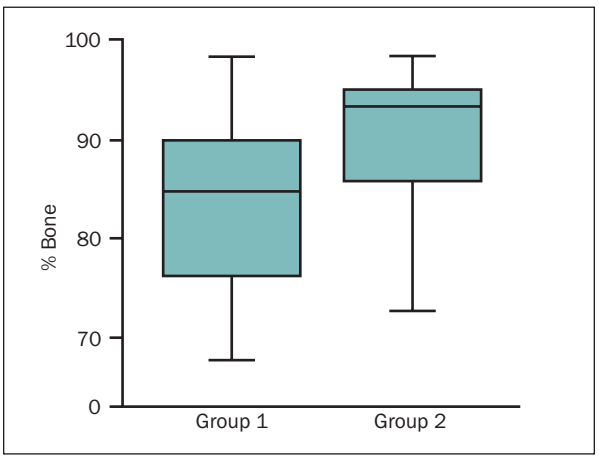


**Fig 8** Mean percentage of the area within the threads that consisted of bone. A statistically significant difference between the 2 groups was not demonstrated.

**Table 4 Percentage of Area Within the Threads Consisting of Bone**

| Implant no. | Group 1 | Group 2 |
|---|---|---|
| 1 | 93.08 | 96.03 |
| 2 | 82.33 | 93.77 |
| 3 | 67.76 | 85.80 |
| 4 | 88.03 | 72.61 |
| 5 | 76.26 | 94.17 |
| 6 | 90.05 | 98.42 |
| 7 | 98.14 | 92.86 |
| 8 | 77.18 | 92.40 |
| 9 | 87.25 | 70.03 |
| 10 | 73.17 | 95.08 |
| Mean | 83.32 | 89.12 |
| SD | 9.61 | 9.94 |

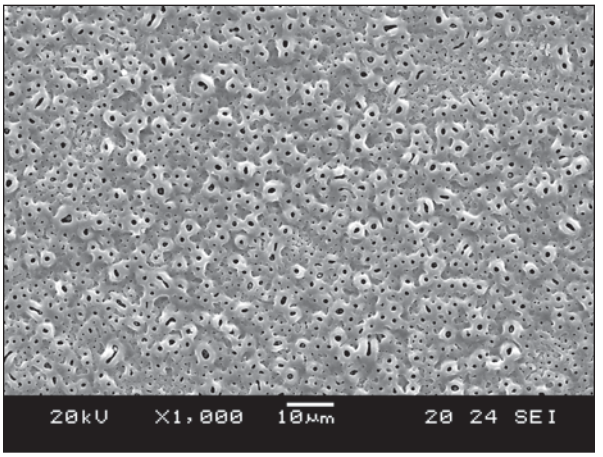


**Fig 9** SEM surface morphology of titanium surface treated with micro-arc oxidation under constant 300 V.

bone was 83.32% ± 9.61% for the nontreated anodized implants and 89.12% ± 9.94% for the anodized implants treated with fusion protein (Table 4).

## DISCUSSION

The organic coating of inorganic surfaces has been widely used to enhance the biocompatibility of implanted materials.[11] The coverage of scaffold materials with matrix proteins such as fibronectin frequently has been applied to direct biological responses.[22] Coating inorganic carriers with RGD peptides in in vitro studies with osteoblasts has resulted in the adhesion of greater numbers of cells and increased mineralization.[23,24] The present researchers reported previously that using $hFNIII_{9\text{-}10}$, which contains the binding site for integrin, and FGF enhanced fibronectin-mediated adhesion in human osteoblastlike MG63 cells.[16]

In the present study, anodized implants with or without a FGF-FN fusion protein coating were placed in rabbit tibia and the bone response to these implants was histomorphometrically analyzed. In addition, bone healing was evaluated by measuring the removal torque value. In the construction of fusion proteins, a recombinant human $FNIII_{9\text{-}10}$ fragment made up of 2 contiguous type III modules containing an RGD polypeptide sequence in $FNIII_{10}$ and a PHSRN synergistic polypeptide sequence in $FNIII_9$ was used. A recombinant human FGF-1 fragment was ligated onto this fragment.

Although synergistic action of FGF and fibronectin on cell attachment and cell spreading was evident in the authors' in vitro study,[16] FGF-FN fusion protein coating on titanium implant itself may be vulnerable to washout by saliva flow under in vivo conditions. For this reason, anodized implants were used. Anodic oxidation of titanium implants has been demonstrated to cause changes of various oxide properties—not only oxide thickness but also surface morphology, pore configuration, crystallinity, chemical composition, and surface roughness.[25,26] Implants prepared in this study had a uniformly porous oxide layer, which was actually composed of small craters with holes at the center. The thickness of oxide layer was 3.1 µm, and the mean pore size was 1 µm in diameter (Fig 9).[18] The pores of the anodized oxide layer were the result of micro arcs which occurred on the surface of the titanium anode. These pores can be utilized as carriers of the growth factors involved in the cellular differentiation and proliferation at the surgical site. In this study, implants of group 2 were soaked in a solution containing fusion protein for 24 hours. The soaked implant may have retained fusion protein on its surface for a certain period of time.

The use of a 12-week healing period was based on the study of Schliephake and associates.[11] They reported that under in vivo conditions, the process of attachment and differentiation of osteoprogenitor cells with the subsequent formation of bone matrix and mineralization may not have been evident after 4 weeks but could have accounted for the significant increase of bone-implant contact after 12 weeks. For this reason, in the present study, the animals were sacrificed after 12 weeks.

For the removal torque test, an electronic device incorporating a strain gauge transducer enabled controlled torque analysis of the peak loosening torque. Hand-controlled devices may introduce operator error; therefore, a conventional device incorporating gravity was used in this study.

Histomorphometric analysis of the bone-implant interface can be carried in different ways, considering various parameters. Investigators often present bone-implant contact as a percentage of the total length or as a percentage of the length of the 3 consecutive "best threads." Depending on bone quality, the ratio of cortical versus cancellous bone, and the length of the implant, significant differences may exist between the "total length" and "3 best threads" results.[21] Thread volume fill and the number of cells in contact with the implant surface are 2 other variables frequently reported in histomorphometric analysis.[27]

The present study demonstrated that coating anodized titanium implants with FGF-FN fusion protein resulted in increased bone-implant contact and removal torque values in comparison with uncoated implants. Greater removal force can generally be

interpreted as an indicator of better bone healing around the implants and improvement in osseointegration. In the histomorphometric analysis, the percentage of bone-implant contact over the total length of the implant, the percentage of bone-implant contact for the 3 best threads, and the bone area inside the thread were measured.[19,27] The bone-implant contact of the group treated with FGF-FN fusion protein was significantly greater than that of the control group. The increase in bone-implant contact can be interpreted as an enhancement of the bone response around the anodized titanium implants coated with FGF-FN fusion protein.

Distance osteogenesis has been described to explain the phenomenon of osseointegration of machined metallic implants. The initiation of mineralization of the healing bone tissue did not occur on the implant surface, but bone grew toward the implant subsequent to the death of the intervening tissue. In contrast, new bone forms first on the implant surface in the process of contact osteogenesis. A combination of the recruitment and migration of osteogenic cells and bone formation by those cells on the implant surface occurs. In the case of poor bone quality, optimizing contact osteogenesis by implant surface design is important to ensure early stability. Implant surface design is closely connected with osteoconduction. It plays a role not only in modulating the levels of platelet activation, but also in maintaining the anchorage of the temporary scaffold through which these cells reach the implant surface.[3]

This study demonstrated that an FGF-FN coating on anodized implants may have an effect on converting bone response from distance osteogenesis to contact osteogenesis, enhancing the stability and predictability of implant treatment in cases of poor bone quality and insufficient quantity. Future experimental and clinical studies should be designed to investigate the influence of FN- and FGF-treated rough surfaces on long-term prognosis and the propagation of inflammation.

## CONCLUSIONS

The objective of this study was to evaluate the bone response around anodized titanium implants treated with FGF1-hFNIII$_{9\text{-}10}$ fusion protein and untreated titanium implants. Such implants were placed in rabbit tibiae and evaluated 12 weeks later using histomorphometric analysis and measurement of the removal torque values. In the present study, anodized titanium implants treated with FGF1-hFNIII$_{9\text{-}10}$ fusion protein demonstrated significantly higher removal torque values and significantly higher bone-implant contact (in analysis of bone-implant contact for the best 3 threads and for the total length of the implant) than the untreated anodized implants. However, the 2 groups did not differ significantly with respect to the percentage of the implant threads filled with bone.

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