Anodic Oxidized Nanotubular Titanium Implants Enhance Bone Morphogenetic Protein-2 Delivery

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Abstract: Implant failure has been attributed to loosening of an implant from the host bone possibly due to poor osseointegration. One promising strategy for improving osseointegration is to develop a functional implant surface that promotes osteoblast differentiation. In this study, a titanium (Ti) surface was functionalized by an anodic oxidation process and was loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2). The following four groups of Ti surfaces were prepared: machined (M), anodized machined (MA), resorbable blast medium (RBM), and anodized RBM (RBMA). The surfaces were characterized by scanning electron microscopy and contact angle measurements. The results showed that a Ti oxide layer (TiO_2) was observed in the anodized surfaces in the form of nanotubes, $\sim$100 nm in diameter and 500 nm in length. The hydrophilic properties of the anodized surfaces were significantly improved. The adsorbed rhBMP-2 loaded on the nonanodized surfaces and lyophilized showed spherical particle morphology. However, the adsorbed rhBMP-2 showed a dispersed pattern over the anodized surfaces. The velocity of the rhBMP-2 released from the surfaces was measured to determine if the anodized surface can improve in delivery efficiency. The results showed that the release velocity of the rhBMP-2 from the anodized surfaces was sustained when compared with that of the nonanodized surfaces. In addition, the rhBMP-2 released from the surface was found to be bioactive according to the alkaline phosphatase activity and the level of calcium mineral deposition. These results suggest that the TiO_2 nanotubular structure formed by anodizing is a promising configuration for sustained rhBMP-2 delivery. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 93B: 484–491, 2010

Key Words: titanium implant, anodic oxidation, nanotube, bone morphogenetic protein-2 delivery, osseointegration, osteoblast differentiation

INTRODUCTION

In recent years, titanium (Ti) and its alloys have been developed into key materials for biomedical applications, such as orthopedic and dental implant surgery, because of their biocompatibility with human tissues and excellent mechanical properties.[1] To improve their functional properties, the Ti implant surface is generally modified by increasing the surface roughness on the Ti surface or by altering the crystal structure and chemical composition using various techniques, such as oxidation methods,[2–5] anodic plasma-chemical treatments,[6] and calcium phosphate and hydroxyapatite coatings on the Ti surface.[7–10] Recently, we reported the fabrication of nanotubular structures on a Ti surface by anodic oxidation.[11] Anodic oxidation has many advantages in surface modification, such as its ability to fabricate porous titanium oxide (TiO_2) films through dielectric breakdown, the changeability of the crystalline structure and chemical composition of the oxide film depending on the fabrication conditions.[12] This unique nanotubular structure improves the cellular activity on the surface *in vitro* and bone–implant contact *in vivo*.[13]

Recombinant human bone morphogenetic protein-2 (rhBMP-2) has been used in dental and orthopedic biomaterials to promote bone formation because of its strong osteogenic activity. The rhBMP-2 induces bone formation *in vivo*,[14–19] presumably by stimulating mesenchymal stem cell differentiation toward an osteoblast lineage and by increasing the number of differentiated osteoblasts capable

of forming bone.[20,21] This stimulatory effect of the rhBMP-2 on osteoblastic differentiation is of major importance during bone healing. Because bone growth is a dynamic process, several *in vitro* studies have suggested that a dose response can be produced with the appropriately timed delivery of growth factors to the cells. Moreover, the systemic delivery of rhBMP-2 can be impractical and undesirable because it may have uncontrolled adverse effects such as unwanted ectopic bone formation. Therefore, it is believed that rhBMP-2 should be immobilized on the implant surface to allow sufficient time to promote osseointegration.[22,23] If the cells are exposed to the rhBMP-2 for long duration, they could fully differentiate to osteoblastic traits.[24,25] In this study, it was hypothesized that the nanotubular structure formed by anodic oxidation can provide rhBMP-2 storage room, which might improve the sustained rhBMP-2 delivery. This study examined whether rhBMP-2 can be deposited on a TiO_2 nanotubular surface. In addition, the release profile of rhBMP-2 and its biological activity were assessed.

MATERIALS AND METHODS

Formation of TiO_2 Nanotubes by Anodic Oxidation

The sample materials used were commercially pure Ti [ASTM Grade II, Machined (M) and resorbable blast media (RBM)-treated Ti] in the form of a disc, 15 mm in diameter and 1 mm in thickness. The specimens were used in the anodic oxidation process, as reported previously,[11] and are designated as anodized machined (MA) for M and anodized RBM (RBMA) for RBM. Anodic oxidation was performed at a constant voltage of 20 V for 60 min using a DC power supply (Fine Power F-3005; SG EMD, Anyang, Korea). The electrolyte for anodizing consisted of $1M$ H_2SO_4 and 1.0 wt % HF solutions with a pH of 2–3. A platinum plate (3 mm × 4 mm × 0.1 mm) was used as the anode, and the distance between the anode and cathode was 10 mm. All anodic oxidation processes were carried out at room temperature. After oxidation, the specimens were washed with water for 20 min and were dried for 24 h at 40°C in an oven.

Measurement of Hydrophilic Properties

The hydrophilic properties of the surfaces were evaluated by measuring the contact angle using the sessile drop method.[26] Twenty-five microliters of rhBMP-2 solution (R&D Systems, Minneapolis, MN) was dropped onto each fabricated Ti surface. The drop images were obtained using a video camera (Camscope®, Sometech, Seoul, Korea), and the contact angles were measured using an image analysis system (Dropsnake®, National Institutes of Health, Bethesda, MD). The contact angles of the specimens were obtained by averaging the right and left sides of the drops.

Measurement of Released rhBMP-2

To immobilize the rhBMP-2 on each Ti surface, 100 μL of the rhBMP-2 solution (50 ng/μL) was loaded onto each Ti surface and was lyophilized as reported.[27,28] To measure the amount of rhBMP-2 released from Ti surfaces, the lyophilized specimens were placed in a phosphate-buffered saline (PBS) solution for 1, 4, 7, 14, and 21 days at 4°C

with shaking gently. The amount of rhBMP-2 in the solution was measured using the rhBMP-2-specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions.

Surface Morphology

Scanning Electron Microscopy. The surface morphology of the Ti specimens and the immobilized rhBMP-2 on Ti surfaces were examined by scanning electron microscopy (SEM; Hitachi, Tokyo, Japan) at voltages ranging from 5 to 15 kV after sputter coating the surfaces with platinum. The structure of the TiO_2 nanotubes was observed by transmission electron microscopy (TEM; JEM-2000FXII, JEOL, Tokyo, Japan) using a carbon-coated copper mesh grid.

Surface Roughness Average. The measured surface roughness was represented as roughness average (Ra) after three times testing. The Ra value of each specimen was defined as the arithmetic average value of all absolute distances of the roughness profile from the center line within the measuring length. All measurements were performed using an atomic force microscope (AFM; Nano ScopeIIIa, di Digital Instruments, USA).

UV-Micro Raman Spectroscopy. The characterization of the rhBMP-2 on the Ti surfaces was examined by UV-micro Raman spectroscopy (Renishaw Invia Reflex, Wotton-under-Edge, UK). The samples were acquired with an excitation wavelength of 633 nm and were scanned from 500 to 3500 cm^{-1} at a spectral resolution of 1 cm^{-1}.

Biological Activity of Delivered rhBMP-2

Cell Culture. To allow the release of rhBMP-2 loaded on the Ti surfaces, the specimens were placed in alpha-minimum essential media (α-MEM) for 7 days at 4°C. The medium containing the rhBMP-2 released from each surface was harvested and applied to the cell culture. MC3T3-E1 cell, which is an osteoblast-like cell line derived from the mouse calvarium (passages 3–4), was used to evaluate the biological activity of the rhBMP-2 released from the specimens in osteoblast differentiation. The cell was cultured in the harvested α-MEM supplemented with 2% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The cells were seeded at 5×10^4 cells/cm^2 and were cultured at 37°C in an atmosphere containing 5% carbon dioxide (CO_2) at 95% humidity.

Alkaline Phosphatase Activity. The bioactivity of the rhBMP-2 released from the anodized surface was examined. MC3T3-E1 osteoblast cells were used to evaluate the biological activity of the released rhBMP-2. The alkaline phosphatase (ALP) activity was measured as an early biomarker specific to osteoblast differentiation. The test group received the medium containing rhBMP-2 released from the anodized surface (RBMA), and the control group received the medium in the absence of rhBMP-2 from RBMA. After 6 days of culture, the cells were harvested in a lysis buffer containing 10 mM Tris-Cl (pH 7.4), 0.2% NP40, and 2 mM PMSF,
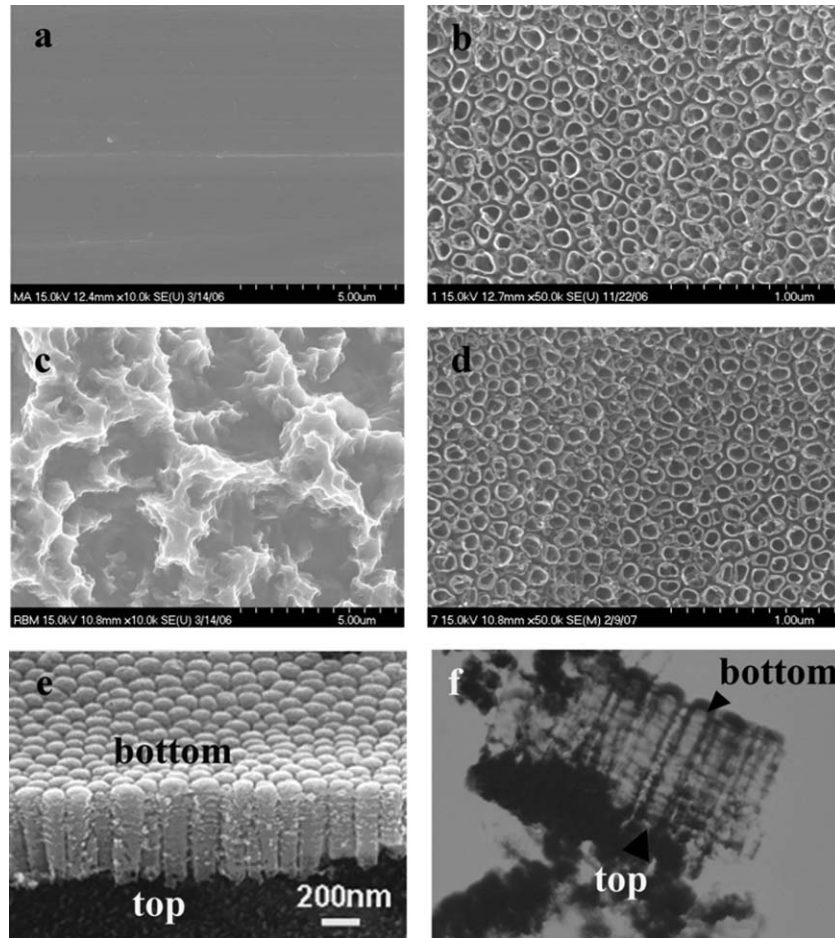
FIGURE 1. SEM and TEM images of Ti surfaces. Surface morphology of (a) machined Ti (M), (b) anodized machined Ti (MA), (c) resorbable blast media-treated Ti (RBM), (d) anodized RBM (RBMA), (e) cross-sectional morphology of a peeled nanotubular layer, and (f) TEM image of a peeled nanotubular layer. The nanotubular layers were open at the top end and closed at the bottom. The anodized nanotubes were ~100 nm in diameter and 500 nm in length.

homogenized by sonication and centrifuged. The lysate was used to measure the ALP activity, as previously reported using a *p*-nitrophenyl phosphate substrate.[29] The absorbance of the solution was measured using a spectrophotometric microplate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 405 nm. The ALP activity was normalized to the total DNA content, which was determined by a PicoGreen® dsDNA quantification kit (Molecular Probes, Eugene, OR).

Alizarin Red Staining. It is well known that the rhBMP-2 promotes osteoblast differentiation. The bioactivity of the rhBMP-2 after releasing from the discs was examined by measuring the level of calcium mineral deposition. MC3T3-E1 cells were seeded on the Ti surfaces. After 3 weeks of culture, the cells were fixed with 70% ethanol, and then rinsed three times with deionized water. For the measurement of the level of calcium mineral deposition, the cultured cell was treated with a 40 m*M* Alizarin Red S (AR-S) solution (pH 4.2) for 10 min. The cultures were washed three times with PBS, and then stained cultures were extracted by 10% (w/v) cetylpyridinium chloride in 10 m*M* sodium phosphate (pH

7.0) for 20 min. The AR-S concentration was determined by the absorbance measurement at 540 nm on multiplate reader using an AR-S standard curve in the same solution.

Statistical Analysis

To compare the significant difference between groups, the results were statistically analyzed using the nonparametric Mann-Whitney ($k = 2$) and Kruskal-Wallis ($k > 2$) examinations (SPSS software Version 17.0; SAS Institute, Cary, NC). The results are expressed as the mean ± standard deviation (SD) from three or more separate experiments, and significant differences between the groups were detected with $p < 0.05$.

RESULTS

Surface Morphology

To compare the drug delivery function between before and after anodizing, four groups of specimens were prepared (nonanodized groups: M, RBM; and anodized groups: MA, RBMA), and the surface morphologies of prepared samples were analyzed by SEM. The anodized Ti surfaces [MA, RBMA; Figure 1(b,d,e)] had a nanotubular array, which was not observed on the nonanodized Ti surface [M, RBM;

Figure 1(a,c)]. The M surface was smooth [Figure 1(a)] but RBM surface was rough [Figure 1(c)], and the apparent morphology of RBMA surface has nanotubular array rather than rough. As SEM is just a qualitative analysis, the surface roughness was measured by AFM. The measured surface roughness was represented as Ra values. The Ra value of M was 12.3 ± 1.3 nm, but the Ra value of MA increased to 21.4 ± 1.3 nm after anodizing. This can be interpreting that surface roughness was increased in nanotubular formation formed by anodizing. However, in the case of RBM/RBMA surface, the Ra value was decreased from 53.63 ± 1.8 nm to 24.9 ± 3.0 nm after anodizing, respectively. The nanotubular morphology was observed in RBMA surface rather than roughened morphology. These results may suggest that the degree of roughness of RBM was decreased during anodizing process, and then nanotubular formation was formed on the surface. TEM analysis of the detached TiO_2 layers confirmed that the array was uniform over the surface with a pore size, ∼100 nm in diameter and 500 nm in length [Figure 1(f)]. The nanotubes were open at the top end and closed at the bottom, indicating that it might serve storage room for drugs such as rhBMP-2.

Hydrophilic Properties of Ti Surfaces

A low water contact angle (i.e., high hydrophilic property) of material surface is generally favorable in biocompatibility. Hence, the hydrophilic properties of the surfaces in this study were evaluated by water contact angle using the rhBMP-2 solution. The rhBMP-2 solution was dropped onto each surface, and the typical shape of the rhBMP-2 solution drops on each surface was demonstrated in Figure 2. The contact angles of the MA and RBMA surfaces, which are anodized surfaces, were 16.2° ± 2.9° and 12.8° ± 2.6°, respectively. On the other hand, the contact angles of the M and RBM, which are the nonanodized surfaces, were 58.5° ± 7.2° and 33.7° ± 7.4°, respectively [Figure 2(a)]. The order of the contact angles is as follows: RBMA = MA < RBM < M [Figure 2(b)]. The contact angles of the anodized surfaces were lower than those of the nonanodized surfaces, indicating that anodized surface has higher hydrophilic properties.

Morphologies of the rhBMP-2 on the Ti Surfaces

As shown in the hydrophilic property test (Figure 2), the shape of the rhBMP-2 solution drop was more flattened in anodized surface when compared with that of nonanodized surfaces. We speculated that the rhBMP-2 compound may be immobilized into or onto nanotubular surface. Therefore, the morphologies of the rhBMP-2 compound on the surfaces were examined by SEM. As shown in Figure 3, the rhBMP-2 existed as spherical particles on the nonanodized surface [RBM; Figure 3(a,c)]. In contrast, on the anodized surface (RBMA), they existed within the nanotubular space with an evenly dispersed pattern [Figure 3(b,d)]. The nature of rhBMP-2 on the Ti surfaces was confirmed by UV-micro Raman spectroscopy. The Raman spectra of the rhBMP-2 on the surfaces were similar to those of the native rhBMP-2 peptide [Figure 3(e)].
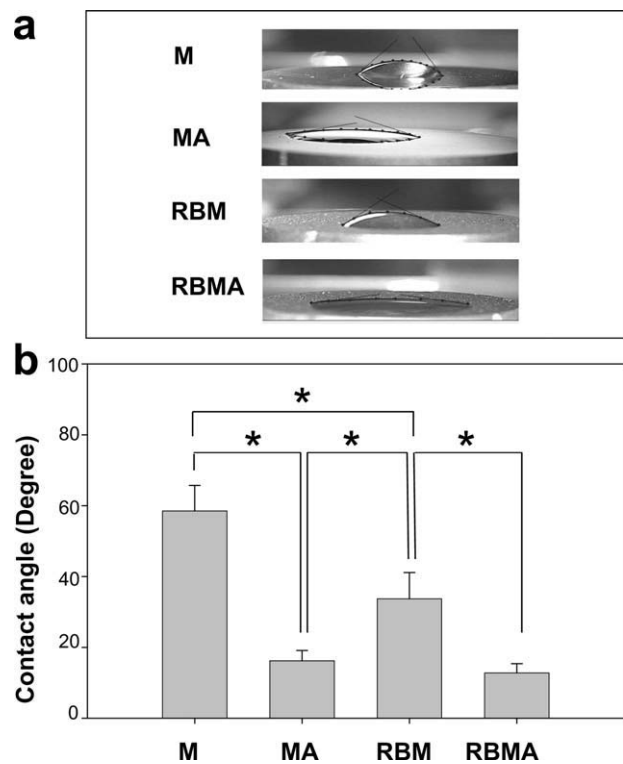

FIGURE 2. Hydrophilic properties of Ti surfaces. Hydrophilic properties were evaluated by measuring the contact angle of the rhBMP-2 solution on each Ti surface. (a) The drop images were captured by a camera, and the contact angles were measured using an image analysis system (b) A histogram of the contact angle is represented as mentioned in "Materials and Methods" section. The angles of the anodized surfaces (MA, RBMA) were lower than those of the nonanodized surfaces (M, RBM). It indicates that anodizing treatment increases hydrophilic properties of Ti surfaces. $n = 3$. Error bars, ±SD. $*p < 0.05$ when compared with contact angles and surfaces.

Release Kinetics of the rhBMP-2 From the Surfaces and Its Biological Activity

Release Kinetics of the rhBMP-2 From the Ti Surfaces. In the morphology study, it was suggested that the rhBMP-2 can be delivered to nanotubular surface. To get the desired effect of the rhBMP-2 on osteoblastic differentiation, the rhBMP-2 should be released from the surfaces at appropriate timing and period. If the cells are exposed to the rhBMP-2 for long duration, they could fully differentiate to osteoblastic traits.[24,25] It may be explained that the sustained release of the rhBMP-2 from the surfaces is essential for osteoblastic differentiation and bone formation. To investigate the rhBMP-2 release kinetics, the rhBMP-2 was loaded on the specimen's surfaces and lyophilized. Subsequently, the specimens were soaked in PBS solution, and then the amount of the rhBMP-2 released at designated time point was measured. As shown in Figure 4, the release velocities were quite rapid and burst in the nonanodized surfaces (M, RBM). The results showed 91% initial release within the first 4 days, followed by an additional 7% release at 7 days, with an almost linear release pattern at 21 days. However, they showed a sustained release pattern in the anodized surfaces (MA, RBMA) when compared with the nonanodized
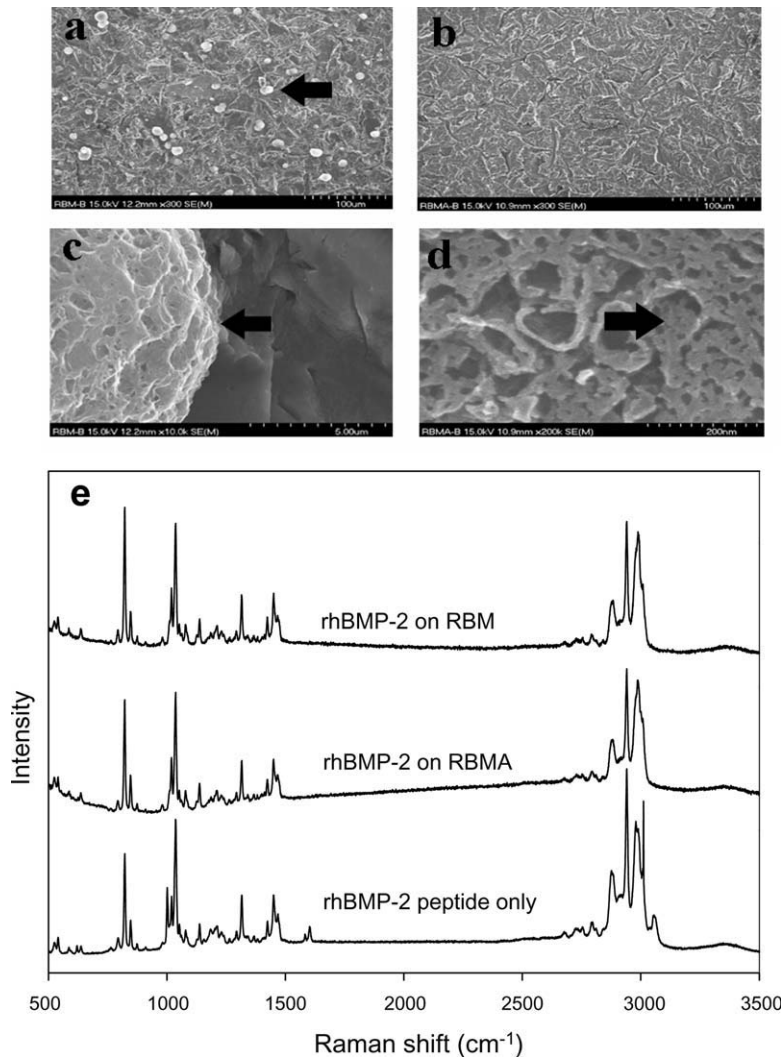
FIGURE 3. Morphology of the rhBMP-2 on Ti surfaces. The lyophilized rhBMP-2 on the nonanodized (RBM; a, c) and the anodized (RBMA; b, d) Ti surfaces are represented. The rhBMP-2 particles on the nonanodized surface showed mainly a spherical morphology (a, ×300; c, ×10,000). However, on the anodized surface (b, ×300; d, ×200,000), they existed within the nanotubular space with an evenly dispersed pattern. The nature of rhBMP-2 on the Ti surfaces was confirmed by UV-micro Raman spectroscopy (e). The arrows indicate the lyophilized BMP-2 particles.

surfaces. This showed that ~60% was released in the first 4 days, followed by an additional 19% and 14% released at 7 days, and 18% at 14 days. The total amount of the rhBMP-2 released from the nonanodized surfaces (M, RBM) was ~50% of that from anodized surfaces (MA, RBMA) at 21 days. Moreover, the amount of the rhBMP-2 released from the M surface was ~30% smaller than that from the RBM surface at 21 days.

Biological Activity of the Released rhBMP-2. The biological activity of the rhBMP-2 released from the anodized surface was examined by the ALP activity and the level of calcium mineral deposition in MC3T3-E1 cultures. It is well known that the rhBMP-2 promotes osteoblast differentiation with increased ALP activity and calcium mineral deposition. The test group received the medium containing the rhBMP-2 released from the anodized surface (RBMA), and the control group received only medium released from RBMA. After 6 days of culture, the ALP activity in the test group was 55% higher than that of the control group [Figure 5(a)]. In this study, the ALP activity was normalized to the total DNA contents that are slightly higher in the rhBMP-2-containing group (22.7 μg/mL) when compared with that of the rhBMP-2-deficient group (18.6 μg/mL). The level of mineral deposition by AR-S assay was also 29% higher in the test group than that of the control group [Figure 5(b)].

DISCUSSION

In general, drugs such as antibiotics, anti-inflammatory drugs, and growth factors are administered either orally or intravenously as a supportive treatment for bone healing. These delivery routes often result in limited bioavailability. Therefore, high doses are needed for them to be effective at the implantation site. The ideal drug delivery route might be local application at the implant and tissue interface. Ti surfaces have been developed into key materials for
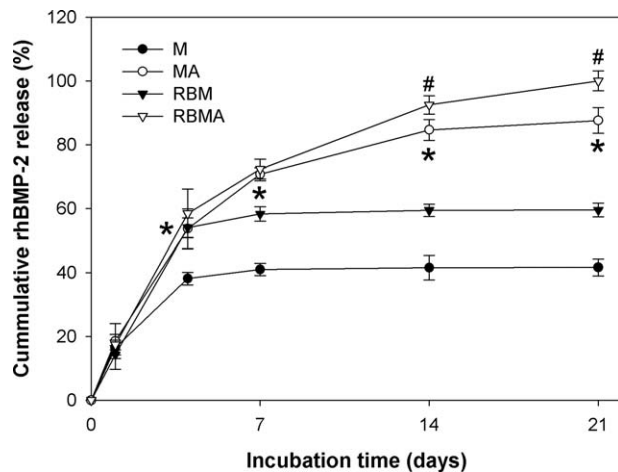
FIGURE 4. Release kinetics of the rhBMP-2 from the Ti surfaces. One hundred microliters of rhBMP-2 solution (50 ng/μL) was loaded onto each Ti surface and lyophilized. Subsequently, the specimens were placed in PBS solution for 1, 4, 7, 14, and 21 days with shaking gently. The amount of the rhBMP-2 in the solution was measured using an rhBMP-2 ELISA kit according to the manufacturer's instructions. Rapid and burst release profiles were observed in the nonanodized groups (M, RBM). However, a sustained release pattern was observed in the anodized groups (MA, RBMA). $n = 3$. Error bars, ±SD. *$p < 0.05$ when compared with the release from M and MA; #$p < 0.05$ when compared with the release from RBM and RBMA.

biomedical applications. The Ti surfaces have been modified by coating or electrochemical anodic oxidation for increasing the surface roughness or altering the crystal structure and chemical nature. Among these, anodic oxidation has many advantages in surface modification. Our previous study demonstrated that anodic oxidation has many advantages in surface modification, such as its ability to fabricate porous TiO_2 films through dielectric breakdown, the changeability of the crystalline structure and chemical composition of the oxide film depending on the fabrication conditions,[12] and have been suggested to provide storage room for the delivery of growth factors, such as rhBMP-2 to enhance osseointegration.[11] There was a precise correlation between the anodizing voltage/time/electrolyte and pore size. Therefore, substrates with different sized nanotubes can be fabricated by varying the anodizing conditions.[30] In this study, a constant voltage of 20 V for 60 min with a $1M$ H_2SO_4 and 1.0 wt % HF electrolyte were used in the anodizing process to form the nanotubular array on the Ti surface. SEM and TEM analyses showed that the nanotubular array was uniformly distributed over the anodized surfaces, and the pore size of the nanotubes were ~100 nm in diameter and 500 nm in length (Figure 1), which is similar to a previous study (anodizing for 10 min at 20 V in a $1M$ H_3PO_4 solution containing 1.5 wt % HF).[11]

SEM results showed that the TiO_2 was observed on the surfaces by anodic oxidation process in the form of nanotubes, ~100 nm in diameter and 500 nm in length (Figure 1). In this study, it was hypothesized that the nanotubular structure can provide rhBMP-2 storage room and focused on whether the nanotubes would allow rhBMP-2 delivery and improve the prevention of the rapid burst

release when the material is implanted to the body. Generally, drug deposition or cell adhesion on a Ti surface is dependent on the surface roughness and wettability.[31] In this study, the anodized surfaces with nanotubular formation showed a lower contact angle in the rhBMP-2 solution than that of the nonanodized surfaces (Figure 2). Because the rhBMP-2 solution is water based, similar to a cell culture media or body fluid, a lower contact angle suggests improved wettability and hydrophilic property, which are expected to lead to better cell attachment and cell proliferation.[31,32] This hydrophilic property on the anodized surfaces was also confirmed by SEM after loading the rhBMP-2 solution onto the Ti surface. The result showed that the rhBMP-2 compound immobilized in the nonanodized surface existed mainly as spherical particles [Figure 3(a,c)]. However, in the anodized surface, the adsorbed rhBMP-2 was dispersed over the surface or immobilized in the nanotubes [Figure 3(b,d)]. Although the rhBMP-2 was a unique additive material to the surfaces, it is important to provide objective evidence as to whether it is natural
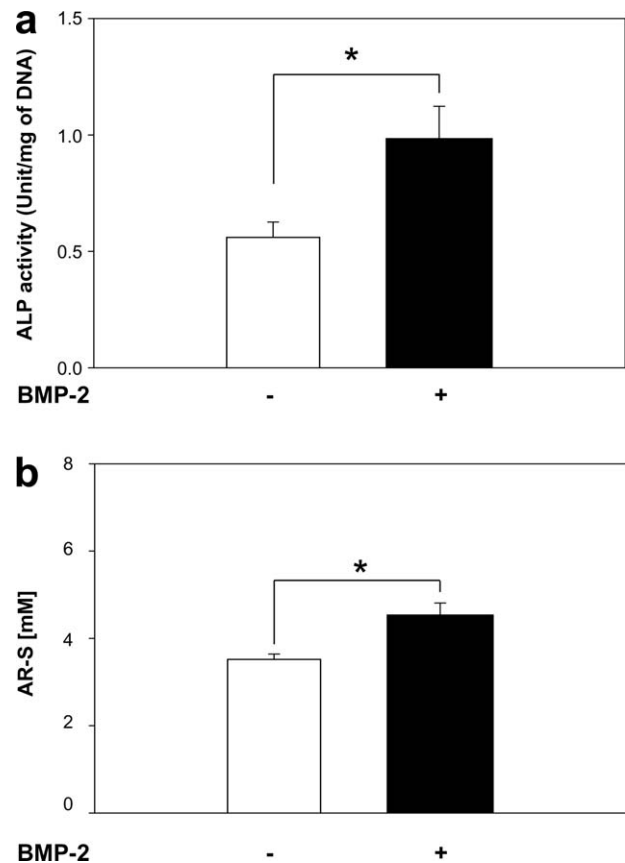
FIGURE 5. Biological activity of released rhBMP-2. To allow the release of rhBMP-2 loaded on the Ti surfaces, the lyophilized rhBMP-2 was placed in α-MEM for 7 days at 4°C. The medium containing the rhBMP-2 released from the surfaces were harvested and allowed to MC3T3-E1 cell culture. (a) The ALP activity of the cells was measured and normalized to the total DNA amount. (b) To examine the level of calcium mineral deposition, the cell culture was stained with Alizarin-red solution after 3 weeks of culture. $n = 3$. Error bars, ±SD. *$p < 0.05$ when compared with ALP activity and the level of calcium mineral deposition in the presence and absence of rhBMP-2 released from RBMA.

rhBMP-2. UV-micro Raman spectroscopy showed that the Raman spectra of the rhBMP-2 on the surfaces were similar to that of the native rhBMP-2 peptide [Figure 3(e)]. These results suggested that anodic oxidation contributed to the significant increases in hydrophilic property of rhBMP2 solution with nanotubular formation as in Figure 2.

In vitro studies have suggested that a dose response can be produced with appropriate period of delivery of the growth factor to the cells.[24,25] For effective drug delivery, exogenous osteogenic molecules, such as rhBMP-2, should be delivered locally and maintained during osteoblastic differentiation. Therefore, this study examined the rhBMP-2 release kinetics from the surfaces and its effect on osteoblast differentiation. The anodized surfaces showed a more sustained release pattern, and the total amount of the rhBMP-2 released from the anodized surfaces was also higher than that of the nonanodized surfaces (Figure 4). Even the total amount and release pattern of the rhBMP-2 from the MA surface was higher and sustained than that of the RBM group (Figure 4). The MA surface was not rough but showed nanotubular formation. On the other hand, the RBM surface was quite rough but did not show nanotubular formation. A comparison of these two surfaces provides information on the surface treatment for drug delivery, indicating advantage in drug delivery of nanotubular formation rather than roughness. The results suggest either more adsorption of the rhBMP-2 into the cylinder type of the nanotube than onto the RBM-treated simple micro-roughness or higher resistance to release into the body fluid from the nanotubular space than from the micro-roughness. In addition, the different surface treatments probably lead to different adsorption capabilities or affinities of these surfaces to the rhBMP-2. Although the quantitative study requires the measurement of initial adsorption of the rhBMP-2, the *in vitro* release results suggest that the anodized Ti surface is a better delivery system for the rhBMP-2 than the nonanodized Ti surface. Furthermore, the rhBMP-2 released from the anodized surface stimulated osteoblast differentiation by the confirmed ALP activity assay and AR-S (Figure 5). Moreover, the nanotubular structure of TiO_2 appears to be advantageous for the adsorption and sustained release of the rhBMP-2. In addition, the rhBMP-2 released from the anodized surface was as bioactive as the native rhBMP-2.

Overall, these results suggest that the nanotubular Ti surface can be used as a suitable delivery vehicle for the rhBMP-2 to enhance osseointegration. Surface modification by anodic oxidation may be a key for developing clinically improved dental or orthopedic implants. Further studies will be needed to test the delivery efficiency *in vivo*.

REFERENCES

1. Pohler OE. Unalloyed titanium for implants in bone surgery. Injury 2000;31 (Suppl 4):7–13.
2. Yang Y, Oh N, Liu Y, Chen W, Oh S, Appleford M, Kim S, Kim K, Park S, Bumgardner J, Haggard W, Ong J. Enhancement of osseointegration using surface modified titanium implants. J Miner Met Mater 2006;58:71–76.
3. Sul YT, Johansson CB, Petronis S, Krozer A, Jeong Y, Wennerberg A, Albrektsson T. Characteristics of the surface oxides on turned and electrochemically oxidized pure titanium implants up to dielectric breakdown: The oxide thickness, micropore configurations, surface roughness, crystal structure and chemical composition. Biomaterials 2002;23:491–501.
4. Liu Y, Park S, Kim H, Lee K, Koh J, Nishimoto SK, Bumgardner J, Haggard W, Yang Y. Engineered Titanium Surface Promoting Osseointegration. Ahmed W, Ali N, Oechsner A, editor. Biomaterials and Biomedical Engineering 2008, 115–134.
5. Nishimoto SK, Nishimoto M, Park SW, Lee KM, Kim HS, Koh JT, Ong JL, Liu Y, Yang Y. The effect of titanium surface roughening on protein absorption, cell attachment, and cell spreading. Int J Oral Maxillofac Implants 2008;23:675–680.
6. Frauchiger VM, Schlottig F, Gasser B, Textor M. Anodic plasma-chemical treatment of CP titanium surfaces for biomedical applications. Biomaterials 2004;25:593–606.
7. Fini M, Cigada A, Rondelli G, Chiesa R, Giardino R, Giavaresi G, Nicoli Aldini N, Torricelli P, Vicentini B. In vitro and in vivo behaviour of Ca- and P-enriched anodized titanium. Biomaterials 1999;20:1587–1594.
8. Yang Y, Kim KH, Ong JL. A review on calcium phosphate coatings produced using a sputtering process—An alternative to plasma spraying. Biomaterials 2005;26:327–337.
9. Wong M, Eulenberger J, Schenk R, Hunziker E. Effect of surface topology on the osseointegration of implant materials in trabecular bone. J Biomed Mater Res 1995;29:1567–1575.
10. Gottlander M, Albrektsson T. Histomorphometric studies of hydroxylapatite-coated and uncoated CP titanium threaded implants in bone. Int J Oral Maxillofac Implants 1991;6:399–404.
11. Kim HS, Yang Y, Koh JT, Lee KK, Lee DJ, Lee KM, Park SW. Fabrication and characterization of functionally graded nano-micro porous titanium surface by anodizing. J Biomed Mater Res B: Appl Biomater 2009;88:427–435.
12. Schreckenbach JP, Marx G, Schlottig F, Textor M, Spencer ND. Characterization of anodic spark-converted titanium surfaces for biomedical applications. J Mater Sci: Mater Med 1999;10:453–457.
13. Sul YT, Johansson CB, Roser K, Albrektsson T. Qualitative and quantitative observations of bone tissue reactions to anodised implants. Biomaterials 2002;23:1809–1817.
14. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci USA 1990;87:9843–9847.
15. Wang EA. Bone morphogenetic proteins (BMPs): Therapeutic potential in healing bony defects. Trends Biotechnol 1993;11:379–383.
16. Ozkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H. OP-1 cDNA encodes an osteogenic protein in the TGF-beta family. EMBO J 1990;9:2085–2093.
17. Sampath TK, Coughlin JE, Whetstone RM, Banach D, Corbett C, Ridge RJ, Ozkaynak E, Oppermann H, Rueger DC. Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-beta superfamily. J Biol Chem 1990;265:13198–13205.
18. Ozkaynak E, Schnegelsberg PN, Jin DF, Clifford GM, Warren FD, Drier EA, Oppermann H. Osteogenic protein-2. A new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. J Biol Chem 1992;267:25220–25227.
19. Elima K. Osteoinductive proteins. Ann Med 1993;25:395–402.
20. Rosen V, Thies RS. The BMP proteins in bone formation and repair. Trends Genet 1992;8:97–102.
21. Reddi AH, Cunningham NS. Initiation and promotion of bone differentiation by bone morphogenetic proteins. J Bone Miner Res 1993;8 (Suppl 2):S499–S502.
22. Bessho K, Carnes DL, Cavin R, Chen HY, Ong JL. BMP stimulation of bone response adjacent to titanium implants in vivo. Clin Oral Implants Res 1999;10:212–218.
23. Stenport VF, Roos-Jansaker AM, Renvert S, Kuboki Y, Irwin C, Albrektsson T, Claffey N. Failure to induce supracrestal bone growth between and around partially inserted titanium implants using bone morphogenetic protein (BMP): An experimental study in dogs. Clin Oral Implants Res 2003;14:219–225.
24. Puleo DA, Huh WW, Duggirala SS, DeLuca PP. In vitro cellular responses to bioerodible particles loaded with recombinant

human bone morphogenetic protein-2. J Biomed Mater Res 1998; 41:104–110.

25. Puleo DA. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. J Cell Physiol 1997;173:93–101.

26. Sawase T, Jimbo R, Wennerberg A, Suketa N, Tanaka Y, Atsuta M. A novel characteristic of porous titanium oxide implants. Clin Oral Implants Res 2007;18:680–685.

27. Foraker AB, Walczak RJ, Cohen MH, Boiarski TA, Grove CF, Swaan PW. Microfabricated porous silicon particles enhance paracellular delivery of insulin across intestinal Caco-2 cell monolayers. Pharm Res 2003;20:110–116.

28. Salonen J, Laitinen L, Kaukonen AM, Tuura J, Bjorkqvist M, Heikkila T, Vaha-Heikkila K, Hirvonen J, Lehto VP. Mesoporous silicon microparticles for oral drug delivery: Loading and release of five model drugs. J Control Release 2005;108:362–374.

29. Manolagas SC, Burton DW, Deftos LJ. 1,25-Dihydroxyvitamin D3 stimulates the alkaline phosphatase activity of osteoblast-like cells. J Biol Chem 1981;256:7115–7117.

30. Mor GK, Varhese OK, Paulose M, Mukherjee N, Grimes CA. Fabrication of tapered, conical-shaped titania nanotubes. J Mater Res 2003;18:3331–3334.

31. Kakoli D, Susmita B, Amit B. Surface modifications and cell-materials interactions with anodized Ti. Acta Biomater 2007;3:573–585.

32. Webb K, Hlady V, Tresco PA. Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization. J Biomed Mater Res 1998;41:422–430.