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Original

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

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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 and probe microscopy were 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[1]. The surface characteristics of the implant material affect the rate and extent of osseointegration[2]. Vandrovcova et al.[3] 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. Accordingly, it is necessary to enhance early bone differentiation of mesencymal cells and achieve better osseointegration around titanium surface[4-9]. A previous study has showed that nanostructural modifications can accelerate tissue engineering for hard tissue through increased initial cell attachment on the surface[10]. 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[11]. Another study found that the degree of osseointegration can be changed by controlling the size of nanostructure on a titanium surface[12].

Osseointegration of titanium dental implants depends on surface characteristics, such as the surface morphology and chemical composition[13,14]. 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

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shown that ALP has an activating effect on the surface modification of materials[15-26]. There is also evidence that surface modification of nanostructures can increase calcium deposition[15, 16, 18, 20, 26]. Recent research has showed that improving the surfaces of nanostructures can increase the production of OCN[18, 27].

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[28] 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[29], 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[30], and SEM images of our modified disks demonstrated that the TNS modified surface had good roughness, without any cracks. A previous study[31] 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 nM 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 Industrials 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.

Table 1. Ra and Rz

	Control	5min	1h	3h	9h	24h
Ra	4.784	16.542	45.806	31.823	15.868	13.026
Rz	45.604	107.56	447.305	280.012	157.77	137.773

Ra: average roughness; Rz: maximum height

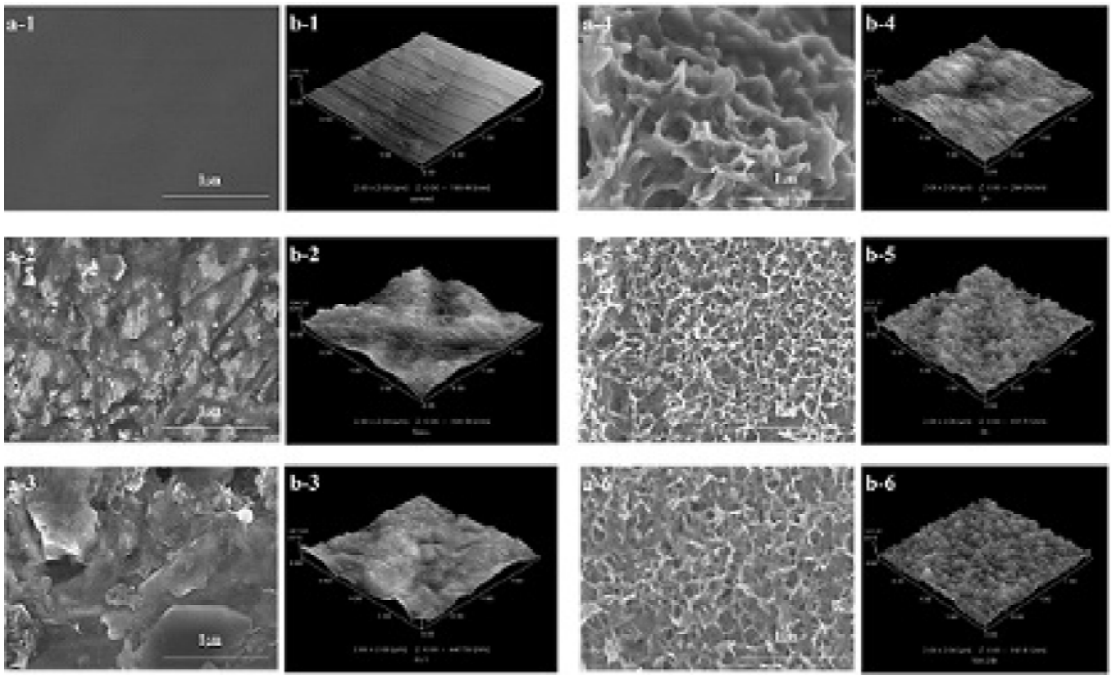


Figure 1. SEM and SPM images of the surface topography of the titanium disks in the control and test groups. A fine network structure at the nanoscale formed on the surface of NaOH-treated titanium, which was well established at 9 h. Qualitative and quantitative measurements of specimens were made by 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).

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

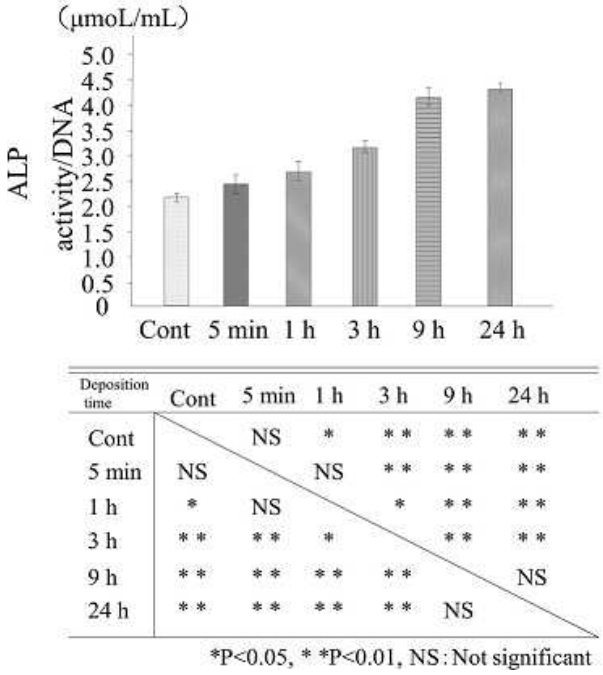
Fig. 1 shows the SEM images and three- dimensional images of an unprocessed specimen and specimens modified by NaOH treatment for 5 min, and 1h, 3h, 9h, and 24 h. A fine network structure at a nanometer scale formed on the surface after NaOH treatment, which began to form when treated for 1 h and was well established at 9 h. Average roughness (Ra) and maximum height (Rz) were obtained from SPM analysis and are described in Table 1.

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

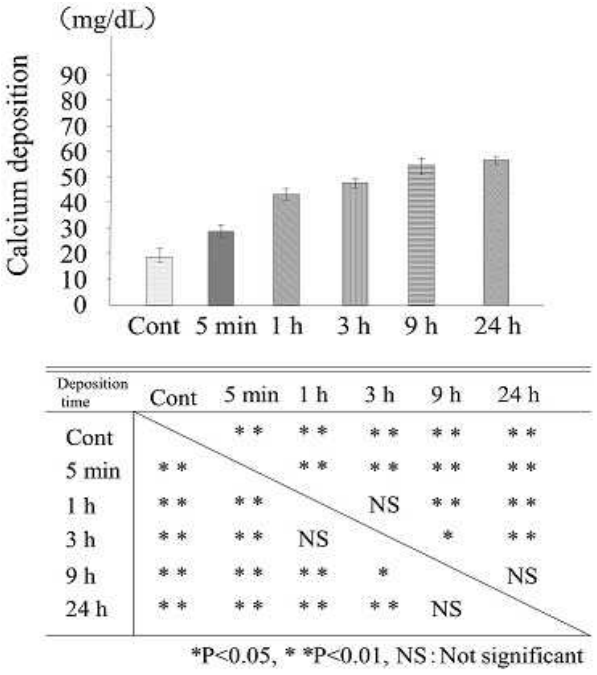
Fig. 3 shows the calcium deposition of cells cultured on the various specimens for 21 days. Calcium deposition of unprocessed specimens and specimens soaked for 5 min were significantly



Deposition time	Cont	5 min	1 h	3 h	9 h	24 h
Cont		NS	*	**	**	**
5 min	NS		NS	**	**	**
1 h	*	NS		*	**	**
3 h	**	**	*		**	**
9 h	**	**	**	**		NS
24 h	**	**	**	**	NS	

*P<0.05, * *P<0.01, NS : Not significant

Figure 2. ALP activity at 14 days of culture in test and control groups as measured by an ALP ELISA. The differences among specimens of the control, 5 min and 1 h treatment groups were not statistically significant. ALP activities of specimens soaked for 1 h or 3 h in a NaOH solution, as well as specimens soaked for 9 h or 24 h were different. However, the difference between specimens soaked for 9 h or 24 h was not statistically significant.



Deposition time	Cont	5 min	1 h	3 h	9 h	24 h
Cont		**	**	**	**	**
5 min	**		**	**	**	**
1 h	**	**		NS	**	**
3 h	**	**	NS		*	**
9 h	**	**	**	*		NS
24 h	**	**	**	**	NS	

*P<0.05, * *P<0.01, NS : Not significant

Figure 3. Calcium deposition after 21 days in test and control groups estimated by a Calcium-E test kit. Calcium deposition of specimens of control and soaked for 5 min in a NaOH solution was significantly different, and that of specimens soaked for 5 min or 1 h, as well as specimens soaked for 1 h or 3 h was not significantly different. The difference between specimens soaked for 9 h or 24 h was statistically significant. However, the difference between the specimens soaked for 9 h or 24 h was not statistically significant.

different, whereas that of specimens soaked for 5 min or 1 h, as well as specimens soaked for 1 h or 3 h were not. The difference between specimens soaked for 3 h or 9 h was statistically significant; however, the difference between specimens soaked for 9 h or 24 h was not.

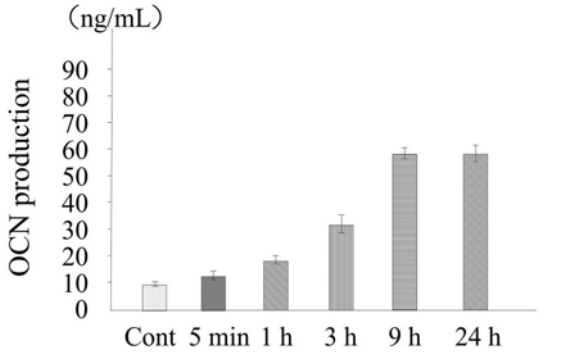
OCN production

Fig. 4 shows the production of OCN by cells cultured on the various specimens for 21 days. OCN production of unprocessed specimens and specimens soaked for 5 min were significantly different, whereas that of specimens soaked for 5 min or 1 h, as well as specimens soaked for 1 h or 3 h were not. The difference between specimens soaked for 3 h or 9 h was statistically significant; however, the difference between specimens soaked for 9 h or 24 h was not.

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 3h. 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[32]. 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



Deposition time	Cont	5 min	1 h	3 h	9 h	24 h
Cont		NS	*	* *	* *	* *
5 min	NS		NS	* *	* *	* *
1 h	*	NS		NS	* *	* *
3 h	* *	* *	NS		* *	* *
9 h	* *	* *	* *	* *		NS
24 h	* *	* *	* *	* *	NS	

*P<0.05, * *P<0.01, NS : Not 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.

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 correlated with some differences in the biological responses of RBM cells cultured on specimens treated for different times. Although the alkaline-treated titanium increased the bioactivity of RBM cells, titanium surfaces soaked for 9 h or 24 h showed significantly better results than those of other titanium surfaces. These results suggest that bone differentiation is achievable in a relatively short time. Indeed, the nanonetwork structure, as revealed by SEM analysis, and a surface roughness between 13 nm and 16 nm, as revealed by SPM analysis, were optimal for RBM cell culture. It is reported that NaOH treatment led to the formation of a Ti-O-Na titanate layer on the Ti surface and covered with a titanium oxide layer with a thickness of approximately 1000 nm. This suggests that the nanonetwork structure on the titanium surface soaked for 9 h in NaOH was made in a Ti-O-Na titanate layer.

In contrast, ALP activity in this study was observed to be surface dependent. ALP activity is an early marker of osteoblastic differentiation. Generally, surface roughness is believed to affect cell proliferation and differentiation. Other studies have reported the occurrence of elevated ALP activities in cells cultured on NaOH-treated surfaces[31]. Considering the differences in ALP activity of cells cultured on TNS-modified titanium disks soaked in NaOH for 9 h or 24 h and their similar surface roughness, the results cannot simply be attributed to differences in surface roughness. The effects of microtopography and surface chemistry must also be considered. OCN production and calcium deposition were all elevated by TNS formed in NaOH for 9 h or 24 h. Importantly, the functional phenotypes expressed in the middle and late stages of culture, such as ALP activity and mineralization, were considerably increased in RBM cells. It has been a challenge to create nanofeatures by controlling the soaking time in NaOH. The improvement of the surface topography could be indirect; the adsorption of proteins or ions might act as a bridge between the nanosurface structure and the cells[15]. The nanonodular features presented in this study resemble globular molecules suggestive of calcium-binding proteins, and are extensively seen in tissue and matrix surfaces during biomineralization. Besides, the size of nanostructures controlled by deposition time allowed us to compose the molecular structures that are seen during the later stages of biomineralization, which may provide an opportunity to make biological surfaces for implant materials. The biological parameters of osteoblasts generally correlated with the roughness values and peaked at 13 nm, indicating that higher counts of nanonodules on the titanium surface resulted in higher rates of cell proliferation and differentiation.

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[9]. 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[28,33].

In conclusion, by controlling the deposition time in a NaOH solution, the titanium surface shows different nanonetwork 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.

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