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<th>Effect of Surface Roughness of Titanium Dental Implant Placed under Periosteum on Gene Expression of Bone Morphogenic Markers in Rat.</th>
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Abstract

Various bone matrix proteins are produced during the process of osteogenesis. Many previous studies suggested that the topography of an implant surface might affect the expression of osteoblast-mediated cytokines. However, these earlier studies were performed using in vitro cell culture. This study investigated the influence of the surface topography of a titanium implant placed under the periosteum on the gene expression of bone morphogenic markers in rat. Six custom-made implants with a rough upper surface and 6 custom-made implants with a smooth machined upper surface were placed subcrestally with the upper surface facing up in the femurs of 6 adult male rats. Five rats were sacrificed 7 days after the implant placement, and the periosteum above the embedded implant was obtained and analyzed by quantitative real-time RT-PCR for the target genes: alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN). The other rat was sacrificed at day 7, and both implants and the surrounding tissue were embedded in paraffin. For light microscopic observations, paraffin sections were stained with toluidine blue. Gene expression of ALP, BSP and OCN at the rough surface implant was significantly higher than that at the smooth machined surface implant. At day 7, both types of implant were covered with soft tissue, but a lower number of cells stained with toluidine blue was observed on the machined surface compared with on the rough surface. It is considered that rough surfaces may stimulate osteoblasts, and that ALP activity is increased indirectly. Furthermore, the two other markers were also increased by the rough surface in vivo, and different distributions of cellular and extracellular components on the upper surface of the implants were observed at day 7. These results suggest that a rough surface implant under the periosteum promotes higher gene expression of ALP, BSP and OCN in rat.

Key words: Dental implants — Periosteum — Gene expression — Surface properties
Introduction

The attachment of osteoblasts and regulation of their differentiation are important factors for the osseointegration of dental implants. Previous studies\(^1\) on the topography of the implant surface have shown that a rough titanium surface promotes osteoblast differentiation. In the process of their differentiation, various bone matrix proteins are produced\(^2\). In particular, alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN) are bone matrix proteins expressed during calcification and are markers of osteogenesis. Rausch-fan et al.\(^11\) cultured osteoblasts on various rough titanium surfaces, and then measured ALP activity as well as OCN production, and reported differences in expression. Masaki et al.\(^9\) also cultured osteoblasts on various rough titanium surfaces and then analyzed the gene expression of ALP, and reported the largest increase occurred on the sandblasted with large grit and acid-etched (SLA) surface. Therefore, the topography of the implant surface may affect the expression of osteoblast-mediated cytokines. However, all those earlier studies were performed in cell culture.

In several in vivo studies examining the reaction of bone to titanium, synthesis of the extracellular matrix by osteoblasts and calcification of the matrix were altered compared with normal bone healing\(^3,15\). As for the integration of rough and machined surfaces in vivo, rough surfaces were found to produce better bone fixation than smooth machined surfaces\(^16\). Donos et al.\(^7\) analyzed the influence of the topography of the implant surface in conjunction with guided bone regeneration (GBR) using molecular biology approaches in vivo.

Recent studies\(^5,10,17\) have shown that implants located subcrestally and application of abutments thinner than the implant diameter can reduce postoperative crestal bone resorption. The surfaces of these implants faced the periosteum during the healing period. There have been no studies evaluating the responses at the titanium surface contacting the periosteum using real-time PCR. Therefore, the aim of this study was to clarify the influence of the topography of the implant surface on the expression of genes encoding bone morphogenic markers. We placed two types of implant that differ in surface topography at the submarginal area, and then evaluated the expression of genes encoding ALP, BSP and OCN 1 week later. Furthermore, we observed bone formation on the surfaces histologically at 1 week.

Materials and Methods

1. Animals and anesthesia

Six adult male Sprague-Dawley rats ranging from 350 to 400 g each were used for this study. This study protocol was approved by the Committee for the Guidelines for Treatment of Experimental Animals at Tokyo Dental College. Each rat was anesthetized with 0.5 ml of 2.5% thiopental sodium (Ravonal\(^8\), Tanabe Seiyaku, Osaka, Japan) injected intraperitoneally, and both femurs were prepared by shaving and disinfection of the exposed skin.

2. Implant design and manufacture

Solid cylindrical implants (2.5 mm in diameter and 2.5 mm in length) made of commercially pure titanium were manufactured. A roughened, sandblasted, large-grit, acid-etched surface (rough surface) was applied to all surfaces of 12 implants, after which the 6 top surfaces of 6 implants which would face the periosteum were polished (machined surface). The SLA implants were prepared as follows: first, the implants were perpendicularly sand-blasted from a distance of 10 mm with 150 μm Al₂O₃ at 0.4 MPa air pressure; after sandblasting, the implants were then etched with an equal mixture of hydrochloric and sulphuric acid at 70°C for 5 min.

3. Surgical procedure

Surgery was performed on both sides of each femur. An incision was made in the skin
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and dissection was continued through the subcutaneous tissue and muscle. The periosteum was carefully reflected and the lateral aspects of the femur were exposed. Drilling with a pilot-drill (2.0, 2.5 mm) was performed with chilled sterile saline. A rough surface implant and a machined surface implant were inserted in each of the 6 rats. Subcrestal placement was performed where the top surface of the implant was located 0.5 mm below the bone crest. The soft tissue was sutured with 4-0 suture material (PERMAHAND, Ethicon Inc., Johnson & Johnson, Belgium).

4. Quantitative RT-PCR

Five rats were sacrificed 7 days after implant placement with an overdose of thiopental. The sutures were removed and an incision was made. The dissection was carefully performed to expose the periosteum. The periosteum (2.5 mm diameter) and soft tissue above the submerged implant were obtained.

RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan® Gene Expression Assays (Applied Biosystems; Foster City, CA, USA) for the target genes: ALP, BSP and OCN. The TaqMan® Endogenous Control (Applied Biosystems) for the target gene β-actin was used as a control. All PCR reactions were performed using a real time PCR 7500 fast system. Gene expression quantitation using TaqMan® Gene Expression Assays was performed 3 times in each specimen as the second step in a two-step RT-PCR. Assays were done in 20μl singleplex reactions containing TaqMan® Fast Universal PCR Master Mix, TaqMan® Gene Expression Assays, distilled water and cDNA, according to the manufacturer’s instructions (Applied Biosystems). Reaction conditions consisted of a primary denaturation at 95°C for 20 sec, then cycling for 40 cycles at 95°C for 3 sec and at 62°C for 30 sec.

5. Histological observation

A rat was sacrificed respectively at day 7 with an overdose of thiopental. Two specimens with the implant were cut from the femur and soft tissues were removed. Ten percent neutral-buffered formalin was used to fix the specimens. After 3 days, the specimens were dehydrated in ethanol before being embedded in paraffin. Paraffin sections were cut in the sagittal plane using a rotary thin-sectioning instrument (Leitz, Stuttgart, Germany). For light microscopic observations, paraffin sections were stained with toluidine blue and the areas above the implant surface facing the periosteum were observed.

6. Statistical analysis

Means of each expression level were expressed. The ratios of ALP, BSP and OCN mRNAs were normalized against β-actin. PCR data are reported compared to the corresponding control at the machined surface. Data of rough and machined surfaces for ALP, BSP and OCN were compared using the student t-test. In all tests, p<0.01 was considered significant.

Results

The expression level of ALP mRNA in the rat periosteum at the rough surface was significantly higher than that at the machined surface (p<0.01, Fig. 1). The expression level of BSP mRNA in the rat periosteum at the rough surface was significantly higher than that at the machined surface (p<0.01, Fig. 1). The expression level of OCN mRNA in the rat periosteum at the rough surface was significantly higher than that at the machined surface (p<0.01, Fig. 1).

In the histological observations (Fig. 2), the surface at the rough surface implant was covered with many cells stained by toluidine blue (Fig. 2b) compared to the machined surface (Fig. 2a).

Discussion

Titanium is widely utilized in commercially available implants and the properties of its surface are considered as key factors for
its integration in the alveolar bone. Osteoblasts, which play an important role in osseointegration, release various types of osteoblast-mediated cytokines during differentiation\(^5\), which induces osteogenesis. Some authors\(^{14,16}\) have evaluated osteoblast responses to the topography of the titanium implant surface using osteoblast-mediated

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**Fig. 1** Expression levels of ALP, BSP and OCN

Means of each expression level are shown in these figures. (n = 5, I = S.D.) Ratios of ALP, BSP and OCN mRNAs were normalized against β-actin. PCR data are reported compared to corresponding control at machined surface. M: Machined surface, R: Rough surface.

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**Fig. 2** Histological observations at day 7

a: Machined surface, b: Rough surface.

Surface of rough surface implant was covered with many cells stained by toluidine blue (Fig. 2b) compared with machined surface (Fig. 2a). Bar indicates 100µm.
cytokines as markers. However, those studies were performed only in cell culture. In recent years, some authors have reported that subcrestal placement is a key factor in long-term stability. Therefore, in this study, we placed two types of implant subcrestally that differed in surface topography, and measured the expression of genes encoding ALP, BSP and OCN.

Boyan et al. analyzed ALP activity on various rough surfaces and reported that rougher titanium surfaces elicited higher ALP activity in vitro. Shapira and Halabi compared the proliferation rate of osteoblast-like cells on rough and machined surfaces, and concluded that the proliferation rate on a rough surface was more rapid than that on a machined surface. Schneider and Burridge compared sandblasted and grooved surfaces, and observed more marked calcification on the sandblasted surfaces. They concluded that rough surfaces may stimulate osteoblasts and thus indirectly increase ALP activity. In this study, we had similar results in that the gene expression of all 3 markers examined was higher on the rough surfaces. We consider that the stimulation of osteoblasts by rough surfaces also increases ALP mRNA expression under the periosteum in rat.

Wang et al. compared the effects of SLA surfaces and hydroxyapatite surfaces on ALP and OCN activities in vitro and reported that surfaces with high ALP activity also showed high OCN activity. Our results showed higher ALP and OCN mRNA expression on the rough surfaces than on the smooth machined surfaces. We consider this to be a sequential reaction of osteoblasts during calcification.

Masaki et al. characterized cultures of osteoblasts on different types of rough surface and analyzed key osteogenic factors using molecular techniques. They reported that surfaces with high ALP activity also have high BSP activity. We obtained a similar result and consider that osteoblasts provide high ALP and BSP mRNA expression during differentiation and calcification.

Donos et al. analyzed the influence of the SLA and polished titanium surfaces in conjunction with GBR using molecular biology approaches in rat cranial bone, and reported that SLA surfaces regulated the more specific regenerative process of skeletal system development at day 14. Our results showed higher ALP, BSP, OCN mRNA expression on the rough surface at day 7. We consider that the usage of membrane for GBR led to a lag in the regenerative process.

Howlett et al. reported that surface topography may affect cell recruitment. In our histological observations, we observed many cell components at the rough surface compared to at the machined surface. Therefore, our results also showed higher gene expression of bone morphogenic markers at the rough surface.

**Conclusion**

A rough surface implant located under the periosteum in rat produced higher gene expression of bone morphogenic markers than a machined surface implant.

**References**


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