J Mater Sci: Mater Med (2008) 19:1851–1859
DOI 10.1007/s10856-007-3265-5

In vivo and in vitro response to electrochemically anodized Ti-6Al-4V alloy

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Received: 21 December 2006 / Accepted: 14 August 2007 / Published online: 4 October 2007

Abstract Tissues' reactions to metals depend on a variety of properties of the metal, most notably surface structure. Anodizing has been shown to alter the surface properties of metal, thus eliciting a change in the biocompatibility of the metal. In order to evaluate the biocompatibility of unoxidized titanium alloy (Ti-6Al-4V) and anodized titanium alloy samples, the samples were implanted in murine abdominal subcutaneous tissues, and maintained for 2 and 4 weeks. The reaction of the abdominal subcutaneous connective tissues to the samples was then assessed. Fibrous connective tissue capsules were observed around the vicinity of the sample, and these capsules were shown to harbor fibroblasts, fibrocytes, and other cells, including neutrophils, macrophages, and giant multinucleated cells. The average thickness of the fibrous capsules observed around the anodized alloy samples was less than that of the capsules seen around samples of the unoxidized titanium alloy. Blood was obtained from the tails of the experimental mice, and blood cell analyses were conducted in order to assess the levels of leukocytes, red blood cells, and thrombocytes. The blood analysis results of the unoxidized control group and treatment group were all within normal ranges. In addition, the biocompatibility of the titanium alloy samples was evaluated using cell culture techniques. The numbers of MG-63 cells cultured on oxidized samples tended to be greater than those in the controls; however, these increases were not statistically significant. The alkaline phosphatase activity of the sample oxidized at 310 V evidenced significantly higher activity than was observed in the control group. These results indicate that the anodized Ti-6Al-4V alloy will be of considerable utility in biomedical applications.

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1 Introduction

The properties of implant materials used in humans may have important influences on the outcomes of clinical treatments. Such materials have been utilized in a variety of applications, including dental treatments, orthopedic surgical treatments, blood vessels, heart valves, and the management of other cardiovascular diseases [1]. Recently, titanium and titanium alloys have been extensively employed as in vivo implant materials, due to their generally favorable biocompatibility, high resistance to erosion, and relatively low purchase cost [2, 3].

On the other hand, even when using chemically identical materials, the biocompatibility of an implant or its stability depends heavily on its surface structure, as well as the thickness and properties of the surface oxide film [4]. As the characteristics of the implant surface have been reported to play an important role in the in vivo reactions of implants, a great deal of interest has recently been focused on different surface treatment methods. Currently, there are a variety of methods with which titanium implant

surfaces are treated, including the thermal oxidation method, the plasma-sprayed anodic method, and the recently developed anodizing surface-treatment method [5, 6]. The anodizing method is an electrochemical technique which forms a rough, thick oxidized capsule, with micropores on the implant surface [7].

The biocompatibility of implant materials, such as those used in dental treatments or orthopedic surgical treatments, has been evaluated in some cases by transplanting the implants to bone. However, in the pre-clinical stage, the biocompatibility of the implants can be conveniently evaluated in the subcutaneous tissues of animals [8]. Using guinea pigs, white rats, and humans as subjects, the healing process or reactions of the connective tissues against transplanted implants evidence similar trends; thus, the implant reaction results from animals can be considered to be basic clinical data [9]. The Ti-6Al-4V alloy has been successfully used as a metallic biomaterial, due to its excellent biocompatibility and mechanical strength [10]. Therefore, several techniques have been applied to Ti-6Al-4V alloy surfaces in order to increase their biocompatibility, including coating with an identical alloy and thermal anodization at 500 °C and 700 °C [10, 11]. However, the in vivo and in vitro responses to the surface roughness of electrochemically anodized Ti-6Al-4V alloy have yet to be fully analyzed.

In this study, the biocompatibility of titanium alloy surfaces treated with anodizing methods at various voltages (260 V, 280 V and 310 V) via surface treatment methods were evaluated. The voltages were selected on the basis of previously reported results from our studies of the anodizing method. To evaluate the in vivo biocompatibility, samples were subcutaneously implanted in murine abdominal wall connective tissue, and the mice were sacrificed after 2 and 4 weeks. Tissue reactions against the samples were then histologically evaluated. Generally, the first biological reaction observed after in vivo implantation is the formation of fibrous connective tissue capsules surrounding the implant, and subsequent severe and chronic inflammatory changes.

However, the formation of such fibrous capsules is crucial to the success of the implant graft [12]. If the biocompatibility of an implant is poor, chronic inflammation occurs, coupled with the formation of a capsule of thick fibrous connective tissue [13]. Therefore, we evaluated the biocompatibility of samples via examinations of the fibrous capsules, which appeared after the implantation of titanium alloy samples, with a light microscope. We also simultaneously evaluated the biocompatibility of the titanium alloy in vitro by measuring cell numbers and alkaline phosphatase activity. In addition, blood was obtained from the tails of the experimental mice, and the blood cells were analyzed.

2 Materials and methods

2.1 Experimental animals

Forty-eight 9-week-old female ICR mice were placed in animal cages, at 6 animals per cage. The animals were maintained in an animal room with a temperature of 23 ± 2 °C, a relative humidity of 50 ± 10%, and a 12-h light:dark cycle.

2.2 Preparation of titanium samples and treatments

Commercial titanium alloy (Ti-6Al-4V) plates were cut to a size of 4 mm × 6 mm × 1 mm (width × length × thickness) and used as samples in these experiments. The prepared samples were sanded with #800 ~ #1500 emery paper and washed ultrasonically in 20% NaOH at 60 °C for 30 min to remove organic materials from the surfaces. All samples were acid-etched in 100 mL of mixed acid solution (15 mL of 63% HNO_3 solution, 3 mL 55% HF, and 82 mL distilled water) for 5 min, and subsequently washed ultrasonically in distilled water. After ultrasonic washing, the titanium alloy samples were anodized in order to evaluate the relative biocompatibility of the samples according to the surface treatment utilized. Samples maintained at 20 mA were connected to a voltage of 260 V, 280 V, or 310 V, and each surface was treated for 10 min using 0.03 M glycerol 2-phosphate disodium salt hydrate and 0.2 M calcium acetate as the electrolyte solution. After the surface treatment, the samples were washed in distilled water and dried for 24 h at 60 °C. Samples without oxidation treatment were used as a control group.

2.3 Scanning electron microscope and atomic force microscopic examination

The control group and the anodized titanium alloy samples were placed in a small chamber and coated with gold. The surfaces of the samples were then examined at 25 kV with a scanning electron microscope (Hitachi S-3500N). In addition, the surfaces were also examined with an atomic force microscope (Model: Autoprobe CP Research, Thermo Microscope, USA), and analyzed with the Proscan 1.7 program to determine the surface roughness.

2.4 The implantation of titanium samples

All samples (control and surface-treated) were autoclaved to prevent bacterial contamination. Subsequently, surgical

preparations were made prior to the insertion of the samples into the abdomens of mice. Each mouse was anesthetized with 50 mg/kg sodium pentobarbital injected into the peritoneal cavity. The completely anesthetized mice were laid down, and abdominal hairs were removed using a depilatory agent (Niclean cream, Ildong Pharmaceutical); the exposed skin area was then sterilized with alcohol. Using a pair of sterilized scissors, a 3-mm incision was made in the skin, and one sterilized metal sample was inserted into the connective tissue between the skin and the abdominal muscles. The incision area was then closed with sutures. Six animals were allocated to the control group and to each group anodized at 260 V, 280 V, or 310 V, and the samples were maintained in the abdomen for 2 and 4 weeks. A total number of 48 mice was used in this study.

2.5 Blood cell analysis and in vivo tissue treatment

Two or four weeks after the insertion of the titanium alloy, the mice were anesthetized with sodium pentobarbitol, depilatory cream was applied, and the regrown hairs were removed. In order to analyze blood cells, the tips of the tails of anesthetized mice were cut with a razor, and blood was collected in Minicollect® tubes treated with 0.5 mL heparin. The numbers of granular leucocytes (neutrophil, eosinophil and basophil), agranular leucocytes (lymphocyte and monocyte), erythrocytes, and thrombocytes were determined using a HEMA VET® 850 system (CDC Technologies Co.). Measurements were obtained for 6 mice from each group and averaged to analyze the blood cells. For microscopic examination, the areas containing the sample, abdominal skin, and muscles were centered and cut together, then fixed for a minimum of 48 h in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) solution. Subsequently, the metal sample in the connective tissues was carefully removed with a pair of forceps. The tissue samples were washed at 4 °C in 0.01 M phosphate buffer (pH 7.4) for 24 h, and after the ethanol dehydration process, were embedded in glycol methacrylate resin. 2.5-μm thick sections were prepared with a microtome and mounted on slides. The tissues were then stained with hematoxylin-eosin and examined with a light microscope.

2.6 Image treatments and the measurement of the thickness of fibrous connective tissue capsule

The stained abdominal tissue sections were examined with a light microscope (Olympus, BX50); pictures were obtained with a SPOT digital camera (Model No. 11.2 Color Mosaic, Diagnostic Instruments) attached to the microscope. To evaluate their biocompatibility, the thickness of the fibrous connective tissue capsules surrounding the sample, as detected via microscopy, were measured. The fibrous capsule thickness was measured using the SPOT INSIGHT™ program (Version 4.0, Diagnostic Instruments). The thickness was determined by measuring the shortest distance from the inside to the outside of the fibrous tissue capsules. When measuring the fibrous capsule thickness, 10 abdominal tissue section sites from each animal were first measured and averaged and then the mean and standard deviation for the group was derived from 6 specimens.

2.7 Cell culture reagents

Dulbecco's modified Eagle's medium with added antibiotic-antimycotic and fetal bovine serum (FBS) was obtained from Gibco BRL (USA). 2-ME (2-mercaptoethanol) and sodium bicarbonate ($NaHCO_3$) were obtained from Sigma (USA). In addition, the trypsin-EDTA required for cell dissociation was obtained from Gibco BRL (USA). The *p*-nitrophenol standard solution (10 mM) used for alkaline phosphatase and the alkaline phosphatase yellow (*p*NPP) liquid substrate system for ELISA were obtained from Sigma (USA).

2.8 MG-63 cell culture and cell number counting

The MG-63 human osteosarcoma cell-line (ATCC CRL-1427, USA) was cultured with Dulbecco's modified Eagle's medium (DMEM) with added sodium bicarbonate ($NaHCO_3$, 3.7 g/L), antibiotics (10,000 units/mL penicillin G sodium, 10,000 units/mL streptomycin sulfate, 25 μg/mL amphotericin B), 2-ME (50 μM), and 10% FBS in a 37 °C, and 5% CO_2 incubator, and the medium was changed every 3 days. In order to assess the effects of the samples on cell numbers, cultured MG-63 cells were treated for 2 min with 0.05% trypsin-EDTA at 37 °C. Ten percent FBS/DMEM was added, and the cells were recovered. The recovered cells were then added to 48-well micro-plates, which contained the metal samples, at a concentration of 2×10^4 cells/sample, then cultured for 3 days in an incubator at 37 °C in a 5% CO_2 atmosphere. To count the number of cells grown on each metal sample, the metal samples were isolated and treated with 0.05% Trypsin-EDTA; the cells were then recovered and counted using a cell counting chamber (Paul Marienfeld GmbH&Co. Germany). The viability of the recovered cells was identified with trypan blue. Each experiment was performed in triplicate, and the mean values were obtained and expressed as the results. Differences between the control group samples and each of the treated titanium samples were assessed via ANOVA.

2.9 Alkaline phosphatase (ALP) activity

To measure the differentiation markers of osteoblast ALP activity, cultured MG-63 cells were treated for 2 min with 0.05% trypsin-EDTA at 37 °C. Ten percent FBS/DMEM was then added, and the recovered cells were used. The recovered cells were seeded in 48-well micro-plates, which contained the metal samples, at a concentration of 2×10^4 cells/sample, then cultured for 7 days in an incubator at 37 °C in a 5% CO_2 atmosphere. Cells grown on each of the metal samples were subsequently recovered and the ALP was measured. Metal samples cultured for 7 days were detached from the wells and treated with 0.05% trypsin-EDTA. The recovered cells were then washed in phosphate buffer solution and lysed with 0.1% Triton X-100. The lysate was then centrifuged for 20 min at 14,000 rpm and the supernatant was obtained. The proteins in the supernatant were quantitated via Bio-Rad DC protein assay. The quantitated protein and the standard diluted to an appropriate concentration were added to 96-well micro-plates, at 100 μL/well. Fifty μL of the yellow substrate was then added to each well, allowed to react in an incubator at 37 °C and 5% CO_2, and the O.D. at 405 nm was measured at 5-min intervals with a Microplate reader (Titertek Multiscan Plus, Finland). When the slope of the standard achieved maximum levels, the reaction was terminated via the addition of 50 μL of 2 N NaOH. By applying the measured O.D. value, the concentration of ALP was calculated as μM/hour/mg protein/mm^2. At that time, the threshold of the measurement of ALP activity was 3.125 μM. Differences between the control group samples and each treated titanium sample were assessed via ANOVA.

2.10 Statistical analysis

To assess significant differences between the untreated control samples and the anodized samples, the ANOVA function of Microsoft Office Excel 2007 was used, and cases with $p < 0.05$ were considered to be significantly different.

3 Results

3.1 Scanning electron microscopic examination of the titanium sample surface

The results of the surface examination of the titanium alloy sample by scanning electron microscope are shown in Fig. 1. In the untreated Ti64 control group sample, no multiporous structures were detected on the surface (Fig. 1A). On the 260 V oxidization-treated sample, volcano-shaped multiporous structures were observed (Fig. 1B), and as the voltage increased, as in the samples oxidized at 280 V (Fig. 1C) and 310 V (Fig. 1D), the diameter of the hole in the multiporous structure increased.

3.2 Atomic force microscopic analysis of the titanium sample surface

The untreated titanium alloy and the alloy samples treated with anodization at various voltages were subjected to atomic force microscopic examinations. In addition, the surface roughness values for these samples were determined. The surface roughness of the untreated control

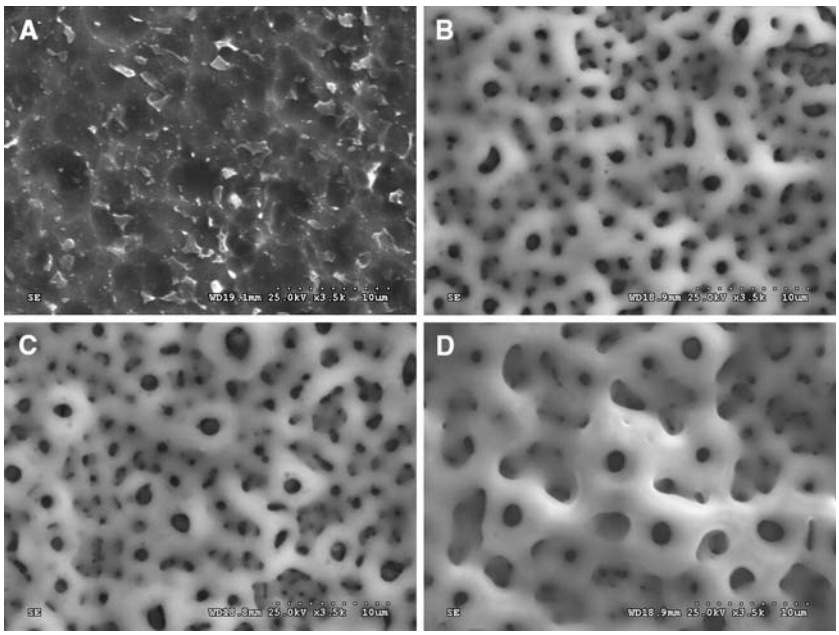

Fig. 1 Scanning electron micrographs of untreated Ti-6Al-4V surface (**A**), and anodized Ti-6Al-4V surfaces at 260 V (**B**), 280 V (**C**) and 310 V (**D**) (original magnification 3500×)

sample was 0.053 μm, and the surface roughness values of the samples anodized at 260 V, 280 V, and 310 V were 0.349 μm, 0.353 μm, and 0.440 μm, respectively; the roughness increased directly with the applied voltage.

3.3 Light microscopic examination of abdominal connective tissues

The space in the abdominal connective tissue into which the metal sample was inserted remained empty, and could thus be readily distinguished (Fig. 2). In the old connective tissues, fibers and cells were sparsely arranged and stained weakly with hematoxylin-eosin. However, the newly formed fibrous connective tissue capsule in the vicinity of the sample was dense and stained strongly with hematoxylin-eosin; thus, it was easily distinguishable from the old connective tissues (Fig. 3). In these connective tissue capsules, fibroblasts and fibrocytes were generally present together, and in some cases, blood vessels were detected in the capsules (Fig. 4). These blood vessels were sometimes empty, but blood vessels filled with red blood cells were detected in other cases (Fig. 4A). The thickness of the capsule was even in some cases; however, different thicknesses were also observed. On the side of the capsule that was in contact with the surface of the sample, giant multinucleated cells were occasionally detected (Fig. 4A). Necrosis, granuloma, and dystrophic calcification were not detected in the connective tissue area into which the sample was inserted.

The thickness of the newly formed fibrous connective tissue capsule was measured in the vicinity of the sample, 2 and 4 weeks after the insertion of the titanium samples. In the untreated control group sample, the thickness of the connective tissue capsule at 2 and 4 weeks was 59.59 μm and 53.06 μm, respectively (Table 1). In particular, in the Ti64 310, the thickness of the capsule after 2 and 4 weeks was 39.45 μm and 38.07 μm, respectively. As compared with the control group sample, this was the thinnest of the connective tissue capsules. In addition, fibroblasts and fibrocytes, as well as neutrophils, macrophages, giant multinucleated cells and unidentified cells, were present in these fibrous connective tissue capsules (Fig. 4B).

3.4 In vitro assessment of MG-63 cell number and ALP activity

The sample with the largest number of cells cultured on top of the sample was Ti64 310, with 0.119×10^4 cells; by way of contrast, the sample with the smallest number was Ti64 260, with 0.101×10^4 cells. The Ti64 control group sample contained 0.102×10^4 cells, and no significant differences were detected between the control group and other treatment groups (Table 2). In the case of ALP activity, the Ti64 control group sample yielded a value of 0.025, the Ti64 260 sample was 0.030, the Ti64 280 sample was 0.026, and the Ti64 310 sample was 0.046. Therefore, compared with the control group, the Ti64 310 sample evidenced significantly increased ALP activity (Table 2).

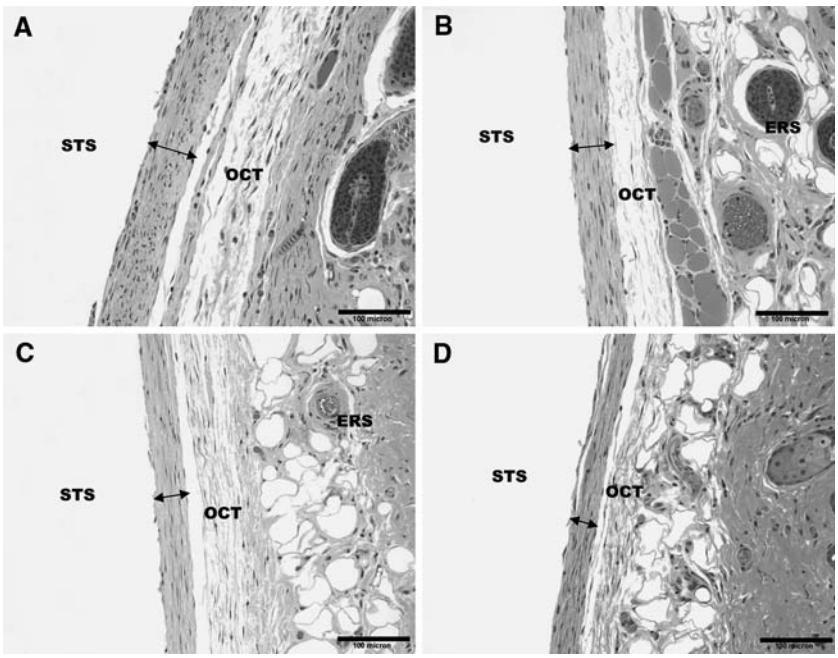

Fig. 2 Photomicrographs of abdominal connective tissue from untreated (**A**), anodized at 260 V (**B**), 280 V (**C**) and 310 V (**D**) Ti-6Al-4V samples after 2 weeks of implantation. Double arrows indicate the thickness of fibrous capsule around the titanium alloy sample. Abbreviations used: ERS, external root sheath; OCT, old connective tissue; STS, space formerly occupied by the sample. Bars size = 100 μm

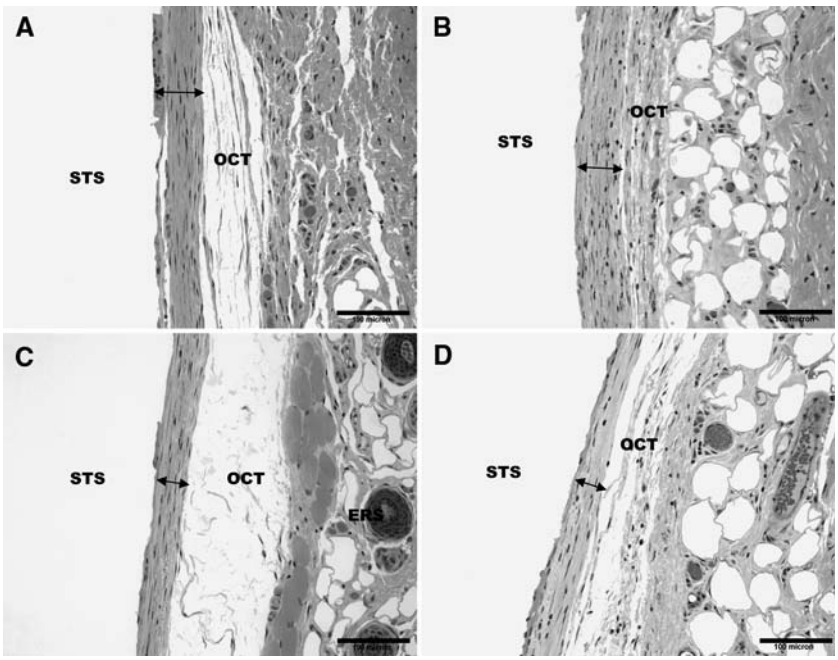

Fig. 3 Photomicrographs of abdominal connective tissue from untreated (**A**), anodized at 260 V (**B**), 280 V (**C**) and 310 V (**D**) Ti-6Al-4V samples after 4 weeks of implantation. Double arrows indicate the thickness of fibrous capsule around the titanium alloy sample. Abbreviations used: ERS, external root sheath; OCT, old connective tissue; STS, space formerly occupied by the sample. Bars size = 100 μm

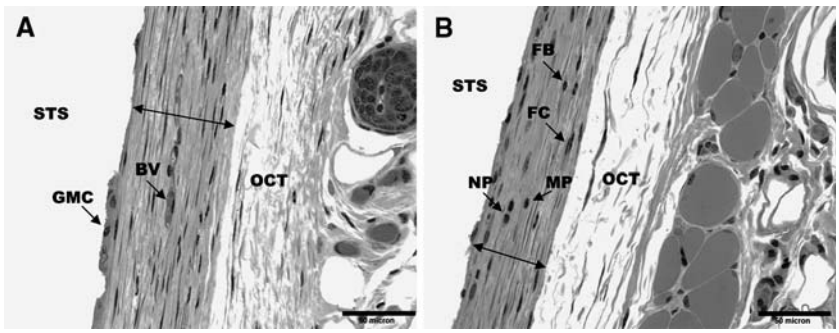

Fig. 4 Photomicrographs of several cell types and newly formed blood vessel from untreated (**A**) and anodized at 260 V (**B**) Ti-6Al-4V samples after 2 weeks of implantation. Double arrows indicate the thickness of fibrous capsule around the titanium alloy sample. Abbreviations used: BV, blood vessel; FB, fibroblast; FC, fibrocyte; GMC, giant multinucleated cell; MP, macrophage; NP, neutrophil; OCT, old connective tissue; STS, space formerly occupied by the sample. Bars size = 50 μm

3.5 Blood cell analysis

The blood analysis of mice implanted with the untreated control group samples and the oxidization-treated samples revealed that almost all values were within normal ranges in both groups, and no distinct differences were detected between the control and treatment groups (Table 3). However, the numbers of granular leukocytes, including neutrophils, eosinophils, and basophils were significantly different, and tended to decrease at 4 weeks as compared with 2 weeks, especially in the samples treated with oxidization at 260 V. With regard to thrombocytes, although not significantly different, the number of thrombocytes tended to be elevated at 4 weeks as compared with 2 weeks.

Table 1 Thickness measurements of the fibrous capsule surrounding the untreated (Ti64), and anodized at 260 V (Ti64 260), 280 V (Ti64 280) and 310 V(Ti64 310) titanium alloy samples after 2 and 4 weeks of implantation in connective tissue of abdomen

Sample type	Thickness (μm)	
	2 weeks	4 weeks
Ti64	59.59 ± 23.25	53.06 ± 14.29
Ti64 260	55.04 ± 21.48	56.25 ± 26.08
Ti64 280	43.17 ± 4.46*	44.22 ± 8.75*
Ti64 310	39.45 ± 8.80*	38.07 ± 4.80*

Values represent mean ± SD

*$p < 0.05$ compared with untreated Ti64 (control) sample

4 Discussion

Presently, commercially utilized pure titanium and titanium alloy have been well established as ideal implant materials in orthopedics or as dental prostheses [1]. The

Table 2 Cell number and alkaline phosphatase activity of MG-63 cultured on untreated (Ti64), and anodized at 260 V (Ti64 260), 280 V (Ti64 280) and 310 V (Ti64 310) titanium alloy samples

Sample type	Cell number ($\times 10^4$)	Alkaline phosphatase activity (μM/h/mg protein/mm^2)
Ti64	0.102 ± 0.039	0.025 ± 0.006
Ti64 260	0.101 ± 0.028	0.030 ± 0.004
Ti64 280	0.118 ± 0.030	0.026 ± 0.005
Ti64 310	0.119 ± 0.039	0.046 ± 0.007*

Values represent mean ± SD

*$p < 0.05$ compared with untreated Ti64 (control) sample

reaction of subcutaneous tissues to titanium or other metal implants is an important component of the evaluation of biocompatibility [9], and cells present in the connective tissue capsules might be a good marker for the evaluation of biocompatibility [14]. This capsule is composed of fibroblasts, inflammatory cells, and collagen fibers [1], and new blood vessels may also be formed in this capsule [15]. Thus, the fibrous capsule surrounding an implant may be utilized to evaluate biocompatibility [16]. It has been determined that the thinner the connective tissue capsule surrounding the implant is, the better the biocompatibility is [17, 18].

In the current study, the biocompatibilities of untreated titanium alloy and the surface-anodized titanium alloy were compared. In general, after anodization, the oxide layer present on the titanium surface became even, thick, and dense, and, when high voltage was applied, volcano-shaped microporous structures were observed to form [7, 19]. With increases in voltage, the size of the holes in the microporous structure become larger, and the oxide film formed on the titanium surface by oxidation treatment thickens [20]. Depending on the thickness of the oxide layer, the microporous structure and its distribution differ, as do the reactions of in vivo tissues [5, 21]. In our experiment, samples prepared via the oxidation of the surface at various voltages, including 260 V, 280 V, and 310 V, were inserted into murine abdominal connective tissues. As is shown in Table 1, the thickness of the murine fibrous connective tissue capsule surrounding the samples gradually decreased according to the increase in voltage; hence, their biocompatibility improved. These results are relative, and the fibrous layer may be relatively thin with a low density of cells, including fibroblasts and fibrocytes, as compared to those produced by some other biomaterials. The fibrous connective tissue formed in the vicinity of titanium, hafnium, or Ti-6Al-4V injected into the abdominal or dorsal tissues of rats was found to be thinner after four weeks as compared to the measurements taken at two weeks [22, 23]. However, in our experiments, the thickness of the capsule surrounding the sample after 2 and 4 weeks was similar, and no significant differences were detected.

Hur and coworkers previously reported that the proliferation of osteoblasts in vitro was more abundant in the presence of titanium that had been subjected to oxidization treatment at 200 V, 250 V, and 300 V than in the presence of untreated pure titanium, and thus, the anodized oxidation method proved to be an effective treatment for the implant

Table 3 Blood cell analysis of untreated (Ti64), and anodized at 260 V (Ti64 260), 280 V (Ti64 280) and 310 V (Ti64 310) titanium alloy samples after 2 and 4 weeks of implantation

Blood cell		Week post implantation	Sample type			
Type	Normal range		Ti64	Ti64 260	Ti64 280	Ti64 310
Neutrophil	0.1–2.4 (K/μL)	2	1.33 ± 0.47	1.09 ± 0.34	1.50 ± 0.39	1.73 ± 0.35
		4	0.92 ± 0.31	1.68 ± 0.01*	0.91 ± 0.24	0.72 ± 0.13
Eosinophil	0.0–0.2 (K/μL)	2	0.15 ± 0.22	0.10 ± 0.06	0.14 ± 0.11	0.20 ± 0.10
		4	0.04 ± 0.04	0.30 ± 0.01*	0.05 ± 0.03	0.01 ± 0.01
Basophil	0.0–0.2 (K/μL)	2	0.09 ± 0.10	0.05 ± 0.04	0.07 ± 0.06	0.08 ± 0.05
		4	0.03 ± 0.03	0.11 ± 0.04*	0.01 ± 0.02	0.01 ± 0.01
Lymphocyte	0.9–9.3 (K/μL)	2	4.36 ± 0.48	3.51 ± 1.17	3.83 ± 0.62	4.33 ± 1.11
		4	2.87 ± 0.42	3.86 ± 0.55	4.67 ± 0.93	3.09 ± 0.99
Monocyte	0.0–0.4 (K/μL)	2	0.31 ± 0.06	0.20 ± 0.10	0.24 ± 0.05	0.20 ± 0.11
		4	0.07 ± 0.03	0.12 ± 0.04	0.05 ± 0.02	0.09 ± 0.04
Erythrocyte	6.3–9.4 (M/μL)	2	8.64 ± 0.46	8.33 ± 0.42	8.17 ± 0.82	8.83 ± 0.83
		4	6.65 ± 1.31	7.70 ± 0.16	7.93 ± 1.18	7.61 ± 1.27
Thrombocyte	592–2972 (K/μL)	2	1092.5 ± 138.4	772.8 ± 750.4	944.0 ± 257.7	732.0 ± 199.9
		4	1031.1 ± 516.2	912.8 ± 55.2	1213.3 ± 131.6	1403.2 ± 166.7

Values represent mean ± SD

*$p < 0.05$ compared with untreated Ti64 (control) sample

surface [20]. Similarly, in our experiments, microporous structures were not detected in the untreated control titanium alloy sample; however, on the surface of the oxidized alloy samples, microporous structures were clearly observed. In addition, increases in the voltage to 260 V, 280 V, and 310 V resulted in increases in the diameter of surface micropores and the roughness on each titanium alloy sample, as distinctly detected on scanning electron micrographs.

In this cell culture experiment, the Ti64 260, Ti64 280, and Ti64 310 samples treated with various voltages appeared to result in the growth of larger numbers of cells than were observed with the control titanium alloy, Ti64; however, no significant differences were detected as compared with the control samples. The activity of the ALP enzyme has been identified as a marker for osteoblast differentiation. Therefore, higher levels of activity could be considered indicative of better biocompatibility. In our experiment, the ALP value of the Ti64 260 and the Ti64 280 samples did not differ significantly from the value of the control Ti64 sample at 0.025; however, the ALP value for Ti64 310 was 0.046, which indicated significantly higher activity than was observed in the control group. Therefore, the Ti64 310 sample with abundant micropores was more biocompatible than the control sample.

In our experiment, the numbers of leukocytes, red blood cells and thrombocytes were all distributed within normal ranges, according to analyses of blood collected from the tip of the tails of mice in the control group implanted with titanium alloy for 2 and 4 weeks. No distinct differences were detected between the samples from 2 and 4 weeks. Furthermore, when comparing the oxidation-treated samples and the untreated control samples, no noticeable differences were detected, except in the Ti64 260 sample after 4 weeks of implantation. Dalu and coworkers defined the inflammation grade by implanting samples in female mice, then sacrificing the mice after 2, 14, 42, 70 or 105 days, and finally identifying inflammatory cells (macrophages and neutrophils) in the connective tissues found in the vicinity of the sample [24]. According to their results, the inflammation grade increased until 14 days after implantation, and subsequently decreased gradually until 42 days after implantation, although these variations were not significant. After this point, the same inflammation grade persisted until the end of experiment, at 105 days after implantation. On the other hand, when samples were implanted in the middle ears of rats, and evaluated for the number of neutrophils, macrophages, and lymphocytes in the middle ear mucosa, the proportion of these cells was shown to achieve maximal levels 1 day after implantation. Until 3 days post-implantation, the cell numbers rapidly increased, after which the levels of macrophages and lymphocytes decreased very slowly from 3 days until the end of the experiment, at 300 days post-implantation [25]. Considering the results of previous studies, it seems that, in our experiment, the inflammation grade was already reduced when blood cells were analyzed 2 and 4 weeks after the sample implantations, and thus, no significant differences were detected at 4 weeks, except with the Ti64 260 sample. A review of our results suggests that the evaluation of biocompatibility is difficult when analyzing blood cells collected from the tail after inserted samples have remained in the mice for relatively long periods—in this case, for 2 and 4 weeks.

To summarize the above in vivo and in vitro results, we found that the fibrous connective tissue was thinner in mice implanted with titanium alloys subjected to anodized treatment at various voltages, as compared with that observed in the untreated control group; thus, biocompatibility in the anodized samples was enhanced. In the in vitro tissue culture experiments, higher numbers of cells were detected in the presence of all oxidation-treated samples than in the presence of the untreated control samples, although this difference was not significant. In the ALP experiments, activity was significantly higher in the sample subjected to oxidation treatment at 310 V; hence, its biocompatibility was increased. The results of the cell analysis of the blood collected from the mouse tails revealed that blood components in both the control group and the treatment group were within normal ranges. The evaluation of biocompatibility via measurements of the thickness of the connective tissue capsule surrounding the vicinity of the sample in vivo and the ALP activity in vitro revealed relatively consistent results. In conclusion, the application of oxidation treatment to the surface of titanium alloy appears to effectively and positively affect its biocompatibility.

Acknowledgment This work was supported by a grant from the Basic Research Program of the Korea Science and Engineering Foundation (R01-2004-000-10238-0).

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