Original

Bone Adaptation of Fibronectin-Immobilized Titanium Implants Using a Tresyl Chloride-Activated Method

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Abstract: The aim of the present study was to evaluate the bone adaptation of fibronectin-immobilized titanium implants *in vivo* using a tresyl chloride-activated method in implantation experiments with rabbit tibiae. Cylindrical pure titanium (3.5 mm in diameter and 7 mm in height) was treated with tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride, $\text{CF}_3\text{CH}_2\text{SO}_2\text{Cl}$) and then coupled with fibronectin to give fibronectin-immobilized cylindrical titanium (Fn/Ti) implants. Non-treated cylindrical titanium (Ti) implants were used as a control. Ti and Fn/Ti implants were implanted into bone defects in the tibiae of rabbits. The implants were excised 12 weeks later and non-decalcified thin sections were prepared. After staining each section with methylene blue and basic fuchsin, the implant-bone interface was histologically evaluated and the bone-to-implant contact ratio (BIC) and bone mass (BM) were histomorphometrically calculated using an image analysis system. A larger amount of new bone formation was recognized for Fn/Ti implants and histomorphometrical evaluations showed that BIC and BM were significantly higher for Fn/Ti implants than for Ti implants. Fn/Ti implants also showed better bone adaptation. These results demonstrated that the immobilization of fibronectin onto cylindrical titanium implants effectively enhanced bone regeneration around implants.

Key words: Titanium implant, Fibronectin, Tresyl chloride, Osteoblast, Bone-to-implant contact

Introduction

In the last few years, several studies have been devoted to investigating surface modifications to titanium dental implants. Various surface modifications to titanium implants have been postulated to improve the bone healing response. For example, sandblasting¹⁾, alkali treatments²⁾, anodizing³⁾, laser irradiation⁴⁾, micro-arc oxidation⁵⁾, and hydroxyapatite coating⁶⁾ were previously identified as effective surface modifications.

Biochemical surface modifications have been attracting increasing attention. The extracellular matrix, cell adhesive proteins, or cytokines have been coated or immobilized onto titanium surfaces⁷⁾. Previous studies reported that a fibronectin, laminin, or collagen coating on titanium implants effectively enhanced new bone formation⁸⁻¹¹⁾.

However, proteins have merely been coated and not adhered chemically with covalent binding on titanium substrates. During implantation, the implant body is strongly torqued and drilled into hard bone. The tight bonding of biomolecules to titanium is desired

by dental implant clinics. Culp *et al.*¹²⁾ examined the chemical immobilization of fibronectin to titanium surfaces using a silane coupling agent. In contrast, Hayakawa *et al.*¹³⁾ developed an easy method for immobilizing proteins or cytokines onto a titanium surface, named the tresyl chloride-activated method. This method is a modification of that previously reported by Nilsson and Mosback¹⁴⁾. This technique is very simple, inexpensive, and does not require special facilities. The immobilization scheme was shown in Fig. 1. Basic terminal hydroxyl groups on the titanium surface were reacted with tresyl chloride and tresyl chloride-activated titanium was coupled with proteins or cytokines. A quartz-crystal microbalance technique and molecular orbital calculation for the model compound suggested the mechanism underlying tresyl chloride-activated methods¹⁵⁻¹⁷⁾. Fibronectin or collagen immobilization onto titanium has been shown to enhance initial cell attachment¹⁸⁾. The arrangements of attached cells were also found to be influenced by fibronectin immobilization¹⁹⁾. The gene expression levels of some genes related to the mineralization process were reported to be up-regulated during an osteoblast-like cell assay on fibronectin or GRGDSP peptide-immobilized titanium²⁰⁾. Alkaline phosphatase or transforming growth factor

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Figure 1. Schematic drawing of protein immobilization using the tresyl chloride-activation method

(TGF- β) has also been immobilized onto titanium using the tresyl chloride-activated method^{21,22}, and the immobilization of TGF- β 2 was shown to enhance bone regeneration around implants.

The aim of the present study was to evaluate the bone adaptation of fibronectin-immobilized titanium implants using the tresyl chloride-activated technique in implantation experiments with rabbit tibiae.

Materials and Methods

Titanium substrate

Pure titanium cylindrical implants (Ti cylinder; 99.9 mass % Ti, Grade 2, 3.5 mm in diameter and 7.0 mm in height, Furuuchi Chemical Co., Tokyo, Japan) were used after ultrasonically cleaning in ethanol and distilled water.

Fibronectin immobilization by the tresyl chloride-activated method

Fibronectin was immobilized onto the cylindrical titanium implant surface using the previously described tresyl chloride-activated technique¹³. Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride, $\text{CF}_3\text{CH}_2\text{SO}_2\text{Cl}$, Fluka, Buchs, Switzerland) was dropped on the cylindrical Ti implant surface from the top to bottom in order to cover the entire area completely. Treated cylindrical Ti implants were then stored at 37°C for 48 h. Tresylated Ti implants were washed with acetone followed by a water: water-acetone solution (50: 50), then dried and stored in a desiccator. Fibronectin (Upstate Biotechnology, Lake Placid, NY,

Figure 2. N1s spectra of the Fn/Ti surface by a XPS analysis

USA) was dissolved at a concentration of 0.1 mg/ml in double distilled water. Tresylated Ti implants were dipped into fibronectin solution and stored at 37°C for 24 h. After rinsing with double distilled water, fibronectin-immobilized cylindrical Ti implants were stored in a desiccator at room temperature. Fibronectin-immobilized cylindrical titanium (Fn/Ti) implants were used as the experimental group, whereas non-treated cylindrical Ti implants were employed as the control group.

Characterization of the Fn/Ti cylinder

A titanium disk (99.9 mass % Ti, Grade 2, 5.5 mm in diameter and 1.0 mm in thickness, Furuuchi Chemical Co., Tokyo, Japan) was used to characterize fibronectin on the titanium surface. The

Figure 3. Histological appearances of cylindrical Ti and Fn/Ti implants 12 weeks after their implantation into bone defects in the rabbit tibia. (a), (b) Ti implant; (c), (d) Fn/Ti implant.

Ti disk was treated with tresyl chloride and coupled with fibronectin as described above to give a fibronectin-immobilized titanium (Fn/Ti) disk. The Fn/Ti disk was characterized using an X-ray photoelectron spectroscope (XPS, ESCA-3400, Shimadzu Corp, Kyoto, Japan) equipped with an MgK α X-ray source operated at 10 kV and 20 mA. The existence of fibronectin was confirmed by the N1s peak of amide bonds of fibronectin. The binding energy for each spectrum was calibrated against the C1s peak at 284.8 eV.

Animal experiment

The animal study was approved by the Animal Experimental Ethical Guidelines of Tsurumi University School of Dental Medicine (certificate no. 12018). Prior to surgery, non-treated Ti and Fn/Ti implants were sterilized using ethylene oxide gas. Cylindrical implants were placed in the cortical bone of the left and right tibiae according to a previously described procedure⁶⁾. Each rabbit received one Ti and one Fn/Ti implant (one in the left and the other in the right). A total of 6 implants, 3 Ti and 3 Fn/Ti implants, were inserted.

Surgery was performed under general inhalation anesthesia with a 5 % isoflurane and oxygen mixture, which was reduced to 2 % isoflurane during surgery. After shaving and disinfecting the operating field, xylocaine was injected as local anesthesia. A

longitudinal incision was made on the tibia and the bone was exposed by blunt dissection. A 2.0-mm pilot hole was drilled and the hole was gradually widened to 3.5 mm using a drilling speed of 800 rpm and continuous internal cooling. Cylindrical Ti or Fn/Ti implants were inserted into bone defects in the tibia by a press fit. The soft tissue was closed in separate layers using non-absorbable sutures and a prophylactic antibiotic equivalent to latamoxef sodium (0.01 mg/kg Shimalin; Shionogi & Co., Ltd., Osaka, Japan) was administered to prevent infection. Rabbits were sacrificed 12 weeks after implantation by a peritoneal injection of an overdose of thiamylal sodium (Isothol; Nichi-Iko, Toyama, Japan). The implants and surrounding bone were excised and excess tissue was removed.

Histological and histomorphometrical observations

After removing the implants and surrounding tissue, specimens were fixed in 10 % buffered formalin solution, dehydrated through a graded series of ethanol, and then embedded in methylmethacrylate. Non-decalcified thin sections with a thickness of approximately 50 μ m were made in a transverse direction perpendicular to the axis of the implants using a cutting-grinding technique²³⁾. Sections were stained with methylene blue and basic fuchsin and evaluated using a light microscope (Eclipse Ni; Nikon, Tokyo, Japan; magnification $\times 40$, $\times 100$).

Histomorphometrical evaluations of new bone formation were performed as described in our previous study⁶⁾. The bone-to-implant contact ratio (BIC) and bone mass (BM) around the implant were measured using an image analysis system (WinROOF, Visual System Division; Mitani Corporation, Tokyo, Japan). BM was defined as new bone formation in the region of interest (ROI), which was within the circumference of 110% of the implant.

Statistical analysis

The results of BIC and BM from histomorphometrical measurements were evaluated by a one-way analysis of variance (ANOVA) and the Bonferroni test for multiple comparisons among the means at $P = 0.05$ with SPSS for Windows (SPSS Statics 17.0, SPSS, Chicago, USA).

Results

Surface characterization

The surface of the Fn/Ti disk was analyzed by XPS. Fig. 2 shows an XPS narrow scan of the Fn/Ti disk. The N1s peak, derived from the amide bond of immobilized fibronectin, was detected around the binding energy of 400 eV.

Histological and histomorphometrical evaluations

Rabbits remained in good health during the experimental period. No clinical signs of inflammation or adverse tissue reactions were observed when animals were sacrificed and all cylinders were still *in situ*.

Fig. 3 shows the histological appearances of Ti and Fn/Ti implants in the tibia (cortical bone). New bone formation was observed around implants after 12 weeks of implantation. Bone remodeling proceeded and mature bone formation was detected. Furthermore, the permeation of inflammatory cells was not observed. New bone formation was greater with Fn/Ti implants than with Ti implants and tight bone-to-implant bonding was observed for Fn/Ti implants.

Table 1 shows the percentages of BIC and BM after 12 weeks of implantation. BIC and BM were significantly higher for Fn/Ti implants than for Ti implants ($p < 0.05$).

Discussion

In the present study, we immobilized fibronectin onto a titanium implant using the tresyl chloride-activated method and evaluated the bone response against fibronectin-immobilized Ti implants. Implants were inserted into bone defects in rabbit tibiae for 12 weeks.

New bone formation was greater around Fn/Ti cylinder implants and histomorphometrical evaluations revealed significantly higher BIC and BM for cylindrical Fn/Ti implants than for Ti implants. Fibronectin is a well-known cell-adhesive

protein and has not only a RGD sequence, but also approximately 30 independent binding domains for collagen, proteoglycan, heparin, and others. The factors affecting osteoblast activity may depend on these functional domains²⁴⁾. Osteoblast attachment to titanium implants and/or osteoblast activity around implants was assumed to be enhanced and better bone responses were obtained by fibronectin immobilization; however, the underlying mechanism needs to be elucidated in more detail.

The advantage of the tresyl chloride-activated method is its ease and wide applicability. There is no need for any pretreatment of the titanium surface such as an acid or alkali treatment. Many types of proteins or cytokines may be immobilized on the titanium surface. Suzuki *et al.*²²⁾ immobilized TGF- β 2 onto titanium by the tresyl chloride-activated method and found that its immobilization effectively enhanced bone regeneration around implants.

The tresyl chloride-activated method is a wet process. Thus, another advantage of this method is that proteins or cytokines may be immobilized on material with a complex surface such as a porous scaffold. In the present study, fibronectin was easily immobilized onto the cylindrical shape of the titanium implant. Amemiya *et al.*²⁵⁾ immobilized fibronectin and collagen onto a three-dimensional titanium fiber structure using the tresyl chloride-activated method. They found that the immobilization of fibronectin or collagen was effective for internal bone augmentation, but not for external bone augmentation.

It currently remains unknown whether immobilized fibronectin is released *in vivo* following implantation. Suzuki *et al.*²²⁾ speculated that a small amount of TGF- β 2 may be released from TGF- β 2-immobilized implants, which may, in turn, stimulate bone formation around the implants. Therefore, the stability of immobilized fibronectin needs to be determined *in vivo*.

In conclusion, the immobilization of fibronectin onto cylindrical titanium implants effectively enhanced bone regeneration around implants. The tresyl chloride-activated method is an innovative system to immobilize various proteins onto titanium without reductions in their bioactivities. The immobilization of other proteins or cytokines will be the next subject of our research. Furthermore, the combined immobilization of different proteins or cytokines may lead to the development of a new type of biofunctional titanium implant.

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