Cell Differentiation on Nanoscale Features of a Titanium Surface: Effects of Deposition Time in NaOH Solution

Tomoko Fujino¹, Yoichiro Taguchi², Satoshi Komasa³, Tohru Sekino⁴ and Masahiro Tanaka¹

¹ Department of Fixed Prosthodontics and Occlusion, Osaka Dental University
² Department of Periodontology, Osaka Dental University
³ Department of Removable Prosthodontics and Occlusion, Osaka Dental University
⁴ Institute of Multidisciplinary Research for Advanced Materials(IMRAM), Tohoku University

Abstract: The present study aimed to investigate cellular behavior on nanoscale features of a titanium surface by controlling the deposition time in NaOH. These effects were then evaluated for osteogenic differentiation of rat bone marrow cells to potentially increase the success rate of titanium implants. Titanium disks were left untreated or soaked in 10 M NaOH for 5 min, and 1h, 3h, 9h and 24 h. Scanning electron microscopy was used to evaluate the nanoscale features. Rat bone marrow cells were seeded on the specimens in osteogenic differentiation medium. Alkaline phosphatase activity, osteocalcin production, and mineralization were then analyzed. Statistical significance was analyzed using one-way ANOVA followed by the Tukey test.

Nanofeatures were detected at 1 h after NaOH treatment and were well established at 9 h. Alkaline phosphatase activities of specimens soaked for 1 h or 3 h were significantly different from specimens soaked for 9 h or 24 h after 14 days of differentiation. Osteocalcin production and calcium deposition between untreated specimens and specimens soaked for 5 min, as well as between specimens soaked for 9 h and 24 h, were significantly different after 21 days. It was found that the nanoscale modification of a titanium implant surface by NaOH treatment affects osteoblastic differentiation of bone marrow cells and enhances mineralization. This study found that modification of titanium surfaces with NaOH could be an effective method of improving their biological properties. Further developments in nanotechnology may help improve osseointegration of titanium implants.

Key Words: Nanoscale features, Osseointegration, Mesenchymal stem cell

Introduction
There has been a concerted effort among materials scientists and clinicians worldwide to improve the performance of dental implants with the aim of accelerating and maintaining their integration into hard and soft tissues and/or extending their range of application. The surface characteristics of the implant material affect the rate and extent of osseointegration. Vandrovcova et al. have recently reviewed the growing evidence demonstrating that surface-modified materials are highly effective for adhesion, growth, and osteogenic differentiation of cells.

Osteogenic cells are known as anchorage-dependent cells. According to Osteogenic differentiation of bone marrow cells and achieve better osseointegration around titanium surface. A previous study has showed that nanostructural modifications can accelerate tissue engineering for hard tissue through increased initial cell attachment on the surface. In terms of the initial cellular response on rough surfaces, there has been a report indicating that surface roughness influences the differentiation of human osteoblastic MG63 cells through α5 integrin interactions. Another study found that the degree of osseointegration can be changed by controlling the size of nanostructure on a titanium surface.

Osseointegration of titanium dental implants depends on surface characteristics, such as the surface morphology and chemical composition. Implant surface modification can enhance and improve the surrounding hard tissue. Therefore, understanding the nanoscale topographical effect on the differentiation of bone can help understand the basic principles of stem cell behavior, and can be designed to speed up the osseointegration. Therefore, the relationship between early and late cellular responses is very important for current research. It is believed with certainly that osteogenic differentiation can be assessed by alkaline phosphatase (ALP) activity, calcium deposition, and osteocalcin (OCN) production. Many studies have
shown that ALP has an activating effect on the surface modification of materials. There is also evidence that surface modification of nanostructures can increase calcium deposition. Recent research has shown that improving the surfaces of nanostructures can increase the production of OCN.

The structures used in this study are nanostructures similar to the TiO$_2$ nanotubes created by titanium deposition using the process of TiO$_2$ sputtering and are named titanium nanosheets (TNS). Recently, it was shown that nanotubes and TNS structures can be obtained on a titanium metal surface using a treatment with a 10 M NaOH aqueous solution at 30 °C, which we therefore employed this method herein to create TNS structures on the modified disks. Recent research has shown that treatment with a NaOH aqueous solution produces a rough, nanoscale surface, and SEM images of our modified disks demonstrated that the TNS modified surface had good roughness, without any cracks. A previous study reported that TNS produced via chemical processing promoted the osteogenic differentiation of rat bone marrow cells. The surface properties and structures of materials play important roles in the adsorption of proteins, which might influence cell behavior. However, the numerator structure of TNS is unclear and the manner in which this structure influences bone differentiation must be evaluated for application as an advanced implant material.

The aim of the present study was to investigate cellular behavior on nanoscale features by controlling the deposition time of NaOH on the titanium surface, and evaluate the ability of these modified surfaces to affect osteogenic differentiation of RBM cells and potentially increase the success rate of titanium implants. The first null hypothesis was that there would be no difference in cellular behavior between untreated and NaOH treated specimens. The second null hypothesis was that there would be no difference in cellular behavior on titanium surfaces as a result of different deposition times for NaOH treatment.

**Materials and Methods**

**Specimen preparation**

Titanium disks (15 mm diameter) were punched from sheets of 1 mm thick grade 2 unalloyed titanium (Daido Steel, Osaka, Japan). These disks were immersed in 10 M NaOH (aq) and placed in an oil bath maintained at 30 °C for 5 min, and 1 h, 3 h, 9 h and 24 h. Unprocessed titanium disks were used as the control. The solution in each flask was replaced with distilled water (200 ml), and this procedure was repeated until the solution reached a conductivity of 5 μS/cm. Specimens were then dried at room temperature. The specimen surface topography was qualitatively evaluated under a scanning electron microscope (SEM, S-4000; Shimazu, Kyoto, Japan) and a scanning probe microscope (SPM, SPM-9600; Shimadzu, Kyoto, Japan). Qualitative and quantitative measurements of specimens were performed by SPM using phase mode. The scanner range was 2 μm in X and Y directions but changed in the Z direction.

**Cell culture**

RBM cells were isolated from the femurs of 7-week-old Sprague-Dawley rats. This study was performed under the Guidelines for Animal Experimentation of Osaka Dental University (Approval No. 11-03038).

At confluence, RBM cells were seeded at a density of 4×10$^4$ cells/cm$^2$ onto each titanium disk in 24-well tissue culture plates (Falcon). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO$_2$.

**Cell differentiation**

The cells on each titanium disk were incubated until they reached confluence. The medium was then removed and replaced with differentiation medium containing 10% fetal bovine serum and osteogenic supplements (10 mM β-glycerophosphate (Wako, Tokyo, Japan), 80 mg/mL ascorbic acid (Nacalai Tesque Inc, Kyoto, Japan), and 10 mM dexamethasone (Nacalai Tesque Inc). Differentiation medium was replaced every second day.

**Alkaline phosphatase (ALP) activity**

After 14 days of culture, cells were washed with phosphate-buffered saline and lysed with 200 μL of 0.2% Triton X-100 (Sigma, St. Louis, MO, USA). The lysate was transferred to a microcentrifuge tube containing a 5 mm hardened steel ball. Tubes were agitated on a shaker (Mixer Mill Type MM 301; Retsh Gmbh & Co., Haan, Germany) at 29 Hz for 20 s to homogenize the specimen. ALP activity was measured using an Alkaline Phosphatase Luminometric ELISA Kit (Sigma) according to the manufacturer's protocol. The reaction was terminated with 3 M NaOH at a final concentration of 0.5 M NaOH, and p-nitrophenol production was measured by the absorbance at 405 nm using a 96-well microplate reader (SpectraMax M5; Molecular Device Inc., Sunnyvale, Calif, USA). DNA content was measured using a PicoGreen dsDNA Assay Kit (Invitrogen) according to the manufacturer's protocol. To normalize ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate.

**Mineralization**

Calcium deposited in the extracellular matrix was measured after dissolution with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako Pure Chemical Industrial Ltd). After 21 days of culture, 1 mL Calcium E-Test reagent and 2 mL kit buffer were added to 50 μL of collected medium, and the absorbance of the reaction products was measured at 610 nm using a 96-well microplate reader (SpectraMax M5). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve.
Osteocalcin (OCN) measurement

The commercially available ELISA kit (Rat Osteocalcin ELISA Kit DS; DS Pharma Biomedical Co., Ltd., Osaka, Japan) used in this study is specific for rat OCN, and measures its levels directly in cell culture supernatant. The OCN levels in cell culture supernatants after 21 days of culture were measured according to the manufacturer’s instructions.

Statistical analysis

All experiments were performed in triplicate. Data are described as the mean ± standard deviation. In all analyses, statistical significance was analyzed using one-way ANOVA followed by the Tukey test.

Results

Specimen preparation

Fig. 1 shows the SEM images and three-dimensional images of an unprocessed specimen and specimens modified by NaOH treatment for 5 min, 1h, 3h, 9h, and 24 h. A fine network structure at a nanometer scale formed on the surface after NaOH treatment, which began to form when treated for 1 h and was well established at 9 h. Qualitative and quantitative measurements of specimens were made by SPM using phase mode. a-1: control groups; a-2: soaked for 5 min; a-3: soaked for 1 h; a-4: soaked for 3 h; a-5: soaked for 9 h; a-6: soaked for 24 h; (Group a shows SEM images) b-1: control groups; b-2: soaked for 5 min; b-3: soaked for 1 h; b-4: soaked for 3 h; b-5: soaked for 9 h; b-6: soaked for 24 h; (Group b shows SPM images).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5min</th>
<th>1h</th>
<th>3h</th>
<th>9h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra</td>
<td>4.784</td>
<td>16.542</td>
<td>45.806</td>
<td>31.823</td>
<td>15.868</td>
<td>13.026</td>
</tr>
<tr>
<td>Rz</td>
<td>45.604</td>
<td>107.56</td>
<td>280.012</td>
<td>157.77</td>
<td>137.773</td>
<td></td>
</tr>
</tbody>
</table>

Ra: average roughness; Rz: maximum height

Table 1. Ra and Rz

Figure 1. SEM and SPM images of the surface topography of the titanium disks in the control and test groups. A fine network structure at the nanoscale formed on the surface of NaOH-treated titanium, which was well established at 9 h. Qualitative and quantitative measurements of specimens were made by SPM using phase mode. a-1: control groups; a-2: soaked for 5 min; a-3: soaked for 1 h; a-4: soaked for 3 h; a-5: soaked for 9 h; a-6: soaked for 24 h; (Group a shows SEM images) b-1: control groups; b-2: soaked for 5 min; b-3: soaked for 1 h; b-4: soaked for 3 h; b-5: soaked for 9 h; b-6: soaked for 24 h; (Group b shows SPM images).

ALP activity

Cell differentiation was assessed by measuring the activity of the osteoblastic differentiation marker ALP in all groups at 14 days. ALP specific activity increased in cells cultured on all surfaces in a time-dependent manner (Fig. 2). The differences among unprocessed specimens and specimens soaked for 5 min or 1 h were not statistically significant. ALP activities of specimens soaked for 1 h or 3 h, as well as specimens soaked for 3 h or 9 h were different. However, the difference between specimens soaked for 9 h or 24 h was not statistically significant.

Mineralization

Fig. 3 shows the calcium deposition of cells cultured on the various specimens for 21 days. Calcium deposition of unprocessed specimens and specimens soaked for 5 min were significantly
disks soaked in 10 M NaOH for 9 h or 24 h promotes the differentiation and activation of RBM cells, which augments calcium deposition. These results also indicate that modification of the implant surface at the nanometer scale for 9 h or 24 h leads to the regulation of the osteogenic differentiation of bone marrow cells and enhances the mineralization. Therefore, nanostructure modification can be easily achieved and we believe that this nanostructure could effectively augment the biointegration of titanium implant materials by accelerating the bone tissue response.

The present study, which used TNS structures created by chemical treatment for various reaction times, demonstrated that different surface roughness induced a biological response in RBM cells among the various specimens. The results suggest that different reaction times affect the nanoscale features. In SEM images, nanostructures began to form when treated with the alkaline solution for 1 h and 3 h. The nanonetwork structure was more obvious when treated for longer time periods and formed an advanced nanonetwork structure at 9 h. This nanonetwork structure is similar to the hierarchical structure in Lingzhou’s study. Hierarchical nano-textured titanium surface topographies with titania nanostructure was produced by simple etching method followed by anodization to mimic the hierarchical structure of bone tissues. Natural tissues are hierarchical structures assembled into a complex structure and the implant surface needs to be modified in the same way. Therefore, we believe that this nanostructure is the key to improving the clinical efficacy of titanium implants.

**Discussion**

Both null hypotheses of this study were rejected. Herein, it was found that the expression of differentiation markers such as ALP and OCN (at later time points) was elevated in specimens containing TNS-modified titanium disks soaked in 10 M NaOH for 9 h or 24 h at 30 °C. It was also found that calcium deposition in the extracellular matrix of RBM cells was increased in these specimens. These results suggest that the TNS structure of titanium disks soaked in 10 M NaOH for 9 h or 24 h promotes the differentiation and activation of RBM cells, which augments calcium deposition. These results also indicate that modification of the implant surface at the nanometer scale for 9 h or 24 h leads to the regulation of the osteogenic differentiation of bone marrow cells and enhances the mineralization. Therefore, nanostructure modification can be easily achieved and we believe that this nanostructure could effectively augment the biointegration of titanium implant materials by accelerating the bone tissue response.

The present study, which used TNS structures created by chemical treatment for various reaction times, demonstrated that different surface roughness induced a biological response in RBM cells among the various specimens. The results suggest that different reaction times affect the nanoscale features. In SEM images, nanostructures began to form when treated with the alkaline solution for 1 h and 3 h. The nanonetwork structure was more obvious when treated for longer time periods and formed an advanced nanonetwork structure at 9 h. This nanonetwork structure is similar to the hierarchical structure in Lingzhou’s study. Hierarchical nano-textured titanium surface topographies with titania nanostructure was produced by simple etching method followed by anodization to mimic the hierarchical structure of bone tissues. Natural tissues are hierarchical structures assembled into a complex structure and the implant surface needs to be modified in the same way. Therefore, we believe that this nanostructure is the key to improving the clinical efficacy of titanium implants.
Tomoko Fujino et al.: Cell Differentiation on Nano-modified Titanium Surface by Controlling Deposition Time in NaOH solution

in a highly organized way composed of nanoscale building blocks. Hierarchical structure composed of nano-components may provide a more suitable surface topography for bone marrow cell functions as it can better mimic the structure of natural tissues. The chemical and nanostructure modification induced by the alkaline treatment were significantly different, and that of specimens soaked for 5 min or 1 h, as well as specimens soaked for 3 h or 9 h was not significantly different. The difference between specimens soaked for 9 h or 24 h was statistically significant. However, the difference between specimens soaked for 9 h or 24 h was not statistically significant.

Figure 4. OCN production after 21 days of culture in test and control groups as measured by an ELISA. OCN production of the control specimen or specimens soaked for 5 min in a NaOH solution were significantly different, and that of specimens soaked for 5 min or 1 h, as well as specimens soaked for 3 h or 9 h was not significantly different. The difference between specimens soaked for 9 h or 24 h was statistically significant. However, the difference between specimens soaked for 9 h or 24 h was not statistically significant.

Titanium implants have become an essential treatment modality for reconstructive surgeries in orthopedic and dental fields. However, there is always a need to reduce patient morbidity and treatment complications, and maximize outcome predictability and treatment indications. Therefore, considerable efforts have been made to develop new technologies to modify the surface of titanium to assist its biointegration with bone. The modification method used here by soaking titanium in NaOH for 9 h is more useful and easily accomplished because the incubation is performed at only 30°C and requires no template.

In conclusion, by controlling the deposition time in a NaOH solution, the titanium surface shows different nano-network and nanoroughness structures. The investigation of different implant surface nanostructures demonstrated that modifying the implant surface at the nanometer scale by soaking in NaOH for 9 h leads to the regulation of osteoblastic differentiation of bone marrow cells and enhances mineralization. We believe that further development of advanced implant materials using nanotechnology will improve osseointegration.

Acknowledgments
The authors thank Prof. Shoji Takeda and Dr. Hisataka Nishida.
for their encouragement and helpful suggestions. We are also grateful to the members of the Department of Fixed Prosthodontics and Occlusion, Department of Removable Prosthodontics and Occlusion, Department of Periodontology, and the Department of Biomaterials for their kind advice and assistance. Supported by The Science Research Promotion Fund from the promotion and mutual aid corporation for private schools of Japan and Grant-in-Aid for Scientific Research (No. 23107506) on the Innovative Areas: Fusion Materials (Area no. 2206) from MEXT and a Grant-in-Aid for Scientific Research (24792345) from the Japan Society for the Promotion of Science and a Research Promotion Grant (13-05) and Oral Implant Research Grant (12-3, 13-04) from Osaka Dental University.

References


10. Webster TJ and Ejiofor JU. Increased osteoblast adhesion on nanophase metals: Ti, Ti6Al4V, and CoCrMo. Biomaterials 25: 4731-4739, 2004


