Original

Improved Cell Adhesion and Osseointegration on Anodic Oxidation Modified Titanium
Implant Surface

Ying Li1, Yapeng You1, Baoe Li2, Yunjia Song1, Aobo Ma1, Bo Han1 and Changyi Li1

1 Stomatological Hospital, Tianjin Medical University, Tianjin, China
2 School of Materials Science and Engineering, Hebei University of Technology, Tianjin, China
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Abstract: Although titanium material is currently widely used in dental and orthopedic application, the bio-inertness of Ti impairs its further use. We previously reported the good bioactivity and biocompatibility of TiO2 nanotube layer fabricated by anodic oxidation technique using in vitro tests. In the present work, we further clarify the cell adhesion and osseointegration properties of anodized surface through both in vitro and in vivo tests. After anodic oxidation (AO), the structure of nanotubes was confirmed by scanning electron microscopy. The surface roughness and hydrophilic properties of the AO and pristine Ti surfaces were evaluated by atomic force microscopy and contact angle measurement test, respectively. It was found that the AO surface displayed moderately higher surface roughness and obviously increased wettability, compared to the original Ti surface. In vitro cellular activity tests demonstrated that osteoblast cells cultured on the AO surface exhibited a much well-spread cytoskeleton organization with more stretched actin filaments, compared to those cultured on the Ti surface. The adherent cell number was also higher on the AO surface than the pure Ti substrate. In addition, we explore the molecular basis of mechanism by analyzing gene expression levels of adhesion and osteogenesis-related genes in MC3T3-E1 cells cultured on different surfaces using quantitative real-time PCR. Increased mRNA levels of vinculin, collagen type 1, osteopontin and osteocalcin were found on the AO surface, compared with the control group. Furthermore, in vivo animal experiment using a rat model revealed that anodized implant surface promotes osseointegration and demonstrated higher bone bonding strength compared to the pure Ti substrate. Our study revealed the superior cell adhesion property, increased adherent and osteogenesis-related gene expressions and enhanced osseointegration by anodized surface, thus implying its enlarged application in future.

Key words: Anodic oxidation, Cell adhesion, Nanotube, Osseointegration, Titanium

Introduction

Titanium (Ti)-based alloys are frequently used in dental and orthopedic clinical applications because they have excellent mechanical properties, high corrosion resistance and good biocompatibility1,2. However, the bioinertness of pure Ti hinders its rapid osseointegration to surrounding tissue, delayed the implant loading, and finally lead to implant failure3. Therefore, design novel titanium implant materials to improve its cell responses in vitro and osseointegration ability in vivo is of great importance in clinic.

Recently, various efforts have been applied to modify the surface topography, hydrophilicity and biological properties of Ti implant surfaces4-9. Among the different strategies for surface modification, anodic oxidation has attracted much attention. Anodic oxidation (AO) is an easy, economical way to fabricate controllable nano-scale surface topography9. We have previously reported that the TiO2 surface created by anodic oxidation induced bone-like apatite layer precipitation in simulated body fluid soaking test and increased the gene expression of bone-specific marker osteoprotegerin compared to the control Ti surface9. Since cell adhesion serve as the first step when tissue contacts with biomaterial, it plays a crucial role in the subsequent cell responses such as cell proliferation, differentiation and final osseointegration9,10. Thus, the cell adhesion property of anodized surface still needs to be further clarified and the in vitro cell culture results requires to be confirmed by in vivo experiments.

Therefore, in the present work, the surface topography of TiO2 nanotube layer was characterized by SEM. The surface roughness and hydrophilicity properties of the AO surface were assessed by AFM and water contact angle measurement, respectively. The cell adhesion property of the AO and pristine Ti surfaces was evaluated using murine preosteoblast MC3T3-E1 cells. The adherent cell counting and immunofluorescence staining of actin were performed and observed by confocal laser scanning microscopy (CLSM). In addition, we explore the molecular basis of mechanism for the AO surface by analyzing gene expression levels of adhesion and osteogenesis-related genes including vinculin, collagen type 1 (COL), osteopontin (OPN) and osteocalcin (OCN) using quantitative real-time PCR. Moreover, an in vivo animal experiment was utilized to examine the effects of the TiO2 nanotube structured surface on bone binding strength and early osseointegration ability using a rat model.

Materials and Methods

Preparation of samples

Commercially available pure Ti plates (10 mm×10 mm×1 mm) and
titanium cylindrical implant rods (diameter of 2 mm, height of 2 mm) were used in this experiment for sample preparation. Samples were polished with abrasive paper #1000 SiC and then successively cleaned in acetone, ethanol and deionized water for 5 min, respectively. The graphite (40 mm×40 mm×5 mm) serve as cathodic electrode, while Ti plates were used as anode. Anodic oxidation procedure was carried out in the electrolyte of a 1M NaF solution under a constant voltage of 10V provided by a direct current voltage source (WYK-150, Annai Electronic Technology Co., Wuxi, Jiangsu, China) at room temperature for 1 h. The samples treated by anodic oxidation were denoted as AO surface, while for comparison, the as-polished Ti samples were used as the control.

**Surface characterization**

In this work, surface morphology was observed by scanning electron microscopy (SEM, Hitachi S-4800, Tokyo, Japan) using implant rods. The roughness was measured by atomic force microscopy (AFM, 5500, Agilent Technologies, Santa Clara, CA, USA). The wettability was assessed through measuring the contact angle of deionized water at room temperature. Data are expressed as mean ± standard deviation (SD), n = 3.

**Cell Culture**

The MC3T3-E1 mouse derived preosteoblast-like cells (CRL-2593, ATCC, Rockville, MD, USA) were cultured in α-MEM medium (Gibco BRL, Grand Island, NY, USA) supplied with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 3% penicillin/streptomycin. Cultures were maintained in an incubator with a fully humidified atmosphere of 5% CO₂ at 37°C.

**Cell Adhesion**

MC3T3-E1 cells were seeded at a density of 2×10⁴ cells/ml on both anodized and Ti surfaces for 4 hours. After that, the non-adherent cells were washed to remove, then fixed and stained with 4', 6'-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA). Five fields of view were pictured by an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan) randomly. Three separate samples were calculated for both groups, with cell numbers on each image calculated using the Image-pro Plus J software (ver. 5.0, Media Cybernetics, Silver Spring, MD, USA).

**Immunofluorescent staining of cytoskeleton actin**

In order to evaluate the cytoskeleton organization on the anodized surface, cells (2×10⁵ cells/ml) were cultured for 4 hours before observation. At designated time point, cells were first rinsed with PBS for three times, then fixed with 4% paraformaldehyde for 10 min. Afterwards, samples were further permeabilized by 0.1% Triton X-100 (Sig-
ma-Aldrich Inc., St. Louis, MO, USA) at) for 5 min. After blocking with BlockAid (Thermo Fisher Scientific, Waltham, MA, USA), samples were incubated with Rhodamine Phalloidin and labeled by DAPI (Thermo Fisher Scientific, Waltham, MA, USA) before mounting on glass slides for observation. Prepared samples were photographed by a confocal laser scanning microscopy (CLSM, TCS SP5, Leica Microsystems, Wetzlar, Germany).

**RNA extraction and quantitative real-time PCR**

The MC3T3-E1 cells were cultured with AO and Ti substrates for 1, 4 and 7 days with a cell concentration of \(2 \times 10^5\) cells/ml. At each predetermined time point, the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was employed to isolate total RNA from the lysate of cell culture. Then the extracted total RNA was reversely transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Highly purified primers for COL, OPN, OCN, vinculin and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Sangon Biotech Company (Shanghai, China). The sequences of the primer sets of interested genes, including gene bank number and the length of the amplicons, were summarized in Table 1.

To compare the gene expression amounts between different groups, real-time PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, 4.0 µl cDNA template, 0.4 µl forward and reverse primer, 10 µl Real Master Mix SYBR green I (NEWBIO industry, Tianjin, China) were used in a final reaction volume of 20 µl. The thermal profile for all reactions was set as follows: 30 s at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C. Data were collected at 60°C in each cycle, with the threshold cycle (Ct) value calculated by the instrument software. The mRNA value of interested genes was normalized to that of GAPDH.

**Histological Analysis and Push-out Test.**

The in vivo animal experiment was approved by Animal Ethical and Welfare Committee (AEWC) of Tianjin Medical University (No. TMUaMEC 2016006). Sprague Dawley rats (440–470 g, \(n = 3\)) were randomized into Ti and anodized groups, with rods separately implanted into the rat femora. After 4 weeks of implantation, rats were sacrificed and the femora with cylindrical implants were harvested.

The harvested sample blocks were treated with EDTA (Sangon Biotech Company, Shanghai, China) for decalcification. Then, the implant rods were carefully removed from the femora. After that the samples were dehydrated and then embedded in paraffin for section preparation. The prepared sections were stained with Masson’s trichrome (Beijing Solarbio Science & Technology Co., Beijing, China). After that, sections were observed using an optical microscope with a digital camera (Ni-E, Nikon, Tokyo, Japan). Push out test was used to evaluate the bonding strength at the tissue/implant interface using an electromechanical testing machine (5544, Instron Ltd., Canton, MA, USA). The values were measured by determining the peak of a load-displacement curve.

**Statistical analysis**

Data presented in this study were analyzed by SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by the Student-Newman-Keuls post hoc test was employed to determining the statistical significance of the difference between samples. A \(p\)-value below 0.05 was considered to be statistically significant.

**Results**

**Surface characterization**

Fig. 1 showed the surface topographies of the anodized Ti (Fig. 1D) and polished Ti (Fig. 1A) surfaces. The as-polished Ti substrate displayed a relatively smooth surface, with only several parallel oriented scratch marks caused by the polishing steps. While on the anodized Ti surface, a layer of uniform and homogeneous nanotube arrays was observed, with inner diameter of the nanotubes about 70 nm, and the wall thickness of the nanotubes around 5 nm. Fig. 1 also revealed the surface roughness of the polished Ti (Fig. 1B) and anodized Ti (Fig. 1E) surfaces. It can be seen from the images that the anodized Ti surface showed higher surface roughness due to the formation of nanotubes, as expected. However, the difference of surface roughness was relatively little as revealed by the measurement of root-mean-square roughness of the surfaces. The root-mean-square roughness of the polished Ti was 40 ± 5 nm, whereas that of the anodized Ti substrate moderately increased to 52 ± 8 nm. Moreover, the water contact angle values of the samples was 90 ± 1° for the polished Ti (Fig. 1C), and 41 ± 3° for the anodized Ti (Fig. 1F). These results suggest the moderately increased surface roughness and substantially improved hydrophilicity produced by the nanotube structure.

**Cell adhesion**

Fig. 2 showed the adhesion properties of osteoblast cells on different surfaces. Images in Fig. 2A displayed that increased cell number was observed on the anodized Ti surface, compared to the smooth Ti surface. Fig. 2B further revealed that the adherent cell number per field of view on the anodized Ti surface is about 1.7-fold of that on the Ti surface, with statistical significances (\(p < 0.05\)). These results suggest that the AO surface facilitates initial osteoblast cell adhesion, compared to the
polished Ti substrate. The enhanced cell adhesion ability of the AO surface may be explained by the nano-scale surface topography and improved wettability rendered by the TiO_2 nanotube structure.

The cytoskeleton staining of actin further displayed the cytoskeletal organization of osteoblast cells cultured on different surfaces. Fig. 3A
demonstrated that after 4 hours of incubation, osteoblast cells grown on the as-polished Ti surface revealed less actin filament formation; while on the AO surface, cells exhibited much more stretched actin filaments with random orientation, indicating good cell communication than the control (Fig. 3A). Moreover, Fig. 3B characterized the osteoblast adhesion behaviors on different surfaces quantitatively by image analysis. As displayed in Fig. 3B, the overall cellular area to nucleus area (C/N ratio) on the AO surface was about 1.5-fold of that on the polished Ti substrate, with statistical significances ($p < 0.05$).

**Quantitative real-time PCR analysis**

In order to explore the molecular basis of mechanism, we also performed the real-time PCR analysis. As shown in Fig. 4, the gene expressions of $COL$, $OPN$, $OCN$ and $vinculin$ in cells exhibited a continuous increase for both AO and Ti surfaces over the 7-day incubation period. At each predetermined time point, the gene expression levels of $COL$, $OPN$ and $vinculin$ in cells significantly increased on the AO surface, compared to the pure Ti surface ($p < 0.05$). The gene expression level of $OCN$ in cells on the AO surface was about 1.5-fold higher than that on the as-polished Ti surface at the first day of culture, with no statistical significance. After 4 days of incubation, the mRNA level of $OCN$ on the AO surface augmented gradually, and achieved a remarkable increase after 7 days of inoculation, compared to the Ti surface. This phenomenon may be explained by the fact that $OCN$ is the late-phase marker of cell proliferation, thus the gene expression of $OCN$ was not substantially induced by the modified surface until 7 days after incubation. These findings indicated that the adhesion and osteogenesis-related gene expressions were all enhanced in osteoblast cells on the AO surface, compared to the control Ti group.

**Animal experiment**

The images of decalcification samples were evaluated by Masson’s trichrome staining. Fig. 5 displayed that at postoperative 4 weeks, deposition of newly formed osteoid which stained blue color was obviously observed on the AO implant surface, only scattered collagen fibrous tissue staining red remaining. On the other hand, the control Ti group showed less osteoid formation, with more collagen fibrous tissue layer remaining at the material-bone interface 4 weeks after the implantation. Additionally, the push out test further revealed that the bonding strength of anodized Ti surface was 56 ± 6 N, compared to that of pure Ti (21 ± 8 N, $p < 0.05$).

**Discussion**

In order to facilitate the initial osteoblast cell adhesion and accelerate osseointegration of implant materials, various strategies have been employed for surface modification of Ti-based implants$^{15}$). In biomedical field, anodic oxidation method was employed to produce TiO$_2$ nanotube coatings on metallic implants$^{7,22}$. Although several researchers reported the work of anodic oxidation induced nanopatterns on Ti surface, however their research objectives were either the improvement of photocatalytic effect, or the development of drug delivery system$^{13,14}$. The direct influence of the nanotube-structured surface induced by anodic oxidation on biological properties, especially its effect on cell adhesion of Ti based implants is not well described. Therefore, in the current study, we design to evaluate the cell adhesion ability and osseointegration property of the anodized titanium surface by *in vitro* and *in vivo* experiments.

**Surface characterization**

TiO$_2$ nanotube structure was successfully fabricated on the implant rods by anodic oxidation, as revealed by the SEM images. Moreover, the AFM demonstrated moderately increased roughness of the anodized substrate, compared to control Ti surface. In addition, contact angle test exhibited substantially increased hydrophilicity of the AO substrate, compared to the original Ti surface.

It is generally accepted that surface roughness has a beneficial effect on implant osseointegration$^{15,20}$. Additionally, increased surface free energy and hydrophilicity were also proved to facilitate cell response and osseointegration *in vivo*$^{17,18}$. Since biomaterial surface characteristics including topography, roughness and wettability all play an important role in regulating osteoblast cells behaviors$^{26,19,20}$, on the AO surface, the characteristic nanotube morphology, increased surface roughness and improved wettability all have the potential to promote osteoblast behaviors and enhance osseointegration.

**Cell adhesion**

When cells contact with the implant material, the first step of cell response at the tissue-implant interface is initial cell adhesion, which

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**Figure 5.** (A) Masson’s trichrome staining images at the bone-implant interfaces of Ti (a and b) and AO (c and d) surfaces after 4-week implantation. Figure b and d depict zoomed areas of black box in a and c, respectively. Bars indicate 100 µm (a and c) and 50 µm (b and d). (B) Quantitative analysis of the push-out tests results. Data are expressed as mean ± SD (n = 5). * $p < 0.05$. 

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**Table 1.** Summary of the experimental results.

| Surface | Adhesion | Osseointegration |
|---------|----------|------------------|
| Ti      | Good     | Good             |
| AO      | Excellent| Excellent        |

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1. Ying Li et al.: Cell Adhesion and Osseointegration on Anodized Titanium Surface
2. In order to facilitate the initial osteoblast cell adhesion and accelerate osseointegration of implant materials, various strategies have been employed...
plays an important role in subsequent cellular cascade including cell survival, migration, recruitment, proliferation and differentiation. Cell adhesion also serves as a vital prerequisite for successful osseointegration, which is regarded as the key factor for achieving immediate implants loading and long-term implant success.

The findings in the present study demonstrated that cells seeded on the anodized Ti surface presented higher adhesion cell number, compared to control Ti surface. Moreover, cells cultured on the anodized surface exhibited much well-organized cytoskeleton which was revealed by the immunofluorescence staining of actin. It is well-accepted that cell migration and adhesion is highly dependent on rearrangements of the actin cytoskeleton. Our results indicated that the anodized Ti substrate is favorable than the polished Ti surface to cell attachment and adhesion, which was in line with previous reports. Our results indicate that anodized Ti surface is much favorable for cell adhesion, probably due to the nanotube structured topography and increased wettability. The improved cell attachment property implies its better bone binding potential, which can promote rapid and early osseointegration in vivo. The possible mechanism of cell adhesion to the anodized Ti surface was based on the hypothesis that positively charged proteins could be absorbed to implant surfaces, which may serve as mediators for the cell attachment of negatively charged osteoblast cells. The presence of numerous filopodia and longer actin filament extensions observed on the anodized surface implies much stronger cell attachment ability than the pristine Ti surface. The improved cell adhesion on the anodized Ti surface may attribute to the combined effects of nano-scale surface morphology, increased hydrophilicity and enhanced protein absorption produced by anodic oxidation.

Gene expression analysis

Vinculin serve as an important marker to identify focal adhesion (FA) and the binding of vinculin to actin is critical to cell–matrix adhesion. Vinculin can stabilize integrin proteins by providing connections to the actin cytoskeleton, promotes integrin clustering and stabilizes focal adhesions. Thus, vinculin is proposed to play a key role in regulating cell adhesion. Our study revealed a continuously increased gene expression of vinculin on the AO surface during the 7-day incubation period, compared with the control group. The finding is consistent with a similar study which indicated up-regulated gene expression of vinculin on Ti surface with nanotopography.

In addition to adhesion-related genes, the expressions of osteogenesis-related genes were also evaluated on different surfaces. Collagen I, a widely-accepted marker of early osteoblast cell differentiation, serves as the main organic protein during the early stage of osteoid formation. OPN, a marker of middle-stage osteogenic differentiation, is associated with the initiation of ECM mineralization. OCN, a late-stage osteoblast differentiation marker, indicates the beginning of mineralization. Both OCN and OPN are major non-collagenous proteins which involve in bone matrix deposition. They could control bone mass and mineral size during bone formation. Consistent with previous studies, our study revealed a sustained increase of the three vital osteogenesis-related genes (COL, OPN and OCN) on AO surface. Taken the abovementioned findings together, the up-regulated gene expression of the adhesion and osteogenesis-related genes may lead to enhanced cell adhesion in vitro and improved osseointegration in vivo for the AO substrate, compared to the control Ti group.

Animal experiment

In order to confirm the in vitro experiment results, we further performed in vivo experiment using a rat model. Our findings indicated that the anodized Ti group accelerated the new bone formation speed around the implant locally and enhanced implant stability, so as to increased osteogenesis and promote osseointegration. It is well established that surface topography, roughness and wettability are identified as crucial factors which affects osseointegration in vivo. It is also widely accepted that cell adhesion to implant substrates is of fundamental importance in a number of cellular biological processes, new bone formation and osseointegration in orthopedic and dental implants. Thus in the present work, the well-controlled homogeneous nanotube structure, increased wettability and roughness of the anodized surface may attribute to increased cell adhesion property, so as to improve osseointegration in vivo. These in vivo results suggested earlier osseointegration and higher bone binding ability for the anodized Ti group.

In this study, we fabricated TiO₂ nanotubes layer on pristine Ti surface. Our results indicated that the newly fabricated hydrophilic AO surface significantly enhanced initial osteoblast cell adhesion, with improved cell spreading. Moreover, we found that the anodized Ti surface induces up-regulated gene expressions of adhesion (vinculin) and osteogenesis (COL, OPN and OCN) marker genes than the polished Ti surface. In addition, in vivo histological analysis and push out test results using a rat model further confirmed the excellent osteogenic ability of the AO surface at the implant/bone interface than the pristine Ti substrate. In conclusion, the TiO₂ nanotube topography acquired by anodic oxidation could accelerate initial osteoblast cell adhesion and achieve early osseointegration, which showed an intriguing method for obtaining improved cell response and osteogenesis.

Acknowledgments

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Conflict of Interest

The authors have declared that no COI exists.

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