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Title: Enhanced Initial Cell Responses to Chemically Modified Anodized Titanium

Authors: Ryo Jimbo DDS, Takashi Sawase DDS PhD, Koumei Baba MS PhD, Tadafumi Kurogi DDS PhD, Yasuaki Shibata DDS PhD & Mitsuru Atsuta DDS PhD

Authors' affiliations:

Ryo Jimbo, Takashi Sawase, Mitsuru Atsuta, Division of Applied Prosthodontics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;

Koumei Baba, Industrial Technology Center of Nagasaki, Nagasaki, Japan;

Tadafumi Kurogi, Division of Prosthetic Dentistry, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;

Yasuaki Shibata, Division of Oral Pathology and Bone Metabolism, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.

Running Title: Enhanced Cell Responses to Modified Anodized Titanium

Reprint requests and corresponding author: Dr. Takashi Sawase, Division of Applied Prosthodontics, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, 852-8588, Nagasaki, Japan

Tel:+81 95 849 7686, Fax:+81 95 849 7689, email:sawase@nagasaki-u.ac.jp

ABSTRACT:

Background: Previously, we reported that anodized porous titanium implants have photocatalytic hydrophilicity. However, this effect was not always sufficient for the significant improvement of bone apposition.

Purpose: The purpose of this study was to improve the photocatalytic properties of porous titanium implants by the fluoride modification of the anodized titanium dioxide (TiO_2), and to investigate the initial cell response to it.

Materials and Methods: The ideal concentration of ammonium hydrogen fluoride (NH_4F-HF_2) used in this study was determined by a static water contact angle assay. The ideal concentration of NH_4F-HF_2 was 0.175 %, and experimental disks were treated with this concentration. A pluripotent mesenchymal cell line, C2C12, was cultured on the disks in order to investigate cell attachment, morphology and proliferation.

Results: Cell attachment after 30 minutes of culturing was significantly higher for the UV-irradiated fluoride-modified anodized TiO_2 ($p < 0.05$), and the simultaneous scanning electron microscope observation showed a rather flattened and extended cell morphology. The proliferation rate after 24 hours was also significantly higher for the fluoride-modified anodized TiO_2.

Conclusion: Fluoride chemical modification enhances the hydrophilic property of the anodized TiO_2 and improves the initial cell response to it.

KEY WORDS: hydrophilicity, anodization, ammonium hydrogen fluoride, titanium, UV irradiation

INTRODUCTION:

The anatase titanium dioxide (TiO_2) surface is known to acquire photocatalytic properties under ultraviolet (UV) irradiation.[1-3] The mechanisms of the decomposition of various organic compounds by photocatalysts have been well described.[4] As a result of those studies, photocatalytic surfaces are now thought to have various qualities such as bactericidal activity,[1,5] deodorization capability,[6] decontamination capability[7] and hydrophilicity.[8]

Photo-induced hydrophilicity was originally discovered by Wang, *et al.*, who demonstrated that a thin TiO_2 polycrystalline film made of anatase sol exhibited a dramatic decrease in the water contact angle, to 0˚± 1˚, after UV irradiation.[9] The UV irradiation of the photocatalyst theoretically creates surface oxygen vacancies at the bridging sites, resulting in the conversion of the relevant Ti^{4+} sites to Ti^{3+} sites, which is favorable for dissociative water adsorption.[10] These defects presumably influence the affinity of chemisorbed water for the surrounding sites, thereby forming hydrophilic domains.

In the field of implant dentistry, hydrophilicity is an important factor for the initial biological cascade of osseointegration. Several recent reports have focused on the hydrophilic profiles of surface-modified Ti implants.[11-13] A chemically cleaned microstructured surface increases the dynamic wettability of Ti implant surfaces and enhances the initial biological cell response. It has also been clarified by Buser, *et al.*

that implants with hydrophilic properties enhance the initial bone apposition to the implant surface.[14] Since it has been reported that changes in the physico-chemical properties promote protein adsorption and further cell attachment through integrin-mediated mechanisms,[15] the hydrophilic TiO_2 surface is also speculated to first promote protein adsorption, and then to enhance cell behavior and bone apposition.

In our past study, we found that the commercially available TiUnite™ implant (Nobel Biocare AB, Göteborg, Sweden) which was created by anodization, shows photo-induced hydrophilicity after UV irradiation.[16] The contact angle significantly decreased from 44˚ to 11˚ upon UV irradiation, and the methylene blue degradation was also significant. However, we could not spot significant differences by histomorphometrical means between the ordinal TiUnite™ and the UV-irradiated TiUnite™ in animal experiments. We concluded that this may be due to the insufficient level of surface hydrophilicity, meaning that the low content of crystalline anatase was not enough to enhance bone regeneration. Therefore, it was hypothesized that further surface modification of the anodized TiO_2 in order to enlarge the crystalline anatase surface area would improve the hydrophilicity upon UV irradiation. It was further speculated that the improved hydrophilicity would naturally upturn the initial cell response, including cell attachment and proliferation, leading to enhanced bone apposition to the implant surface.

In this study, we focused our attention on fluoride as a means of modifying the anodized

TiO_2 surface, because fluoride has been used in studies to improve the crystallinity of anatase for enhanced photocatalytic activity. Hattori, *et al*. have stated that fluoride modification, the so-called F-doping of photocatalytic TiO_2, resulted in an increase in anatase crystallinity.[17,18] Yu, *et al*. have stated that fluoride ions not only suppress the formation of brookite, but also prevent the phase transition of anatase to rutile.[19] They have also stated that the F-doped TiO_2 samples showed stronger absorption in the UV-visible range. This promising background motivated us to use fluoride as a possible enhancer for the photo-induced hydrophilicity and further observed the initial cell reactions to the fluoride-modified anodized titanium disk in terms of cell attachment, morphology and proliferation.

MATERIALS AND METHODS:

Specimen preparation

Commercially pure Ti disks (grade 2, Furuuchi Co., Tokyo, Japan), 10 mm in diameter and 1 mm in thickness, were ground on a series of silicon-carbide papers (320, 600, and 1000-grit) and were anodized in an electrolytic solution consisting of 1.5 M H_2SO_4, 0.3 M H_3PO_4 and 0.3 M H_2O_2 under direct constant-current electrolysis at 3.0 Adm^{-2} to a final voltage of 200 V for 10 minutes. Finally, the specimens were ultrasonically de-greased in trichloroethylene for 15 minutes, followed by soaking in 95 % ethanol for 15 minutes and distilled water for 15 minutes three times.

Ammonium hydrogen fluoride treatment

The concentration of the $NH_4F\text{-}HF_2$ that showed the highest hydrophilicity (tested range: 0 % to 0.2 %) was determined through contact angle analysis using a FACE contact angle analyzer (Kyowa Interface Science Co., Ltd., Asaka, Japan) in conjunction with the sessile drop technique. These measurements were carried out at room temperature in air with distilled water as the probe liquid. Liquid droplets (8 µl) were deposited onto the sample surface at a rate of 8 µl/s. Six each of the disk-shaped specimens were used for this analysis, and the contact angles reported here represent the averages of at least five measurements. The results are shown in Figure 1. The ideal concentration was 0.175 % and the disks treated with this concentration were used in the cell culture assay.

Surface analysis

Micrographs of the anodized disks with or without the uppermost hydrophilic 0.175 % $NH_4F\text{-}HF_2$ treatment were taken using a scanning electron microscope (SEM, S-3500N, Hitachi High-Tech Corp., Ibaragi, Japan). The surface roughness was analyzed using a color 3D-laser microscope (VK-8700, Keyence, Osaka, Japan). The centerline average roughness (Ra) values for each disk were determined by averaging the values of five random areas per disk. The mean Ra values are an average of 3 disks for both groups.

Cell culture assay

The pluripotent mesenchymal precursor C2C12 cell line was obtained from the Riken

Gene Bank (Tsukuba, Japan). The cells were cultured in alpha-minimum essential medium (alfa-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 10 % fetal calf serum, streptomycin (100 μg/ml), penicillin (100 U/ml) and glutamine (2 mM) in an atmosphere of 100 % humidity and 5 % CO_2 at 37°C. Sub-confluent cultured cells were detached by trypsinization and were then plated on the following groups of disks, which were placed in 24-well polystyrene plates at a dilution of $4x10^4$ cells/ml:

Group 1: Anodized TiO_2 disks without UV irradiation.

Group 2: Anodized TiO_2 disks with UV irradiation for 24 hours.

Group 3: Anodized TiO_2 disks treated with 0.175 % NH_4F-HF_2 without UV irradiation.

Group 4: Anodized TiO_2 disks treated with 0.175 % NH_4F-HF_2 with UV irradiation for 24 hours.

The cell morphology after 30 minutes of incubation was observed by SEM. The samples were washed twice with phosphate-buffered saline (PBS) and then washed in a 0.1-M sodium cacodylate buffer solution, post-fixed with 2.5% glutaraldehyde/30 mM HEPES for 20 minutes, and finally dehydrated in an ascending series of ethanol. Each specimen was sputter-coated with gold prior to SEM.

After 30 minutes of incubation, cell attachment was also evaluated, as follows. The adherent cells were washed twice with PBS, fixed with 4% formaldehyde/PBS for 20 minutes, and nuclear-stained with propidium iodide (50 μg/ml) for 20 minutes. Five points for each disk (n=6, a total of 30 points for each group) were randomly selected, and the number of stained nuclei in an area of 175 × 140 μm was counted using a laser microscope (Axioskop 2; Carl Zeiss, Oberkochen, Germany) and Image J version 1.36b analyzing software (National Institutes of Health, Bethesda, MD).

The proliferation of the cells was evaluated immunocytologically by the uptake of 5-bromodeoxyuridine (BrdU) at 24 hours after incubation. The cells were treated with 1% BrdU (Invitrogen, Carlsbad, CA) for 2 hours prior to fixation with 70 % ethanol, and permeabilized with 2.0 M HCl for 30 minutes, followed by treatment with 0.5 % Tween-20 for 15 minutes. After the above-mentioned pretreatment procedures, the cells were treated with 1 μg/ml fluorescein isothiocyanate-conjugated monoclonal mouse anti-BrdU (Biomeda, Foster City, CA) for 30 minutes. The cells were further stained with propidium iodide (50 μg/ml) for 20 minutes. The number of BrdU-positive cells was estimated using the method described in the cell attachment assay (randomly selected five points for each disk; n=6; 30 points for each group), and the percentage of BrdU-positive cells relative to the number of cells stained with propidium iodide was used to calculate the cell proliferation.

Statistical analysis

All statistical analyses in the present study were performed with the KaleidaGraph software (Synergy Software, Essex Junction, VT). The mean and standard deviation values for the *in vitro* parameters were calculated. The average values were compared by one-way ANOVA, followed by a *post hoc* Tukey-Kramer test with the value of statistical significance set at the 0.05 level.

RESULTS:

Photo-induced hydrophilicity

The water contact angles of the NH_4F-HF_2 -treated specimens ranging from 0% to 0.2 % are shown in Figure 1. The optimum concentration that showed the highest hydrophilicity was 0.175 %. Although NH_4F-HF_2 concentrations higher or lower than 0.175 % showed improved hydrophilicity compared to the non-treated specimens, the 0.175 % NH_4F-HF_2 treatment showed significantly highest hydrophilicity among any other concentrations tested.

The contact angle measurements indicated that Groups 1 and 3 had hydrophobic surfaces, with an average contact angle of 45.5˚ and 47.3˚, respectively. As reported in our past study, the UV-irradiated anodized surface (Group 3), became hydrophilic, with an average contact angle of 11.5˚. Furthermore, Group 4 showed an improved contact angle of 4.2˚.

Surface characteristics

SEM micrographs of the tested substrates can be observed in Figures 2a and 2b. A rather smooth structure could be observed for the NH_4F-HF_2-treated anodized disks as compared to the non-treated anodized disks. The mean Ra value (SD) of the anodized and NH_4F-HF_2-treated anodized disks was 0.94 (0.04) and 0.82 (0.06) respectively. The

NH_4F-HF_2 treatment seemed to have smoothened the anodized disks.

Cell culture assay

The cell morphology after 30 minutes of incubation can be observed in Figure 3. Groups 1 to 3 showed a rather round morphology with a filopodia extensions (Figs. 3a-3c). In contrast, the morphology for Group 4 showed flattened seeded cells with numerous omnidirectional lamellipodia extensions (Fig. 3d).

The results of the cell attachment assay are shown in Figure 4a. The means (SD) of the attachment numbers were as follows: Group 1: 7.35 (2.45) $\times 10^2/mm^2$; Group 2: 8.08 (2.45) $\times 10^2/mm^2$; Group 3: 9.67 (3.35) $\times 10^2/mm^2$; Group 4: 12.7 (3.47) $\times 10^2/mm^2$. There was no significant difference among Groups 1 to 3, whereas the number of attached cells for Group 4 was significantly higher compared to that for the other groups.

The results of the cell proliferation assay are shown in Figure 4b. The means (SD) of the proliferation rates were as follows: Group 1: 30.3 % (4.6); Group 2: 37.5 % (7.1); Group 3: 35.0 % (5.9); Group 4: 47.2 % (7.6). Cell proliferation after 24 hours of incubation indicated a significantly higher proliferation rate for Group 4 compared to that for the other groups, and no significant difference could be observed between Groups 1, 2 and 3.

DISCUSSION:

The central finding of this investigation was that the fluoride modification of the anodized titanium improved its photo-induced hydrophilicity, resulting in a better initial cell response. After a short time of culturing, the mesenchymal cells showed a significant increase in cell attachment. The corresponding cell morphology was flat, with pseudopodial extentions. In addition, the proliferative activity was significantly accelerated after 24 hours.

The water contact angle analysis showed that the anodized surface became hydrophilic after UV irradiation. However, this level of hydrophilicity did not induce a more prominent cell response. On the other hand, the fluoride-treated anodized titanium after UV irradiation showed further improved hydrophilicity.

The SEM examination revealed a rather flattened structure after fluoride modification, a result corroborated by topographical measurements. However, neither the electron spectroscopic surface analysis for the chemical analysis nor the X-ray diffraction analysis showed any difference in the crystal structure before and after fluoride modification (data not shown). The overall mechanism through which the photo-induced hydrophilicity improved after fluoride modification remains unclear at this point.

Although the mechanism remains to be elucidated, the improved hydrophilicity did enhance the initial cell response. One key factor may be the surface energy intensified by the increased hydrophilicity. There are reports stating that hydrophilic surfaces cause an augmentation of the surface energy. Rupp, *et al*. have reported that a highly energized microstructured Ti surface acquired higher biological performance *in vitro*.[12] Buser, *et al*. have also reported that inorganic molecules, such as calcium and phosphate ions, and, equally importantly, organic molecules, such as proteins, lipoproteins and peptides, adsorb onto the hydroxylated/hydrated TiO_2 surface when exposed to the patient's blood, and they have speculated that this phenomenon involves electrostatic interactions.[14]

In addition, the remnant fluoride may have acted in synergy with the hydrophilicity to intensify the cell response. Numerous studies report that fluoride may act primarily on osteoprogenitor cells, undifferentiated osteoblasts and/or mesenchymal stem cells which synthesize an abundance of growth factors, rather than stimulating the proliferation of highly differentiated osteoblasts.[20-23] Furthermore, Ellingsen has stated that fluoride may be the key inductive factor in the transformation of undifferentiated precursor cells into osteoblasts.[24]

Judging comprehensively, the enhanced initial cell attachment and proliferation seen in the present study was speculated to be a result of numerous factors, as indicated above. Hence, it may be difficult to clarify the mechanism that caused this enhancement if we focus only on the hydrophilicity. However, the results of this *in vitro* study are promising, and the *in vivo* effects involved in the possible acceleration of osseointegration will be discussed in future studies.

CONCLUSIONS:

The fluoride chemical modification of the anodized TiO_2 disk surface significantly enhanced its photo-induced hydrophilicity. Although the full clarification of the mechanism involved is yet to be made, the modification clearly and significantly enhanced the initial cell attachment and subsequent proliferation activities.

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FIGURE LEGENDS:

Figure 1. The water contact angle of the NH_4F-HF_2-treated specimens, ranging from 0% to 0.2 %. The optimum concentration was 0.175 %.

Figure 2. SEM micrographs of the (a) ordinal anodized disk and the (b) NH_4F-HF_2-treated anodized disk. The corresponding surface roughness values are shown.

Figure 3. Morphological SEM observations of the cultured cells after 30 min of incubation. (a) Group 1; (b) Group 2; (c) Group 3; (d) Group 4.

Figure 4. Cell attachment and cell proliferation analyses. (a) Cell attachment after 30 minutes of incubation. (b) Cell proliferation evaluated based on the BrdU uptake at 24 hours (mean ± SD; *, $p < 0.01$).

Figure 1.

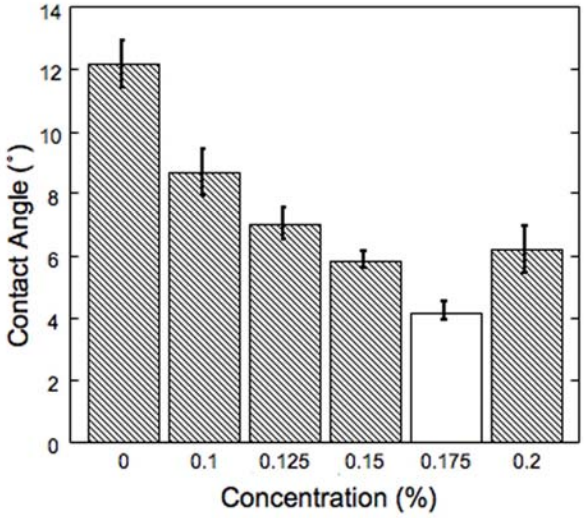

Figure 2.

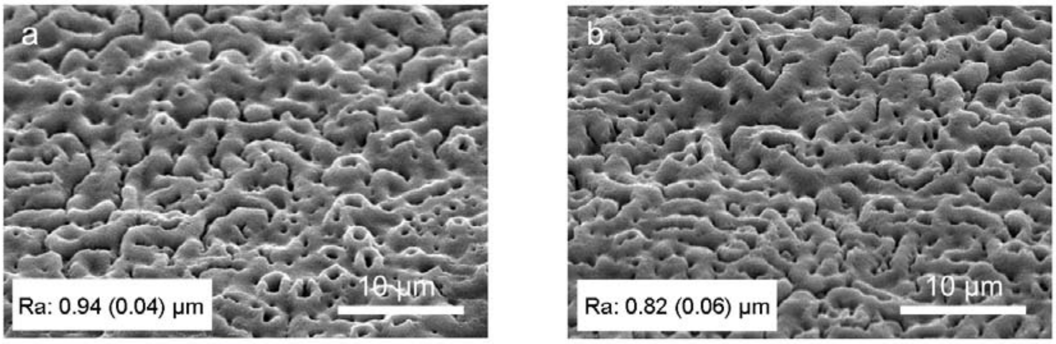

Figure 3.

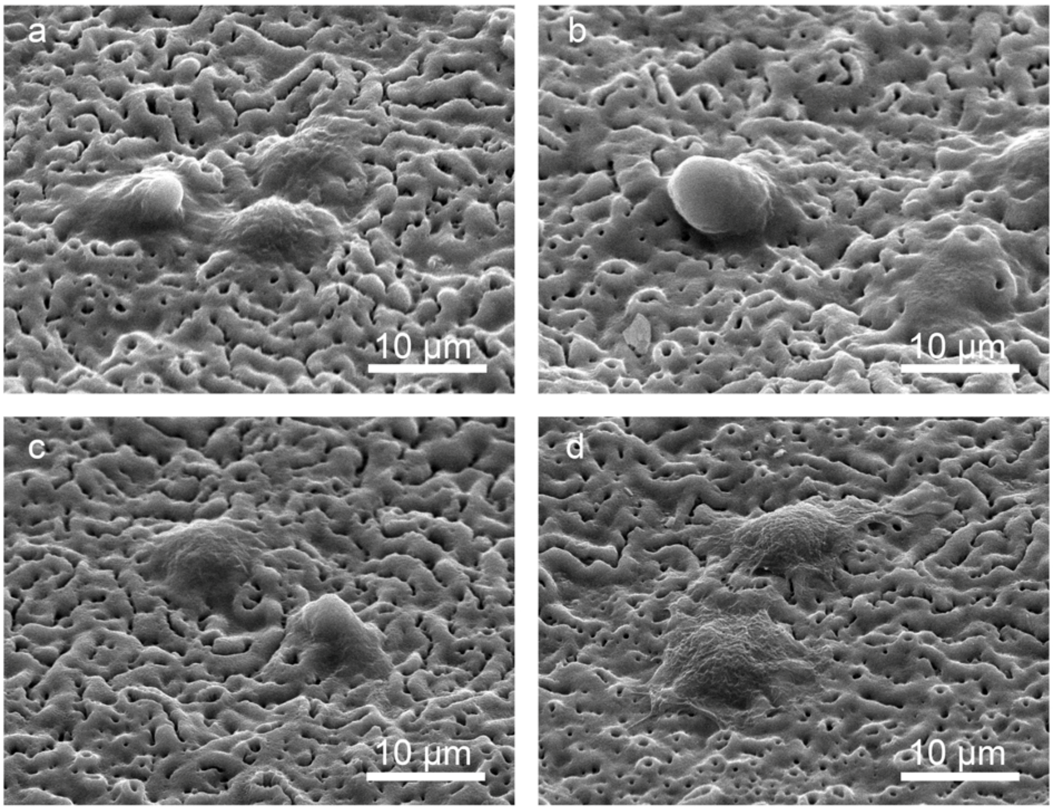

Figure 4.