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Bone Formation of Submicron Poly(lactide-co-glycolide)/TGF-β2 Application on Anodized Titanium Implants by Electrospray: An Animal Study

Submicron Poly(lactide-co-glycolide)/TGF-β2를 전기분사법으로 코팅한 양극산화 타이타늄 임플란트의 골형성능: 동물실험

2016년 8월

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Bone Formation of Submicron Poly(lactide-co-glycolide)/TGF-β2 Application on Anodized Titanium Implants by Electrospray: An Animal Study

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Abstract

Bone Formation of Submicron Poly(lactide-co-glycolide)/TGF-β2 Application on Anodized Titanium Implants by Electrospray: An Animal Study

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(Directed by professor Seong-Kyun Kim, D.D.S., M.S.D.,Ph.D.)

Purpose: Transforming growth factor - β2 has been shown to influence the proliferation and differentiation of osteoprogenitor cells in vitro. However, due to complexity of bone formation mechanism, effects of various growth factors on osseointegration of dental implants are not clearly understood yet. This study aimed to evaluate the bone formation effect on osseointegration of anodized titanium implants coated with poly(D,L-lactide-co-glycolide)(PLGA)/recombinant human transforming growth factor – β2 (rhTGF-β2) submicron particles by electrospray technique in rabbit tibia model.

Materials and Methods: 48 implants were used in 12 New Zealand rabbits for in vivo study, and 14 implants were used for surface analysis. Anodized titanium implants coated with PLGA/rhTGF-β2 submicron particles by electrospray
technique were tested as an experimental group (n=24) compared to anodized
titanium implants as a control group (n=24) in an in vivo rabbit tibia model. Implant
surface examination was done by using field emission-scanning electron microscope,
and the surface roughness of the implants was measured with atomic force
microscopy. 30 µm thick specimens were prepared for histomorphometric analysis,
which was done at 3 weeks and 6 weeks after implants being placed. Measured bone
to implant contact (BIC) and bone area (BA) were statistically analyzed using
Krukal-Wallis test and Mann-Whitney U-test with p-value being adjusted by
Bonferonni correction.

**Results:** FE-SEM analysis of implant surfaces confirmed uniform coating of
PLGA/rhTGF-β2 particles. There were no statistically significant differences in
surface roughness between two groups (p=2.78). Histomorphometric analysis
revealed that BIC% and BA% of three best consecutive threads in rhTGF-β2 coated
titanium implants in 3 weeks were statistically significant compared to control
groups (p=0.045 and p=0.048 respectively). BIC% of experimental group of three best
consecutive threads in 6 weeks was also found to be higher than control groups
(p=0.033) whereas BA% of experimental group of three best consecutive threads in
6 weeks did not show statistically significant results. All of groups tested in total
length of implant did not show any statistically significant differences between
control and experimental groups.

**Conclusion:** With the electrospray technique, a uniform and submicron coating of
rhTGF-β2 was able to be achieved. Effective and optimal concentration of rhTGF-
β2 was identified to enhance BIC and BA during early healing period with the help of the PLGA carrier in a rabbit model.

**Keywords:** recombinant human transforming growth factor- β2, polylactic acid-polyglycolic acid copolymer, osseointegration, anodized implant

**Student Number:** 2013-31188
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I. Introduction

The key to success in implant dentistry is osseointegration between contacting bone tissues and implant surface.\(^1\) Thus, it was great interest to implant dentistry in improving osseointegration in dental implant surface, and one way to achieve is by modifying implants surfaces either physically by roughening the surfaces or chemically altering ionic composition or hydrophilicity of implant surfaces.\(^2\)\(^-\)\(^7\)

Recently, biomimetic coatings of implant surfaces have been extensively researched through various methods to improve osseointegration of dental implants.\(^8\) rhBMPs and rhTGF-\(\beta\) have been known to be key candidate molecules associated with successful osseointegration.\(^9\) In previous study by our group, we have shown that rhBMP-2 could promote cellular response to induce osteogenesis in early period of implant placements.\(^10\)

However, recently it has been reported that there are some limitations in using BMPs. BMPs often have been shown to cause a series of adverse side effects, such as pain, radiculitis, ectopic bone formation, osteolysis, and poor global outcomes.\(^11\) In addition, higher doses of rhBMP-2 have been associated with a greater apparent risk of cyst-like bone void formation, soft tissue swelling, inflammatory and adipogenic induction.\(^12\)\(^-\)\(^14\)

TGF-\(\beta\) on chondrogenesis has been known to be effective in many studies.\(^15\)\(^,\)\(^16\) Bosetti et al. found that when rhTGF-\(\beta\)2 combined either with FGF-4 or FGF-6 induced chondrogenesis of human bone marrow mesenchymal stem cells.\(^17\) Other
studies had consistent results with the effect of rhTGF-β1 and rhTGF-β3 on chondrogenesis.\(^\text{15}\) Whereas rhBMP-2 has been shown beneficial effect on ossteointegration in dental implants, the effect of rhTGF-β2 on ossteointegration was unclear or presented rather conflicting results in previous studies.\(^\text{18-21}\) This is due to its complex interactions with other growth factors and multifunctional regulatory characteristics of rhTGF-β2.\(^\text{19}\) Robey et al. also stated that rhTGF-β2 might be involved in the “coupling” of bone resorption and bone formation to maintain normal rates of bone turnover.\(^\text{21}\) TGF beta has both stimulatory and inhibitory effect on proliferation of osteoblasts.\(^\text{18}\) That depends on the concentration, timing and kinetics of growth factor administration.\(^\text{19}\) This is why it is especially critical controlling the concentration, timing and kinetics of growth factor administration. Thus this complexity of rhTGF-β2 interaction may explain why some studies found that there was no significant and consistent increase of cell proliferation observed with rhTGF-β2.\(^\text{18}\)

Thus, the controlled and stable delivery of growth factors immobilized on titanium implants is very important in order to scrutinize the effect of specific growth factors on osteogenesis. However, conventional coating techniques such as soaking or immersion in aqueous solution often involve limited adhesion and composition of growth factors.\(^\text{8}\)

The electrospray technique allows for easy control of thickness and composition of coating layers. From previous studies conducted using the
electrospray technique, this resulted in a uniform coating within the range of submicron particles.\textsuperscript{10, 22, 23} Therefore, the present investigation used the electrospray technique, which allows controlling even thickness and composition of coating layers.\textsuperscript{23} In addition to that, using biocompatible synthetic polymer PLGA allows to modulate the temporal release of target growth factor.\textsuperscript{24} This technique in turn results in controlled and sustained release of rhTGF-\(\beta\)\textsubscript{2}, and is expected to be a suitable experimental model to examine the effect of specific growth factor on osteogenesis between titanium implant surface and tissues.\textsuperscript{25}

The purpose of this study is to analyze topographical implant surface characteristics of submicron-sized PLGA/rhTGF-\(\beta\)\textsubscript{2} coated by electrospray technique and to identify the effect of PLGA/rhTGF-\(\beta\)\textsubscript{2} coating on osseointegration quality and quantity in 3 and 6 weeks evaluating histomorphometric analysis in an \textit{in vivo} rabbit tibia model.
II. Materials and Methods

Implant preparation

Total 62 threaded commercially pure grade IV titanium implants were prepared (Warantec Co., Seoul, Korea). 48 implants were used for in vivo study and 14 implants were used for surface analysis. The implants had a total length of 7 mm, a diameter of 3.75 mm, and an average thread pitch height of 0.6 mm. They were cleaned ultrasonically in acetone, ethanol, and deionized water. Anodic oxidation of the implants was performed at room temperature at 300 V in an aqueous electrolytic solution of 0.15-mol/L calcium acetate monohydrate and 0.02-mol/L calcium glycerophosphate. Implants were sterilized in ethylene oxide gas prior to surface modification.26, 27

Implant surface modification

4 mL PLGA (PURAC Biochem BV, Gorinchem, Holland), a 50:50 ratio mixed DL-lactide/glycolide copolymer in 0.4% w/v aceton (Duksan pure chemicals Co., Kyungkido, Korea) and 4 mL of 25 μl rhTGF-β2 (Prospec-TechnoGene Co., East Brunswick, NJ) in sterile 4mM HCL containing 0.1% BSA (BSA; Becton Dickinson, Franklin Lakes, NJ) were incorporated to coat the titanium surface by electrospray technique. Total 48 implants were coated by electrospray technique as follows:

Control group: Anodized at 300 V, under 660 Hz DC power for 10 min.26, 28
**Experimental group**: Anodized at 300 V, under 660 Hz DC power for 10 min and then coated with PLGA/rhTGF-β2 submicron particles (3 µg/ml per implant) by electrospray.

**Surgical procedures**

12 New Zealand white female mature rabbits, weighing 3 to 3.5 kg, were used in this study. The animals were kept in separate cages and were fed a standard diet. Selection, care, surgical protocol, and preparation of the animal were abided by the guideline approved by the Institutional Animal Care and Use Committee, School of Dentistry, Seoul National University (SNU-140129-3). General anesthesia was induced by an intramuscular injection of 10 mg/kg Zoletil (Vibac, Carros, France) and 0.15 ml/kg Rompun (Bayer Korea Co., Ansan, Korea). Prior to the surgery, the shaved skin in the proximal tibia was washed with iodine solution and prophylactic intramuscular injection of a 50 mg/kg kanamycin (Dong-A Co., Pochun, Korea) was administered. 2% lidocaine solution (1:100,000 Epinephrine) (Yu-han Co., Seoul, Korea) of 1 ml was also injected in the tibia for surgery. 29

As shown in Figure 1, following sterile surgical techniques, an incision was made in the skin to expose the proximal aspect of each tibia, and muscles were dissected to allow the elevation of the periosteum. The flat surface on the lateral aspect of the proximal tibia was chosen for implant placements. 12 rabbits received four implants each in pre-assigned orders. Two anodized implants (control group) and two implants coated with PLGA/rhTGF-β2 solution (experimental group) were placed. All implants are placed on the crestal level of tibia. Based on the
predetermined randomized design, two implants from each group were installed at contralateral side respectively to make multiple comparisons. The surgical site was sutured separately. Muscular and fascial layers were sutured with resorbable suture material of Vicryl (Woori Medical, Namyangju, Korea), while skin was sutured with black silks (Mersilk, Ethicon Inc., Somerville, NJ) for primary closure.

![Surgical procedure](image)

**Fig.1 Surgical procedures.**

(a) An incision was made on the skin to expose the proximal aspect of tibia, and muscles were dissected to elevate the periosteum. The flat surface on the lateral part of the proximal tibia was chosen for the implant placements. The anodized implant (control group) and an implant coated with PLGA/rhTGF-β2 (experimental group) were placed according to the predetermined randomized design.

**Implant surface examination**

Overall surface morphology was confirmed by a field emission scanning electron microscopy (FE-SEM) (S-4700, Hitachi, Ltd., Tokyo, Japan) at 15kV accelerating voltage. Images taken by FE-SEM were analyzed by an image analysis
program (KAPPA Image LLC, Oakland, CA, USA) at 60 randomly chosen areas on the implant surfaces to measure the coated particle sizes of the titanium implant surface. The surface roughness of the implants was measured with atomic force microscopy (FM XE-10, Park Systems, Suwon, Korea).

**Preparation of specimens and histomorphometric analysis**

6 rabbits after 3 weeks and remaining 6 rabbits after 6 weeks were sacrificed for histologic evaluation and histomorphometric analysis. The rabbits were anesthetized and sacrificed with an intravenous administration of KCl. The implants and surrounding bone were harvested en bloc and fixed in neutral buffered formalin, dehydrated in 70%, 80%, 90%, 95% and 100% alcohol, and embedded in a light-curing resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). The Exakt sawing machine and grinding equipment (Exakt Apparatebau, Norderstedt, Germany) was used to cut and grind. The sections were approximately 30 µm thick and stained with 1% toluidine blue, as described by previous study.30

The histomorphometric analysis was performed with the aid of an Olympus IX71 microscope (Olympus Co., Tokyo, Japan) connected to a computer. Cellsense (Olympus Co., Tokyo, Japan) software was used to measure and calculate bone to implant contact (BIC) under ×100 magnification (×10 objective and ×10 eye-pieces) as shown in Figure 2d. Percentages of BIC and bone area in three consecutive threads (Fig. 2b and 2c) and the total implant length (Fig. 2a) were calculated.31 A higher magnification objective and zoom were used to determine if the bone was directly in contact with the implant surface.
Fig. 2 Quantitative BIC% and BA% measurements in 3 best consecutive threads and total length.

(a) x12.5 magnification view showing total length of interest for BIC and BA measurements (b) 3 best consecutive threads in cortical region is selected in x12.5 magnification view. (c) In x40 magnification view, 3 best consecutive threads’ length and inner thread areas are calculated. (d) Under x100 magnification view, actual bone-to-implant contact and bone area within the threads are determined as shown in red lines and blue shadow area respectively.
Statistical analysis

All statistical analyses were conducted by the SPSS (IBM SPSS Inc., Chicago, IL, USA). For the result of surface roughness and size of sprayed particles, paired t-test were used because the assumption of normality was satisfied. As the assumption of normality and equality of variances was not satisfied for the distributions of histomorphometric data in vivo, the Kruskal-Wallis test was performed to evaluate. When a significant result was obtained, the pairwise comparisons were carried out using Mann Whitney U-test under the type one error rate adjusted by Bonferroni correction. Statistical significant level was set to p-value of 0.05.
III. Results

Characteristics of titanium implant surfaces

FE-SEM results of the anodized Titanium implant surface showed that the surface had rough, porous oxide layers and was composed of small craters with holes at the center. FE-SEM image was analyzed to evaluate mean particle size, and it was shown to be 0.21±0.13 μm ranging from 0.12 – 0.46 μm which is less than 1 μm and could be interpreted as submicron sized particles. By the electrospray coating method, round PLGA/rhTGF-β2 submicron particles were deposited on the anodized titanium surface (Fig. 3). Submicron sized PLGA/rhTGF-β2 particles are distributed fairly uniformly on the anodized titanium surface.

Fig.3 FE-SEM (x5000) image of titanium implant surface coated by electrospray technique (a) Anodized titanium implant (b) Anodized titanium implant coated with PLGA/rhTGF-β2 (3μg/ml per implant) by electrospray technique. The image analysis of titanium implant surface coated with PLGA/rhTGF-β2 revealed that the mean size of the particles was 0.21 ± 0.13 μm (range from 0.12 - 0.46 μm)
AFM test results

AFM results showed that there was no statistically significant difference between control and experimental groups of implant surfaces. The mean roughness (Ra) of control group and experimental group were $1.227 \pm 0.03 \, \mu m$ and $1.229 \pm 0.01 \, \mu m$ respectively with p-value of 2.78 (Fig.4 and Table. 1).

![AFM results](image)

Fig.4  The results of titanium implant surface roughness measured by AFM (10µm x10µm) (a) Anodized titanium implant (b) Anodized titanium implant coated with 3 µg/ml rhTGF-β2.

| Table 1. Results of surface roughness (Ra) of anodized titanium surfaces |
|---------------------------------|-----------------|-----------------|
| **Ra (surface roughness) by AFM (mean value) with standard deviation: Unit (µm)** | n | Mean ± SD | P-value |
| Anodized titanium implant (Control group) | 20 | $1.227 \pm 0.03$ | |
| Anodized titanium implants coated with 50µg/ml rhTGF-β2 (Experimental group) | 20 | $1.229 \pm 0.01$ | $P = 2.78$ |
**Histological Findings**

The implantation sites in the distal and proximal tibia both consist of cortical bone, implying that the two most coronal threads are located within the cortex as shown in Fig. 5. The remaining part of the implant protrudes into the marrow cavity without contacting the endosteal surface of the opposite cortex. Histological analysis showed bone formation around and in direct contact with the implant surface (Fig.5 and Fig.6). Bone tissue had extended into the threads from the surrounding bone tissue appearing to extend apically and inwards. Tendency of formation of new bone in the apical direction seems to be stronger in experimental groups. No morphological signs of adverse events, such as the presence of inflammatory infiltrates, were detected.

Fig. 6 shows light microscopic view of 30-µm-thick toluidine blue-stained ground sections after 3 weeks and 6 weeks. As shown in Fig. 6, there was increased tendency of new bone formation in experimental groups of 3 weeks while not much differences confirmed in 6 weeks. Generally, density and size of osteocyte lacunae seem to be larger around implant contact surfaces indicating new bone formation. This newly formed mineralized tissue extended from the endosteum onto the implant surface of all implant groups. The osteocytic lacunae were smaller in 6 weeks than those in 3 weeks implying more mature bone structure in 6 weeks.
Fig. 5  Histologic images of implant and bone tissue (Toluidine blue) of 3 weeks after implant placement. (a) Implant (I) is placed in a dense cortical bone (CB) and into a bone marrow cavity (BM) of upper region of the tibia. (magnification x12.5) (b) The white arrow(←) denotes a new bone formation region along the implant surface in the apical direction. (magnification x40) (c) The reversal line(←), osteocyte lacuna(*), harversian canals(☜) are shown. (magnification x100) (d) The PLGA/rhTGF-β2 coated titanium implant is placed in a lower region of the right tibia. (magnification x12.5) (e) The arrow(←) denotes a new bone formation region along the PLGA/rhTGF-β2 coated implant surface in the apical direction. (magnification x40) (f) The reversal line(←), osteocyte lacuna(*), harversian canals(☜) are shown. (magnification x100)
Fig. 6  Light micrographs of 30-µm thick sliced and ground sections in x100 magnification

- After 3 weeks. (a) Control group (b) Experimental group
- After 6 weeks. (c) Control group (d) Experimental group
Histomorphometric Analysis

The BIC percentages on best three consecutive threads in crestal bone area (BIC-3) and the total implant length (BIC-T) at 3 weeks and 6 weeks were measured (Table 2), and mean and standard deviation of control groups and experimental groups of BIC-3 at 3 weeks were 54.98 %±10.55 and 67.82 %±10.79 respectively (Fig 7a, Table 2). Mean and SD of control groups and experimental groups of BIC-3 at 6 weeks were 66.28 %±14.00 and 80.29 %±11.11 respectively (Fig. 7c, Table 2). Both BIC-3 at 3 weeks and 6 weeks showed statistically significant differences (p=0.045 and p=0.033 respectively). However, there was no statistically significant difference found on BIC-T at 3 and 6 weeks.

Statistical analysis of bone area on the three consecutive threads (BA-3) at 3 weeks confirmed statistically significant differences between control and experimental groups with mean and SD of 66.17 %±8.90 and 73.12 %±8.10 respectively (p=0.048) (Fig. 7b, Table 2). BA-3 at 6 weeks showed no statistically significant differences between control and experimental groups (Fig. 7d, Table 2). Bone area in the total implant thread area both at 3 weeks and 6 weeks were not statistically significant (p>0.05).
Table 2. Histomorphometric analysis. Bone to implant contact (%) and bone area (%) at 3 and 6 weeks after implant placement.

<table>
<thead>
<tr>
<th>Time/measurement</th>
<th>n</th>
<th>Control</th>
<th>Experimental</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td><strong>Week 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIC% (total)</td>
<td>24</td>
<td>45.52±12.58</td>
<td>45.63±6.63</td>
<td>p=0.488</td>
</tr>
<tr>
<td>BIC% (three consecutive)</td>
<td>24</td>
<td>54.98±10.55</td>
<td>67.82±10.79</td>
<td>*p=0.045</td>
</tr>
<tr>
<td>Bone area (total)</td>
<td>24</td>
<td>45.66±15.79</td>
<td>49.45±7.69</td>
<td>P=0.149</td>
</tr>
<tr>
<td>Bone area (three consecutive)</td>
<td>24</td>
<td>66.17±8.90</td>
<td>73.12±8.10</td>
<td>*p=0.048</td>
</tr>
<tr>
<td><strong>Week 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIC% (total)</td>
<td>24</td>
<td>55.76±10.38</td>
<td>53.73±9.17</td>
<td>p=0.729</td>
</tr>
<tr>
<td>BIC% (three consecutive)</td>
<td>24</td>
<td>66.28±14.00</td>
<td>80.29±11.11</td>
<td>*p=0.033</td>
</tr>
<tr>
<td>Bone area (total)</td>
<td>24</td>
<td>50.92±10.69</td>
<td>47.38±8.99</td>
<td>p=0.488</td>
</tr>
<tr>
<td>Bone area (three consecutive)</td>
<td>24</td>
<td>72.91±12.18</td>
<td>77.34±6.58</td>
<td>p=0.436</td>
</tr>
</tbody>
</table>

*p-value adjusted with Bonferroni correction
Fig. 7  Histomorphometric analysis.

(a) BIC (%) at 3 weeks. The BIC (%) over three consecutive threads and total implant length were measured. There were statistically significant differences (p=0.045; the graph bar represents mean± SD) in BIC (%) measured over three consecutive threads. However, there was no statistically significant difference in BIC (%) measured over total implant length (p>0.05; the graph bar represents mean± SD).

(b) BA (%) at 3 weeks. There were statistically significant differences between groups in three consecutive threads (p=0.048; the graph bar represents mean± SD). However, there was no statistically significant difference between groups in total implant length (p>0.05; the graph bar represents mean± SD).

(c) BIC (%) at 6 weeks. The BIC (%) measured over three consecutive threads at 6 weeks showed statistically significant differences between control and experimental groups (p=0.033) whereas there was no significant difference between groups measured over total implant length (p>0.05; the graph bar represents mean± SD).

(d) BA (%) at 6 weeks. The BA (%) both in three best consecutive threads and total threads at 6 weeks was not statistically significant different (p>0.05)
IV. Discussion

Growth factors can promote replication, differentiation, protein synthesis and/or migration of proper cell types.\textsuperscript{32, 33} If a growth factor binds to a target cell receptor, it produces biological responses thorough an intracellular signal transduction system. TGF-\(\beta\)2 has been known for a positive regulator of bone remodeling accelerating bone repair by coordinating osteoblast and osteoclast activities.\textsuperscript{21}

Polypeptides from the TGF-\(\beta\) family are initially synthesized as pre-pro-TGF-\(\beta\), a monomer of a molecular weight of ca. 55 kDa and consisting of 390 amino acid residues in total, including N-terminal signal peptide of 29 amino acids, a pro-region of 249 amino acids called latency associated peptide, and a C-terminal sequence of 112 amino acids forming the actual active form of TGF-\(\beta\) after relevant modifications.\textsuperscript{34, 35} Active form of homodimers, TGF-\(\beta\)2 interacts with a receptor complex forming a heterotetrameric combination containing two of each of type I and type II subunits.\textsuperscript{36-38} Once receptor complexes activated, various intracellular signal pathway is stimulated to induce osteogenic genes. TGF-\(\beta\)2 upregulates expression of Runx2, which is the osteoblastic phenotypic marker, and is required for proliferation in both osteoprogenitor and chondroprogenitor cells.\textsuperscript{15}

In this study, we have used recombinant human TGF-\(\beta\)2 applied to rabbits. It has been known that Mature human TGF-beta 2 shows 100% amino acid identity with porcine, canine, equine and bovine TGF-beta 2, and 97% amino acid identity with mouse and rabbit TGF-beta 2. It demonstrates cross-species activity.\textsuperscript{34}
Growth factors released from an implant surface can influence the osteoblastic activity of the bone tissue.\textsuperscript{39} However, because of complexity of interactions with various growth factors involved in bone regeneration, optimal growth factor dosage, release kinetics and duration are critical to be able to impact osseointegration of implants.\textsuperscript{40} Based on the results of our previous in-vitro study\textsuperscript{22} of rhTGF-β2 coated implant, we were able to identify optimal concentration of rhTGF-β2 to effectively induce statistically significant changes in histomorphometric analysis of New Zealand rabbit model.\textsuperscript{23}

In the present study, unlike other biomimetic coated implants, a more uniform rhTGF-β2 layer was obtained on the titanium surface with the electrospray techniques.\textsuperscript{41} Electrospray technique yielded a monodispersed layer of PLGA/rhTGF-β2 submicron particles as shown in Fig 3. Catledge et al. stated that in order for the effective attachments of mesenchymal stem cells to implant, implant surface needs to be controlled in submicron level.\textsuperscript{42} Submicron-sized particles which is smaller than the average size of capillary blood vessels which is around 5 µm will be disseminated and metabolized more easily and will influence mesenchymal stem cells around implant more effectively and faster than when particles are not in submicron sizes.\textsuperscript{43}

Another important consideration when coating growth factors on implant surfaces is to choose a proper carrier system to deliver growth factors. The limiting factor regarding the use of growth factors in surface treatment of implants is that the active product has to be released progressively and not in a single burst.\textsuperscript{44} To
maximize efficacy, BMPs must be delivered to the target site gradually, at a low level and in a sustained manner, rather than in a single high-dose burst. One example of the carrier system which has been commercially available these day is collagen sponges functionalized by the adsorption of several milligrams of BMP-2 with the goal of promoting the repair of large bony defects. However, this method of BMP-2 delivery is far from satisfactory because a surface-adsorbed depot of the protein is released too rapidly in a single high-dose burst.\textsuperscript{45, 46}

However, the PLGA polymer carrier, which is being used in this study has been effective in stable and sustained release of growth factors when mixed with PLGA. Theoretically, when PLA and PGA were mixed in 50:50 ratio as designed in our study, the degradation rate and time is expected to be 7-60 days.\textsuperscript{25} In previous study where PLGA was used as the carrier, the anodized titanium disks coated with PLGA/rhBMP-2 released rhBMP-2 over time, and the discharged concentration of rhBMP-2 reached a peak at 7 days and decreased thereafter.\textsuperscript{47}

Our study with increased bone formation findings in rabbit are consistent with previous work done in rat.\textsuperscript{48} In this rat study, titanium implants coated with 10 µg of rhTGF-β2 showed increased BIC and bone volume fraction(BV/TV). However, there were also negative findings reported in sheep and rat.\textsuperscript{49} To the best of my knowledge, this study is the first to test the effect of rhTGF-β2 coated titanium implants experiment in rabbit. Therefore, the concentration tested in this study would provide an acceptable reference for the future rhTGF-β2 related study.

It has been known that mechanically rough-surfaced titanium implants
enhances both bone anchorage and biomechanical stability, and certain range of 
surface roughness favors osseointegration than others.\textsuperscript{50, 51} In this study, we have 
evaluated the surface roughness of control and experimental groups of titanium 
surfaces, and found that there were no statistically significant differences between 
two groups. This finding rules out the possible effect of surface roughness on bone 
responses around dental implants.

In the course of bone regeneration, osteocyte lacunar density and area 
undergo substantial changes. During fracture healing, the osteocyte lacunar density 
is almost twice as high in woven bone compared with mature lamellar bone.\textsuperscript{52} In 
qualitative histological analysis, we have found consistent results with previous 
studies in that there was generally increased tendency of density and size of 
osteoocyte lacunae in close to implant contact surfaces. This implies that newly 
formed bone structures are extended into threads from surrounding pre-existing bone 
tissue.

Increased BIC and BA findings on rhTGF-\(\beta\)2 treated groups in 3 weeks are 
consistent with previous works on positive effect of rhBMP-2 coated dental 
implants.\textsuperscript{53} This suggests that rhTGF-\(\beta\)2 can be a potential positive regulator like 
rhBMP-2 in early healing period. However, whereas there are many studies 
conducted with rhBMP-2 for osseointegration, there are limited or scarce 
information available for the effect of rhTGF-\(\beta\)2 at this time. This may be due to its 
complex interactions with other growth factors and multifunctional regulatory 
characteristics of rhTGF-\(\beta\)2.
While in previous study, rhBMP-2 showed the osteogenic effect exclusively in 3 weeks, in this study, rhTGF-β2 affected BIC% on 6 weeks as well. This suggests that rhTGF-β2 somehow involves in later stage of bone formation as well as early healing period. rhTGF-β2 has been reported that it stimulates BMP activity in the early phases of bone healing just before the BMPs exert their effects. rhTGF-β2 stimulates proliferation of osteoblast precursors. The combination of both growth factors has shown a synergistic effect on implant ingrowth through related but separate signal transduction pathways; TGF-β with control of osteoprogenitor cell proliferation, BMPs with more important influence in osteoblasts differentiation. Therefore, in this study, rhTGF-β2 may have increased number of osteoblast precursors which may in turn stimulate BMP activity differentiating osteoblast precursor cells to osteoblast. Another possible cause of effect of BIC% on 6 weeks in this study would be due to the sustained release of rhTGF-β2. As mentioned earlier, using PLGA carrier allows sustained release rhTGF-β2 not in a single dose burst. It can be assumed that sustained release of rhTGF-β2 after 3 weeks may have somehow affected BIC% results in 6 weeks.

Growth Factors are closely related each other both structurally and functionally, and each has a distinct temporal expression pattern and potentially unique role in fracture healing. Abe et al. stated that multiple BMPs in combination with other growth factors administered in a specific temporal sequence are necessary for the complete process of new-bone formation in vivo. Because of differential temporal effect between rhBMP-2 and rhTGF-β2, evaluating the effect of rhBMP-2
combined with rhTGF-β2 may be interesting subject for the future study.

Because of possibility of individual variations in the rabbits influencing the outcomes of the experiment, we could not directly compare between 3 weeks and 6 weeks statistically, but we were able to find general increasing trend both BIC and BA of 3 consecutive threads from 3 weeks to 6 weeks. However, BA and BIC calculated over the entire implant surface did not show any statistically significant results. This may be due to the fact that regeneration from dense cortical bone could not continue along the length of the implant into the bone marrow. Lee et al. showed in previous work that in a rabbit tibia model, the rate of cancellous bone formation was slower than that of cortical bone regeneration.\textsuperscript{61}

Even if the results of rhTGF-β2 effect on osseointegration seem to be promising, there is still limited information available on undesired host/tissue reactions of certain growth factors. However, this novel approach of combining the electrospray coating technique with effective PLGA carrier method will undoubtedly have a promising effect on biomimetic implant dentistry.
V. Conclusion

With the electrospray technique, a uniform coating of rhTGF-β2 was able to be achieved, and average particle size of rhTGF-β2 found to be submicron as expected. However, there was no statistically significant difference in surface roughness between control and experimental groups, and this could lead to the conclusion that the roughness did not affect the result of our study. From this study, effective and optimal concentration of rhTGF-β2 was identified to enhance BIC and BA during early healing period with the help of the PLGA carrier in a New Zealand rabbit model. In addition to that, rhTGF-β2 affected BIC % in 6 weeks as well as 3 weeks implying rhTGF-β2 may play a role in osseointegration process in late healing period. Within limited scope of this study, this study showed possibility of accelerating the osseointegration rate in titanium implants coated with the submicron-sized PLGA/ rhTGF-β2 during early healing period. This approach could be a viable therapeutic strategy in future implants dentistry.
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Abstract in Korean

Submicron Poly(lactide-co-glycolide)/TGF-β 2를 전기분사법으로 코팅한 앙극산화 타이타늄 임플란트의 골형성을: 동물실험

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목적: 재조합 형질 전환 성장 인자 중 하나인 rhTGF-β 2는 골형성에 효과적이라고 알려져 있다. 그러나 다른 골 형성 단백질과 비교하여 rhTGF-β 2를 이용한 타이타늄 임플란트에 대한 연구는 미비하다. 본 연구의 목적은 New Zealand rabbit model에서 PLGA를 이용하여 rhTGF-β 2를 전기분사법으로 양극산화 타이타늄 임플란트 표면에 코팅한 후 식립했을 때의 골형성을 알아보고자 하는 것이다.

제료 및 방법: 총 48개의 치과용 임플란트가 12 마리의 New Zealand rabbit에 식립되었다. 전기분사법을 이용하여 PLGA/rhTGF-β 2 코팅된 임플란트 실험군 (n=24)과 코팅되지 않은 대조군 (n=24)을 토끼 경골에 식립하여 조직형태학적 분석 및 조직학적 분석을 시행하였다. 전계 방출 주사 전자현미경 (FE-SEM)을 이용하여 코팅된 타이타늄 임플란트의 표면 형태분석을 하였으며, 원자간력 현미경 (AFM)을
사용하여 표면 거칠기를 측정하였다. 임플란트 삽입 후 점적 3주와 6주째 토끼를 희생시켜 30μm 두께의 시편을 제작하였다. 조직학적 분석을 통해 골-임플란트 접촉률과 골조직양을 측정하여 통계분석 하였다. 통계방법은 비모수법 Kruskal wallis test를 사용하였으며 Mann-Whitney U-test로 검정한 후 Bonferonni correction으로 p-value를 조정하였다.

결과: 전계 방출 주사 전자현미경 (FE-SEM) 관찰을 통해, 임공한 submicron 크기로 PLGA/rhTGF-β2 코팅이 되었음을 확인하였으며, 원자간력 현미경 (AFM)을 통한 관찰에서는 대조군과 실험군 사이에 거칠기에 유의한 차이가 없었다 (p>0.05). 3개의 연속된 나사선에서 측정할 때, 3주차의 rhTGF-β2를 코팅한 타이타늄 임플란트에서 골-임플란트 접촉률 (p=0.045)과 골조직양 (p=0.048)이 대조군보다 유의하게 컸으며, 6주차에서의 골-임플란트 접촉률 또한 통계적인 유의함이 관찰되었다 (p=0.033). 그러나 6주차에는, 3개 연속된 나사선의 골조직양에서 두 그룹간에 유의한 차이가 없었다 (p>0.05). 3주와 6주 모두 전체 임플란트안에서 계산한 골-임플란트 접촉률과 골조직양은 두 군간에 유의한 차이가 없었다 (p>0.05).

결론: PLGA/rhTGF-β2를 코팅한 타이타늄 임플란트 표면 거칠기는 양극산화 표면과 통계적으로 차이가 없었다. 특히 3주차에서 임플란트와 골과의 접합 정도, 임플란트 나사 내로 자라 들어온 골면적이 대조군에 비해 실험군에서 유의하게 컸다. 이 실험의 한계 내에서, 국소적으로 방출되는 rhTGF-β2가 생체처유 초기 단계에서 타이타늄 표면과 골 조직 사이의 골형성능을 증가시키는 것으로 보여진다.
주요어: 인간제조합 형질전환 성장인자 - β 2, PLGA, 골융합, 양극산화 임플란트,
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