Antibacterial Titanium Plate Anodized by being Discharged in NaCl Solution Exhibits Cell Compatibility

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INTRODUCTION
Titanium has been widely utilized in biomaterials fields (Albrektsson et al., 1981; Howlett et al., 1994; Gronowicz and McCarthy, 1996), particularly as applied to dental implants, since it has excellent corrosion-resistance and biocompatibility. However, since titanium implants penetrate not only bone but also gingiva, they are partially exposed in an oral environment that includes oral bacteria. It has been reported that titanium itself has no antibacterial activity compared with other metals (Leonhardt and Dahlén, 1995). Since bacterial accumulation surrounding dental implants has pathogens known to cause peri-implantitis, it is important to prevent colonization of oral bacteria on the surfaces of implants to ensure their long-term clinical success. Indeed, there are a few reports regarding surface modification of titanium implants to prevent colonization of oral bacteria (Yoshinari et al., 2000).

We have already demonstrated that titanium plates anodized by being discharged in NaCl (Ti-Cl) exhibited high antibacterial activity. Since Ti-Cl was prepared with a NaCl solution, we hypothesized that Ti-Cl would exhibit low toxicity toward cells. The aims of this study were to characterize the surface of Ti-Cl and investigate the cell compatibility (MC3T3-E1 and L929 cells) of Ti-Cl. The results demonstrated that, since the TiCl₃ formed on the Ti-Cl surface was hydrolyzed into HCl, HClO, and TiOH after immersion in pure distilled water, TiCl₃ contributed to the antibacterial activity of Ti-Cl. On the other hand, TiO formed on the Ti-Cl surface enhanced cell extension and cell growth through a larger adsorption of fibronectin compared with the pure titanium control. These findings suggest that antibacterial titanium is a promising material for use in dental implant systems.

KEY WORDS: titanium, anodizing, oral bacteria, antibacterial activity.

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The crystal phases of Ti-Cl were detected by TF-XRD (XRD-6100, SHIMADZU, Kyoto, Japan) with CuKα radiation. XRD was operated at 40 kV, 40 mA with a scanning speed of 0.02°/4 sec and a scanning range of 20-50°.

**XPS**
The surfaces of Ti-Cl specimens were detected by XPS (ESCA-3400, SHIMADZU, Kyoto, Japan). High-resolution spectra of Ti2p, O1s, C1s, Na1s, and Cl2p were analyzed with the use of MgKα radiation. After analysis, the specimens were immersed in pure distilled water and again subjected to XPS analysis. A 20-mA emission current and 8-kV accelerated voltage were applied in this analysis. The binding energies for each spectrum were calibrated with the use of a C1s spectrum of 285.0 eV.

The results were expressed as the mean ± standard deviation (SD) of 6 specimens (n = 6), and analyzed statistically by Student's t tests. Significant differences were considered to exist when p < 0.01.

**Cell Cultivation**
An osteoblastic cell line, MC3T3-E1, and a fibroblastic cell line, L929, were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in α minimal essential medium (Gibco, Tokyo, Japan) containing 10% fetal bovine serum (Gibco) and 1% antibiotic (penicillin, Gibco) under a 5% CO₂ atmosphere at 37°C. Cells were suspended in the medium at 1 x 10⁵ cells/mL and used for cell adhesion experiments.

A 1-mL quantity of floating cells was plated onto each of the specimens and incubated at 37°C and 5% CO₂ for 1 wk.

**Cell Counting**
A cell-counting kit (Dojindo, Kumamoto, Japan) was used for the measurement of cell adhesion. After incubation, each specimen was moved to another well and washed 3 times with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS; Gibco) to remove non-adherent cells. Adherent cells were mixed with 1 mL of medium and 100 μL of reagent solution. After 1 hr of incubation, the absorbance at 450 nm was measured. The number of adherent cells was calculated from the activity of the original cell suspension. The results were expressed as the mean ± SD of 6 specimens (n = 6), and analyzed statistically by analysis of variance (ANOVA). Significant differences from respective control values were considered to exist when p < 0.01.

**Stress Fiber Formation and Cell Morphology**
Specimens were placed in 24-well culture plates with 1 mL floating cells each. Subsequently, the specimens were incubated at 37°C in 5% CO₂ for 1 hr. Adherent cells on each specimen after 1 hr of cultivation were dehydrated after being washed with PBS. The cells were fixed with 3.7% formaldehyde in PBS and permeabilized by treatment with 0.1% Triton X-100 (Sigma, Tokyo, Japan) in PBS for 1 min. The cells were then incubated for 3 hrs in a rhodamine-conjugated phalloidin solution. After the cells were washed with water, stress fiber formation and cell morphology were observed with the use of a fluorescence microscope (E-600, Nikon, Tokyo, Japan).

**Protein Adsorption**
FITC labeling was used for the visualization of adsorbed fibronectin on each specimen. The specimens were soaked in α minimal essential medium (Gibco) containing 10% fetal bovine serum (Gibco) at 37°C for 30 min. After incubation, the specimens were washed with PBS 3 times and blocked with 5% BSA (SIGMA) for 1 hr. After being washed again with PBS 3 times, they were immersed in a 2000-fold dilution of monoclonal anti-
human fibronectin (TaKaRa, Shiga, Japan) for 2 hrs. After another 3 washes with PBS, they were immersed in a 32-fold dilution of FITC-conjugated anti-mouse IgG (SIGMA) for 1 hr at room temperature. Following another 3 washes with PBS, the adsorbed fibronectin on each specimen was observed with the use of a fluorescence microscope (E-600, Nikon). Quantitative analysis of the images was performed on a Windows® computer equipped with the public domain program NIH Image (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov from the National Technical Information Service, Springfield, VA; part number PB95-500195GEI). The results were expressed as the mean ± SD of 6 specimens (n = 6). The findings were analyzed statistically by Student’s t tests. Significant differences were considered to exist when p < 0.01.

RESULTS

SEM Observation
Small amounts of granular deposits were sparsely observed on the Ti-Cl surface (Fig. 1).

Surface Characterization

XRD
A peak of TiCl$_3$ was detected on Ti-Cl, while the peak of the TiO suboxide layer was obviously increased on Ti-Cl (Fig. 2A).

XPS
The energy position of Cl$_2$p was 198.3 ± 0.1 eV, the chemical state of the chloride compound formed on Ti-Cl was TiCl$_3$. After 1 hr of immersion, the energy position of Cl$_2$p shifted to 201.4 ± 0.1 eV, indicating that the chemical state of TiCl$_3$ had changed and had combined with oxygen (not shown). The three peaks of the curve fit of the O1s spectra on Ti-Cl are shown (Figs. 2B, 2C). Peak 1 was set at 530.1 eV at TiO$_2$, peak 2 at 531.1 eV for H$_2$O, and peak 3 at 532.3 eV for Ti-OH. The peak 3 indicating Ti-OH increased (p < 0.01) on the surface of Ti-Cl after immersion (C). The results of the XPS data are expressed as the mean ± SD of 6 specimens (n = 6). The findings were analyzed statistically by Student’s t tests. Significant differences were considered to exist when p < 0.01.

Cell Cultivation
After 1 wk of cell cultivation, the number of adherent cells for each cell line on Ti-Cl was significantly higher than those on control specimens. Adherent cells were incubated with 100 μL/mL of assay reagent solution for 1 hr, and then the absorbance at 450 nm was measured. The number of adherent cells was calculated from the activity of the original cell suspension. The results are expressed as the mean ± SD of 6 specimens (n = 6). The findings were analyzed statistically by ANOVA. *Significant differences from respective control values were considered to exist when p < 0.01.
Stress Fiber Formation and Cell Morphology

Adherent cells on Ti-Cl had already begun to form stress fibers and had widely extended cytoplasm (Figs. 4A, 4B). On the other hand, no stress fiber formation was observed in the cells on the control specimens, and the cells adhered loosely to the surface compared with those on Ti-Cl (Figs. 4A, 4B).

Protein Adsorption

After 1 hr of incubation, the adsorbed fibronectin on each specimen was evaluated (Fig. 4C). Ti-Cl showed higher adsorption (9.8 ± 0.8%) of fibronectin than that of control specimens (1.3 ± 0.4%) (p < 0.01).

DISCUSSION

The present study characterized the surface and in vitro cell compatibility of Ti-Cl. In our previous study, we examined the antibacterial activity of titanium plates anodized by being discharged in NaCl, NaF, and KI solutions (Ikeda and Igarashi, 2001). Since titanium plates anodized in NaCl demonstrated much greater antibacterial activity than those of the other specimens, we hypothesized that the chemical state of the chloride formed on the Ti-Cl was HClO. However, in the XPS and XRD studies, TiCl₃ was detected on the Ti-Cl. Since chloride itself has no antibacterial activity, the chemical state of TiCl₃ changed chemically with increasing immersion time. In addition, since TiCl₃ combined with oxygen and Ti-OH increased on Ti-Cl after 1 hr of immersion in the XPS study, the process of the antibacterial effect on Ti-Cl can be explained as follows. The large amounts of chloride ions contained in the electrolyte were adsorbed onto the substrate, while the titanium oxide layer grew with anodizing. TiCl₃ was formed on the substrate. Subsequently, chloride was gradually released into the culture medium and hydrolyzed into HCl, HClO, and TiOH. Since HClO itself was not formed on Ti-Cl, but TiCl₃ was slowly hydrolyzed into HClO with increasing immersion time, the antibacterial effect of Ti-Cl was maintained even after 8 weeks’ immersion (Ikeda and...
In the cell cultivation test, since cell growth was not inhibited on Ti-Cl compared with the control specimens, Ti-Cl exhibited no toxicity toward the cells. In addition, the numbers of adherent cells on Ti-Cl were significantly greater than those on the control specimens (p < 0.01). Furthermore, in the initial adhesion test, since cells were extended on Ti-Cl compared with those on the control specimens, Ti-Cl demonstrated good compatibility to cells, at least to MC3T3-E1 and L929 cells.

It is well-known that adhesion of osteoblastic cells and fibroblasts to a substrate depends strongly on fibronectin. In addition, many tissue culture cells need the formation of focal adhesions initiated by specific binding of extracellular matrix proteins and receptors to grow and differentiate. In this study, since higher cell extension was observed on Ti-Cl from 1 hr of cultivation, and fibronectin adsorption on Ti-Cl was much greater than that on control specimens, Ti-Cl promoted cell growth and differentiation. The XRD study showed that the peak of TiO increased with processing. Since the TiO suboxide layer has high ionic activity compared with the original TiO2 layer (Zhu et al., 2001), the forming TiO contributed to the adsorption of cell-binding proteins.

Yoshinari et al. (2001) reported that titanium plates could be modified to be antibacterial by dry process ion plating (Ca+, N+, F−). They also reported that the antibacterial metal has low toxicity to L929 cells. However, this method did not contribute to cell growth. In addition, since the antibacterial metal has been utilized by means of complicated processes, the methods involved are relatively high-cost.

On the contrary, cell growth on Ti-Cl was significantly higher than that on control specimens. In addition, since our anodizing method was processed by means of a simple power supply in a NaCl solution, our anodizing method was relatively low-cost, and appears to be more suitable than other methods reported in earlier studies. From this study, we conclude that Ti-Cl is a promising material for use in dental implant systems.

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