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Original Paper

Surface Properties and Biocompatibility of Acid-etched Titanium

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The purpose of this study was to evaluate the effects of acid-etched titanium on the biological responses of osteoblast-like MC3T3-E1 cells. Four types of treatments (polishing, sandblasting, concentrated H_2SO_4 etching, and concentrated H_2SO_4 etching with vacuum firing) were carried out on the surfaces of commercially pure titanium (cpTi) disks. MC3T3-E1 cells were then cultured on the treated cpTi surfaces. Through surface roughness measurement and SEM analysis, it was found that the acid-etched surfaces showed higher roughness values than the sandblasted ones. Scanning electron microscope analysis showed that the cells on the disks treated with acid-etching and acid-etching with vacuum firing spread as well as the sandblasted ones. There were no significant differences in cell proliferation and collagen production on cpTi among the four different surface treatments. Based on the results of this study, it was concluded that etching with concentrated sulfuric acid was a simple and effective way to roughen the surface of titanium without compromising its biocompatibility.

Key words: Acid, Titanium, Biocompatibility

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INTRODUCTION

When a biomaterial is placed in contact with human tissues, the biocompatibility of the material is determined not only by its physical and chemical surface properties, but also by the initial reaction of cells with the surface of the material. Pure titanium and its alloys have been widely used in clinical medicine and dentistry, chiefly because of their excellent mechanical properties, high corrosion resistance, and adequate biocompatibility[1]. An additional advantage is that titanium can be treated with different bioactive surface modification methods. Amongst which, chemical surface treatments have been frequently applied to dental implant materials in the last decade.

Titanium is a very active material. Upon exposure to air, a stable TiO_2 film will cover the surface immediately. This passive film protects the titanium surface from corrosion. However, when titanium is exposed to a reducing environment, the passive film is unstable and corrosion can occur. Various acids have been used to modify the titanium surface and some have been applied to biological use[2-5]. It has been reported that sandblasted and acid-etched (SLA) titanium promotes greater osseous contact compared with titanium-plasma-sprayed implants[6-9]. This is due to the high surface roughness of SLA produced by grit blasting and acid etching, with the latter resulting in an increase in sub-surface hydrogen concentration and the formation of titanium hydride[10].

Surface topology is an important factor for biological interactions at the interface between implants and living tissue[11,12]. In the same vein, surface wettability is another important factor that influences the interactions between the implant surface and the surrounding environment[13,14]. Various new surface treatments have emerged lately and have been reported[15-18]. As for SLA surface treatment, it has also been improved by modifying the treatment conditions[19]. These surface treatment methods modify the surface properties (surface roughness and wettability), thereby modifying cell attachment and proliferation properties. For dental implants, osseointegration at the bone-implant interface is a prerequisite to succeed in achieving and maintaining their long-term stability. On this ground, it is very important to understand the cellular basis of osteoblast -biomaterial interaction as it should ideally lead to osseointegration.

Etching of titanium in concentrated acid seems to be an attractive method to modify the surface of titanium for biological applications. However, studies in this area are rarely reported in biomaterials science. Our previous study revealed that etching in concentrated sulfuric acid produced a rougher titanium surface than in HCl, H_3PO_4, HF, or HNO_3[20]. It was also demonstrated that the surface roughness and weight loss of commercially pure titanium (cpTi) plates increased with acid temperature and etching time in concentrated sulfuric acid[21]. Moreover,

etching in concentrated sulfuric acid was found to be a simple and effective surface modification method of cpTi for bonding to veneering composite resins[22] as well as an effective surface pretreatment for apatite coating on titanium using an alternate soaking method[23].

An outstanding advantage of acid etching is that it renders the substrate with homogeneous roughening regardless of its shape and size. However, it is very important that the biocompatibility of cpTi is retained — if not enhanced — after acid etching treatment. By means of X-ray diffraction analysis, the aim of this study was to evaluate the properties of acid-etched cpTi disks in concentrated sulfuric acid by investigating their surface roughness and contact angle values, as well as to examine their biocompatibility *in vitro* using osteoblast-like MC3T3-E1 cells.

MATERIALS AND METHODS

Specimen preparation

As shown in Table 1, four types of treatments were carried out on the surfaces of cpTi disks (diameter: 15 mm, thickness: 1 mm; KS40, JIS Class-2, Kobelco, Kobe, Japan): polishing with 1-μm alumina (PO), sandblasting with 70-μm alumina (SB), etching in 48% H_2SO_4 at 60℃ for one hour (A60), and both etching in 48% H_2SO_4 at 60℃ for one hour and firing in vacuum at 600℃ for 10min (A60VF). Six disks were prepared for each group. All the specimens were carefully washed in water and ultrasonically cleaned with acetone, 70% ethanol, and distilled water for 20 minutes each, and then dried in air at room temperature. The specimens were maintained under sterile conditions after ethylene oxide gas sterilization.

After each treatment, the XRD patterns of cpTi disks were recorded with an X-ray diffractometer (RINT-2500, Rigaku, Tokyo, Japan) at 40 kV and 120 mA. Scans were conducted between 30° and 60° in 2θ angle values at 1°/min. In addition, the surfaces of cpTi disks after treatment were observed using a scanning electron microscope (SEM) (JSM-5510LV, JEOL, Tokyo, Japan).

The surface roughness of dried specimens was analyzed using a surface roughness tester (Surfcom 130A, Accretech, Tokyo, Japan). Five measurements were performed for each specimen according to ISO 4287:1997. The arithmetic mean deviation of the profile (*Ra*) and the maximum height (*Rz*) were measured with a cut-off value of 0.8 mm, measurement length of 5 mm, measurement speed of 0.6 mm/s, and a Gaussian filter. Contact angles were obtained using a sessile drop method with a contact angle measurement device (CA-DT Instrument, Kyowa Kaimen Kagaku, Tokyo, Japan). Four different specimens were used for each treatment, and five drops per specimen were analyzed.

Cell culture

Mouse osteoblast-like cells, MC3T3-E1 subclone 4, were maintained in α minimum essential medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (Moregate Biotech, Bulimba, Australia), 2 mM L-glutamine, 70 μg/ml of penicillin, and 100 μg/ml of streptomycin at 37℃ in a humidified 5% CO_2 atmosphere. The cells — placed into individual wells of 24-well plates — were seeded at a density of 5×10^3 cells/cm^2 on the cpTi disks treated using four different surface modification methods.

Evaluation of cell morphology and proliferation

After one-day culture, cells grown on the cpTi disks with different surface modification methods were fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.4) for one hour and postfixed for 30 minutes with 1% osmium tetroxide in 0.1 M cacodylate buffer. After dehydration in graded ethanol, specimens were transferred into *t*-butyl alcohol and freeze-dried. Specimens were sputter-coated with gold, and cell morphology was observed using SEM.

The number of cells grown on the cpTi disks was measured at three, six, and nine days using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). Polystyrene plates (PP) were used as control. The counting technique used a tetrazolium salt that produced a highly water-soluble formazan dye. After one-hour incubation with a reagent according

Table 1 Materials used in this study

Code	Surface modification		
	Finishing	Acid etching	Oxidation
PO	Polishing with 1-μm alumina	–	–
SB	Sandblasting with 70-μm alumina	–	–
A60	–	48%H_2SO_4, 60℃, 1hr	–
A60VF	–	48%H_2SO_4, 60℃, 1hr	600℃, 10min, in vacuum

to manufacturer's instructions, relative cell number was determined by measuring light absorbance at a wavelength of 405 nm by formazan dye product in the cultures. The cell number was calculated, based on a calibration curve which was prepared using the data obtained from the wells that contained known numbers of viable cells.

Collagen production assay

Collagen levels in the culture medium were determined by a Soluble Collagen Assay kit (Biodye Science, Newtownabbey, Northern Ireland) according to manufacturer's instructions.

Statistical analysis

Data were presented as mean±standard deviation. Data were subjected to one-way analysis of variance, and Bonferroni-Dunn test was used for *post hoc* comparison of specific groups.

RESULTS

Surface characteristics after surface modification treatments

Figure 1 shows the XRD patterns of cpTi disks with different surface modification treatments. All the specimens with different surface modification treatments showed diffraction peaks of α-Ti. PO and SB showed only the diffraction peaks of α-Ti. The α-Ti diffraction peaks of SB were smaller and wider than those of PO. The diffraction peaks assigned to TiH_2 were observed on A60. In A60VF, the diffraction peaks assigned to TiH_2 were not observed, while the diffraction peaks of α-Ti were decreased in intensity.

Figure 2 shows the SEM photographs of cpTi disks with different surface modification treatments. The grooves in parallel orientation were observed on the surface of PO. The grooves seemed to be caused by the finishing procedure. SB showed numerous

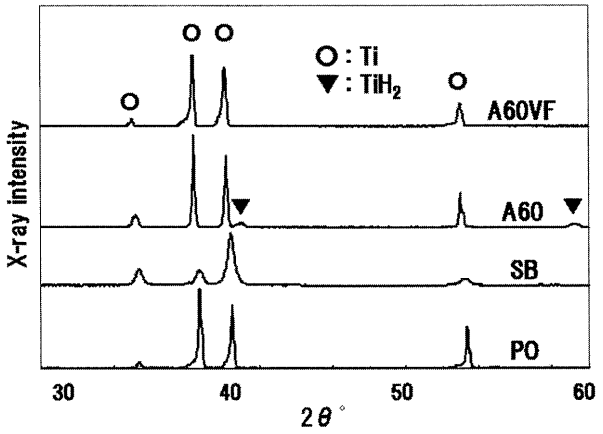

Fig. 1 XRD patterns of cpTi disks with different surface modification methods.

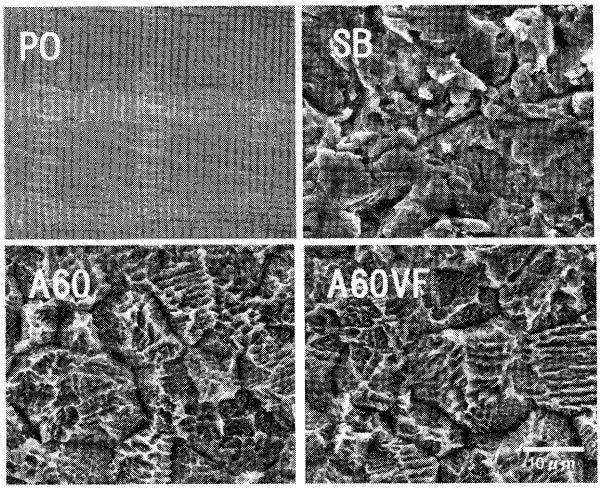

Fig. 2 SEM photographs of cpTi disks with different surface modification methods. Scale bar = 10 μm.

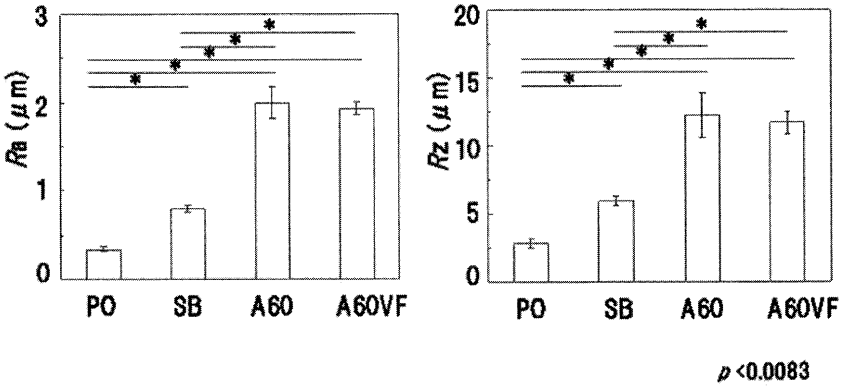

Fig. 3 Surface roughness (*Ra* and *Rz*) values of cpTi disks with different surface modification methods.

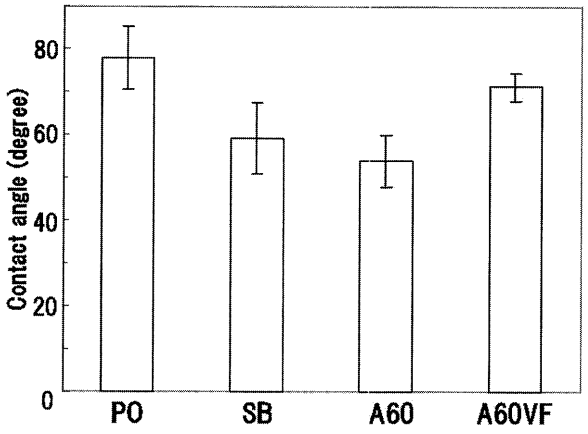

Fig. 4 Contact angles of cpTi disks with different surface modification methods.

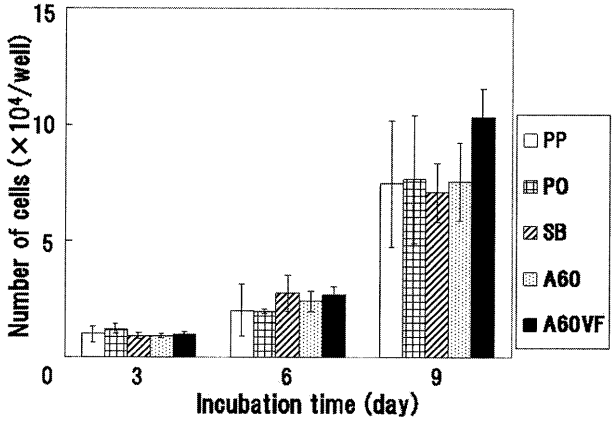

Fig. 6 Proliferation of MC3T3-E1 cells cultured in direct contact on cpTi disks with different surface modification methods for 3, 6, and 9 days.

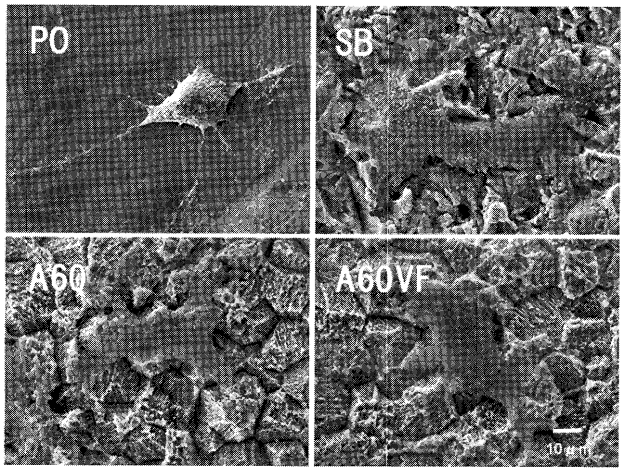

Fig. 5 SEM photograph of MC3T3-E1 cell after 1-day culture in direct contact on cpTi disks with different surface modification methods. Scale bar = 10 μm.

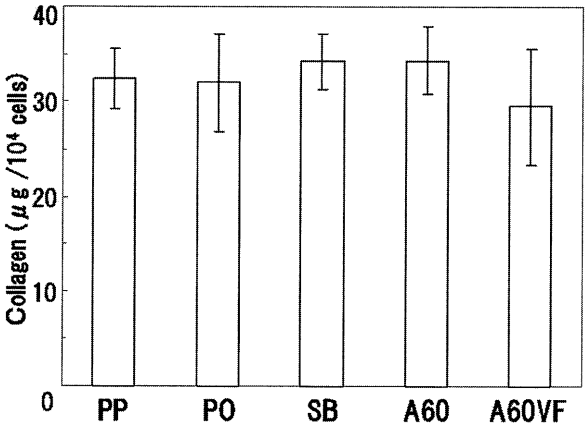

Fig. 7 Collagen produced by cultured MC3T3-E1 cells on cpTi disks with different surface modification methods at 3 days.

irregular cavities and sharp edges, which were quite different from the original smooth surface. Both A60 and A60VF showed a microporous structure consisting of homogeneous micro pits, about 1 μm in diameter, formed by acid etching. The grain boundaries of A60 and A60VF were obvious and the crystal grains were distinguishable. There were no remarkable differences in appearance between A60 and A60VF.

Figure 3 shows the surface roughness values (*Ra* and *Rz*) of cpTi disks with different surface modification treatments. PO showed the lowest roughness values for *Ra* (about 0.34 μm) and *Rz* (about 2.8 μm), while A60 and A60VF showed the highest *Ra* (about 2.0 μm) and *Rz* (about 12.3 μm) values. SB showed moderate values. Between the specimens of A60 and A60VF, there were no significant differences in surface roughness.

Figure 4 shows the contact angles of cpTi disks with different surface modification treatments. SB and A60 showed lower contact angles, thus indicating higher hydrophilicity than PO.

Cell morphology and proliferation

Figure 5 shows the SEM images of MC3T3-E1 cells after one-day culture on cpTi disks with different surface modification treatments. It was revealed that the morphology of attached cells on the disks of A60 and A60VF were flattened with many cytoplasmic extensions and lamellopodia, similar to that which was observed on SB.

Figure 6 shows the proliferation of MC3T3-E1 cells cultured on cpTi disks with different surface modification treatments. The cells proliferated in all

specimens in a time-dependent manner. There were no significant differences among the specimens with different surface modification treatments.

Collagen production

Figure 7 shows the collagen levels produced by MC3T3-E1 cells cultured for three days on the cpTi disks with different surface modification treatments. There were no significant differences in collagen level among the specimens with different surface modification treatments.

DISCUSSION

As shown in Fig. 1, the diffraction peaks assigned to TiH_2 were observed in A60 but not in A60VF. Our previous study[21] demonstrated that TiH_2 was formed on the cpTi plates during etching in H_2SO_4 as follows:

$$TiO_2 + 2H_2SO_4 \rightarrow Ti(SO_4)_2 + 2H_2O$$
$$Ti + 2H_2SO_4 \rightarrow Ti(SO_4)_2 + 2H_2\uparrow$$
$$Ti + H_2 \rightarrow TiH_2$$

With limited experimental data obtained, it was speculated that although a new oxide layer might have formed on the TiH_2 layer owing to contact with moisture in the air, the purported existence of this oxide layer could not be confirmed by XRD. This was because it was only a thin layer with a low crystallinity. In A60VF, the diffraction peaks assigned to TiH_2 were not observed, while the diffraction peaks of α-Ti were decreased in intensity. Based on these results obtained, it was suggested that the surface of A60VF was covered with a thin oxide layer. Although A60VF was heated in a vacuum porcelain furnace, small quantities of oxygen could have reacted with TiH_2 and formed a small quantity of TiO_2 on the surface. Based on these XRD surface characterization results, the structures of cpTi surfaces subjected to four kinds of surface pretreatments are summarized in Fig. 8.

As for SB, its α-Ti diffraction peaks were smaller and wider than those of other surface treatments. It seemed that repeated blasting of high-speed sand particles led to plastic deformation of the cpTi surface, thereby resulting in lattice strain of Ti. Jiang *et al.*[24] have demonstrated that sandblasting resulted in the formation of a severely deformed surface layer and generated compressive residual stresses on the surface.

On contact angles, both SB and A60 showed lower contact angle values (Fig. 4), hence indicating their higher hydrophilicity than PO. The key factors that affect contact angles are surface chemical composition and surface texture (*i.e.*, roughness, presence of pores). Tamada *et al.*[25] suggested that

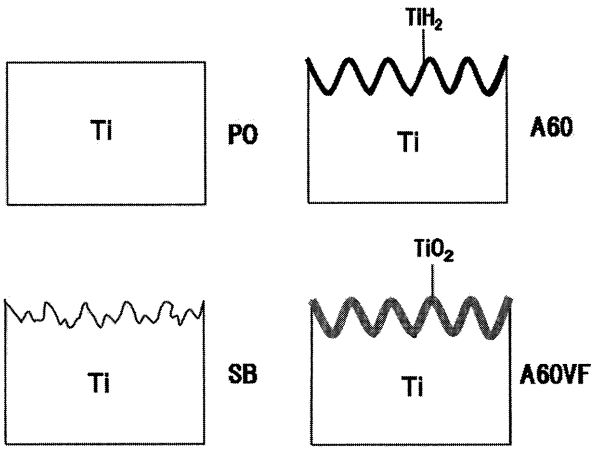

Fig. 8 Schematic illustration of cpTi disks with different surface modification methods used in this study.

surfaces with water contact angles in the range of 60−80° were most favorable for growth and adhesion of fibroblasts. In the present study, the contact angles of all the treated surfaces were almost within that range. Hence, it could be suggested that all the substrates prepared in this study were effective for cell growth.

Cell proliferation assay (Fig. 6) showed that there were no significant differences in cell growth among the surface modification methods used in the present study. We have previously reported that despite differences in surface topography, early cellular response was similar for both sandblasted cpTi plates and those etched in concentrated H_2SO_4[26]. In the same vein, there were some studies which reported that there were no clear effects of surface roughness on proliferation of bone cells[27-29]. However, other studies reported a significant correlation between surface roughness and cell growth, whereby greater proliferation of osteoblasts on smoother or rougher surfaces resulted in better osseointegration[30-34]. Currently, we have no clear explanation for this discrepancy. Nonetheless, Lohmann *et al.*[35] showed — using three types of osteogenic cell lines — that osteoblastic cells responded in a differential manner to changes in surface roughness depending on their maturation state. In other words, the types and conditions of cells in the osteoblast lineage may affect proliferation responses to surface roughness.

Larsson *et al.*[36] reported that machined and electropolished implants with different oxide thicknesses had no clear influences on early tissue response. This finding was compatible with our result that there were no clear differences between A60 and A60VF in cell morphology and proliferation. In other words, it was implied that the presence of TiH_2

had neither influence nor impact on cell proliferation. Perrin *et al.*[12] also reported that the presence of a high and unusual amount hydrogen in etched titanium did not play a significant role in bone response. Therefore, differences in cell proliferation cannot be explained by surface properties such as roughness and composition. Further investigation is needed to interpret and explain these results.

Type I collagen is a major extracellular matrix protein present in bone. It plays a central role in bone mineralization[37] and supports the growth and regulation of osteoblastic differentiation[38]. Kim *et al.*[39] reported that collagen coating of titanium promoted attachment and proliferation of osteoblasts when collagen was assembled into fibrils and with an increase in assembly degree. In the present study, collagen production by cultured osteoblast-like cells on the differently treated cpTi disks at three days (Fig. 7) showed no significant differences in cell response. Taken together with the results of cell proliferation and cell morphology, it was very likely that the biocompatibility of cpTi disks etched in concentrated sulfuric acid was maintained and uncompromised.

In light of the results obtained in this study, it was suggested that etching in concentrated sulfuric acid was an effective surface roughening treatment for titanium for biological applications. Indeed, results showed that it was more effective than sandblasting without compromising the surface biocompatibility of titanium. Furthermore, it might be an effective surface treatment method for titanium implants with complicated shapes.

CONCLUSION

The surface roughness of cpTi after etching in 48% H_2SO_4 increased significantly. Osteoblast-like cells attached, spread, and proliferated well on cpTi disks with different surface treatments. This study concluded that etching with concentrated sulfuric acid was an effective way to roughen the surface of titanium without compromising its biocompatibility.

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