Histomorphometric assessments of peri-implant bone around Ti-Nb-Sn alloy implants with low Young’s modulus

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Many β-Ti alloys have been developed for, and used in, medical devices because of the corrosion resistance, biocompatibility, and exceptionally low Young’s modulus. The aim of the present study was to investigate the histomorphometric aspects of peri-implant bone around Ti-Nb-Sn alloy implants and compare them with those in the case of commercially pure Ti (Ti). Fluorescent morphological observations of ST-2 cells on the substrate were performed and bone morphogenesis around implants in rat femur was evaluated. There was no difference between the cell morphology on Ti and those on the Ti-Nb-Sn alloy during observation for 24 h. A comparison of the Ti-Nb-Sn alloy implant and the Ti implant showed no significant differences between the bone-to-implant contact ratios or the bone fractions. These results suggest that the biological adaptations with Ti-Nb-Sn implants during a healing period are similar to those with Ti. Ti-Nb-Sn is therefore suitable for use in dental implants.

Keywords: Ti-Nb-Sn, Implant, Low Young’s modulus

INTRODUCTION

The replacement of missing teeth with dental implants is an efficacious and reliable treatment for fully and partially edentulous sites, but implant failures still occur because of failure to establish initial osseointegration and long-term bone loss. Many systematic studies have clarified the effects of important factors such as implant design, clinical handling, bone quality, intake of medicines, smoking, and bruxism. Control of biomechanical stress on the peri-implant bone is essential for preventing implant failure because occlusal overloading associated with infection causes marginal bone loss. Biological-data-based finite element studies have been used to study the effects of biomechanical stress and the relationship between prosthetic design and distribution of stress transferred to the supporting bone. Finite element analysis (FEA) showed that maximum mechanical stress distribution occurs in the cortical bone close to the neck of the implant because of the differences between the elastic moduli and tensile strengths of the implant and bone.

β-Ti alloys have been developed for use in orthopedic implants because of their corrosion resistance, biocompatibility, and exceptionally low Young’s modulus. Ti-Nb-Sn alloy is a new β-Ti alloy; its most important characteristics are its low Young’s modulus, which is close to that of human cortical bone, and high tensile strength. Ti-Nb-Sn alloys give greater biocompatibility, bone induction ability, and binding capacity between the metal matrix and hydroxyapatite compared with those obtained with other β-Ti alloys. These excellent properties can affect the stress distribution along the interface between the implant and marginal bone, therefore Ti-Nb-Sn alloy implants might transfer less stress to the peri-implant bone and cause less bone deformation than Ti implants.

We have been working to evaluate a series of biocompatibility, bioactivity and mechanical property of Ti-Nb-Sn alloy implant. We previously reported that Ti-Nb-Sn alloy implants have biocompatibility similar to those of Ti, and give excellent biomechanical strength of bone-implant integration. In this study, we performed the cell morphological observations on substrates and bone morphogenesis around implants because our substrate materials had different surface roughness and Young’s modulus, which may affect the ability of initial cell contact with the substrate surface and the bone quality of new bone formation. The aim of the present study was to evaluate the biological stability of Ti-Nb-Sn alloy implants by examining the histomorphometric aspects of peri-implant bone without load application.

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MATERIALS AND METHODS

Specimen design
Ti and Ti-Nb-Sn alloy disks (diameter 8.0 mm, thickness 1.0 mm) and cylindrical implants without screws (diameter 1.0 mm, length 2.0 mm) were prepared for in vitro and in vivo tests. We used commercially pure Ti (Ti), and Ti-25Nb-11Sn (wt%) alloy which was developed by the Institute for Materials Research, Tohoku University. It has been reported that this Ti-Nb-based alloy containing Nb is easily fabricated by conventional cold working or plastic forming processes followed by heat treatment\(^{21}\). Surface characterization details were reported in our previous paper\(^{20}\). Briefly, these specimens were mechanically polished with a lathe machine regulated at 15 μm intervals. The surface roughness, Sa values, of Ti and Ti-Nb-Sn alloy were 384 and 577 nm, respectively.

Cell morphology
The actin cytoskeletons of cells attached to the Ti and Ti-Nb-Sn alloy disk substrates were examined. Mouse bone marrow stromal ST-2 (ST-2) cells (Riken Cell Bank, Ibaraki, Japan) were cultured in α-MEM (Sigma-Aldrich, St Louis, MO, USA) containing 1% penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) under 5% CO\(_2\) at 37°C; 1×10\(^4\) ST-2 cells were seeded onto each disk. After incubation for 4 or 24 h, the ST-2 cells were fixed in 4% paraformaldehyde solution. Samples were washed with PBS and cells were incubated with 165 nM rhodamine phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min, and followed by washing with PBS. The cells were additionally stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) and immediately washed with PBS. Laser scanning confocal microscopy was used to examine the cell morphology and cytoskeletal arrangement at 405 and 532 nm (TCS-SPE, Leica Microsystems, Wetzlar, Germany). These samples were covered with cover glass using 10 μL ProLong\textsuperscript{TM} Live Antifade Reagent (Thermo Fisher Scientific) to prevent the loss of fluorescent signal.

Animal surgery
Ten-week-old male Sprague-Dawley rats weighing 280–300 g received two implants in the femur on a reported basis\(^{22}\). Surgery was performed under general gas anesthesia with 2.0% isoflurane for induction and 1.5% during surgery. The femur was exposed and a pilot drill was used to prepare implant cavities 7 and 11 mm from the distal edge of the femur under irrigation. Implants were placed into each side of the femur alternately. Surgical sites were closed in layers, and muscle and skin were sutured separately with resorbable suture thread. This study protocol was approved by the Animal Research Committee of Tohoku University.

Histological preparation and analysis
The experimental rats were sacrificed after healing periods of 2 and 8 weeks, and the femurs containing the implants were harvested and immediately fixed in 40% ethanol solution for 24 h and then in 70% ethanol solution for 7 days. Villanueva osteochrome bone stain (Polysciences, Bergstrasse, Germany) was used to visualize new bone formation in the implants. Tissues were dehydrated in ethanol with an ascending series of concentrations and eventually embedded in methyl methacrylate resin and polymerized. These tissue blocks, along with the implants, were cut along the longitudinal direction of the implant's axis with a diamond saw (Exakt BS-300CL, Exakt Technologies, Norderstedt, Germany). The specimens were polished to a thickness of 40 μm (Exakt MG-400CS, Exakt Technologies) and observed by light microscopy (Leica DM3000, Leica Microsystems) and a charge-coupled device camera (Leica DFC 420 C, Leica Microsystems). Histological analysis was performed by digital image processing (Adobe Photoshop CS5, Adobe Systems, San Jose, CA, USA, and ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA). To identify the details of bone formation around the implant surface, the following variables were analyzed:

1) Bone-to-implant contact (BIC)=(the sum of the lengths of all contact regions between bone and implant (μm))/(whole implant length from first to last BIC (μm))×100

Fig. 1 Illustration of boundary for peri-implant bone fraction (green); ROI\(_1\) is the range from implant surface to 50 μm (pink) and ROI\(_2\) is from 50 to 250 μm (yellow); bar=500 μm.
2) Bone fraction (BF) = \( \frac{\text{the area occupied by bone (\( \mu m^2 \))}}{\text{(reference area (\( \mu m^2 \}))} \times 100\%} \)

Two reference areas were defined and included the peri-implant tissues of the cervical and apical cortex, as well as the medullary cavity. The peri-implant reference sites were at various distances from the implant. Range of interest (ROI) 1 was from 0 to 50 \( \mu m \) and ROI2 was from 50 to 250 \( \mu m \). This range was determined by reference to a previous report\(^\text{23} \). Illustrations of the implant sites and histological quantification areas are shown in Fig. 1. The boundary for the peri-implant BF is green; ROI1 specifies the range from the implant surface to 50 \( \mu m \) (pink), and ROI2 is the range from 50 to 250 \( \mu m \) (yellow).

**Statistical analysis**

Five samples were taken (\( n = 5 \)) for the studies. Data were shown as the mean±standard deviation. The digital imaging processing data, BIC, and BF were analyzed by two-factor analysis of variance (IBM SPSS statistics 24, Chicago, IL, USA). If necessary, a post hoc Mann-Whitey test was used to examine differences between the week 2 and week 8 groups; \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Morphological observation of ST-2 cells on Ti-Nb-Sn alloy**

Figure 2 shows the actin cytoskeletons of ST-2 cells on Ti and the Ti-Nb-Sn alloy after incubation for 4 and 24 h. There were many cells on both substrates but some appeared to have spindle or polygonal morphologies after incubation for 4 h (Figs. 2A and B). Spreading and cytoskeletal development were observed along the turned trace on the surface after 24 h (Figs. 2C and D).

**Bone morphogenesis around Ti-Nb-Sn alloy implants**

At week 2, the implants of Ti and the Ti-Nb-Sn alloy were histologically in direct contact with the surrounding bone along their upper part; there were no signs of inflammation or connective tissue interposition in the cortical region. The lower parts were in contact with either osteoid or marrow tissue (Figs. 3A and B). At week 8, new bone formation occurred more extensively along the implants, and a small part of the surface had osteoid or marrow tissue intervening between the bone and the implant (Figs. 3C and D).

**BIC analysis**

Figure 4 shows the BICs (%) for the two healing periods.
Fig. 4 BICs for two healing periods. No significant differences between the BIC values for commercially pure Ti and Ti-Nb-Sn alloy were apparent in weeks 2 and 8.

Fig. 5 BF analysis
The bone areas in both the near zone (ROI1=0–50 μm) and the far zone (ROI2=50–250 μm) showed no significant differences between the two materials in weeks 2 and 8 (Fig. 5). ROI2 was statistically significant regarding the healing periods and the material, with 

No significant difference between the BICs for Ti and the Ti-Nb-Sn alloy were apparent in weeks 2 and 8. The BICs in the Ti implant and the Ti-Nb-Sn alloy implant were 61.1±2.0 and 63.0±1.1, respectively, for week 2, and 72.5±1.5 and 68.9±4.7, respectively, for week 8.

BF analysis
The bone areas in both the near zone (ROI1=0–50 μm) and the far zone (ROI2=50–250 μm) showed no significant differences between the two materials in weeks 2 and 8 (Fig. 5). ROI2 was statistically significant regarding the healing periods and the material, with p<0.05. The BF (%) in the Ti implant and Ti-Nb-Sn alloy implant were ROI1 39.3±9.0 and ROI2 42.4±3.7, and ROI1 39.0±1.3 and ROI2 38.4±8.4, for week 2; and ROI1 51.7±5.8 and ROI2 51.4±2.3, and ROI1 46.8±4.3 and ROI2 54.1±4.9, for week 8.

DISCUSSION
We previously reported that Ti-Nb-Sn alloy implant with a low Young’s modulus has biomechanical properties (hydrophilicity, ability to induce cell proliferation, and alkaline phosphatase activity) similar to those of Ti, and gives excellent osseointegration (values from push-in tests)20. In this study, we performed fluorescent morphological observations of ST-2 cells on a substrate and bone morphogenesis around implants.

Visualization by cytoskeleton staining within 24 h showed the initial contact of ST-2 with the substrate surface. There was no difference between the cell morphologies with Ti and those with the Ti-Nb-Sn alloy within the 24 h observation period. The fluorescence intensities of the actin cytoskeleton were similar on both substrates (Fig. 2). Niobium and Sn are well known to be β stabilizing elements in Ti alloys with a low elastic modulus16,24, and Nb is non-toxic and non-allergic alloying elements to titanium and Sn is regarded as a minimal cytotoxic element14,25,26. It has been reported that the cell proliferation number on the surface of Ti alloys containing Nb is higher than that of Ti, but immunofluorescence staining observation showed the similar cell morphology, mostly well-spread and polygonal with long processes, on both alloys after 3 days incubation27. On the other hand, Li et al. reported that metal powders of Nb showed cytotoxicity compared with that of cells seeded into wells containing only media, however, bulk metals of Nb and Sn exhibited excellent biocompatibility28. These results suggested that ion concentration in local area might be related to cytotoxic. In this study, the alloying elements may not affect the cell morphology because we used the alloy disks polished with a lathe machine.

After implant placement, immature new bone forms rapidly on the implant interface. Woven bone is produced when osteoblasts rapidly produce osteoids. This woven bone is replaced by stronger bone along the interface after several months. In this study, Villanueva osteochrome bone stain was used to visualize new bone formation on the implant surface and around the implant. At week 2, the lower parts of the implant were mainly in contact with either osteoid or marrow tissue (Figs. 3A and B). Although there were no significant differences between the BICs in different healing periods, formation of new bone with a woven bone-like structure occurred more extensively along the implants at week 8 (Figs. 3C and D). The differences of the BF in ROI2 between week 2 and 8 were statistically significant (Fig. 5). The BICs of Ti were reported to be 15 to 50%
at 2 weeks after implantation in a rat study. Our BIC result for the Ti implant was 61.1±2.0% and that for the Ti-Nb-Sn alloy implant was 63.0±1.1% (Fig. 4). A FEA study showed that the BIC is associated with reduced stress on surrounding tissues under vertical and oblique loading. In this study, there was no significant difference between the BICs in different implants, but this relativity high BIC value of Ti-Nb-Sn alloy implant might be of advantage against biomechanical stress.

Human bone follows Wolff’s law, i.e., the bone adapts, and is regularly modeled and remodeled in response to particular mechanical loads. This bone functional adaptation has been studied from the aspect of bone metabolic activity. Frost suggested criteria for bone remodeling, adaptation, or resorption, depending on loading strength thresholds when functioning. However, it is important for the success of dental implant treatment that the micromovement of the bone is within 50 to 150 μm. It is also essential for preventing implant failure to control the biomechanical stress on the peri-implant bone because occlusal overloading associated with infection causes marginal bone loss. Biomechanically, peri-implant bone remodeling is driven by mechanical stimuli. It is therefore important to evaluate the biomechanical properties of implants with low Young’s moduli. However, it has been reported that a low elastic modulus of the implant material results in greater cortical and trabecular bone deformation. In this model, it was assumed that the bone had linear elastic properties to evaluate the stress distribution transferred from an implant with a low elastic modulus to the supporting bone. The deformation magnitude for an implant with a low elastic modulus is higher than that for an implant with a high elastic modulus when a static load was applied in a linear elastic model. This induced a larger stress distribution in the bone. However, internal stress relaxation occurs in a low elastic modulus implant. Use of a linear elastic model in FEA studies of the stress distribution around a low elastic modulus implant may lead to results that differ from the actual behavior.

This study has limitations because we did not apply either a static or a dynamic load to the implants. Evaluation of the implant behavior with load application is needed in the future research.

CONCLUSIONS

Our results suggest that implants fabricated from Ti-Nb-Sn have potential biological applications similar to those of Ti, and could be suitable for use in dental implants.

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