Research Article

UV Photocatalysis of Bone Marrow-Derived Macrophages on TiO₂ Nanotubes Mediates Intracellular Ca²⁺ Influx via Voltage-Gated Ca²⁺ Channels

Seunghan Oh,¹ Eun-Joo Choi,² Munkhsoyol Erkhembaatar,³ and Min Seuk Kim³

¹Department of Dental Biomaterials and Institute of Biomaterials-Implant, College of Dentistry, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

²Department of Oral and Maxillofacial Surgery, College of Dentistry, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

³Department of Oral Physiology and Institute of Biomaterial-Implant, College of Dentistry, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

Correspondence should be addressed to Min Seuk Kim; happy1487@wku.ac.kr

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Titanium (Ti) possesses excellent properties for use in dental implants but has low osteogenic surface properties that result in limiting rapid osseointegration. The physiological interaction between the surface of the implant material and bone cells, especially osteoclasts, is a crucial factor in determining successful osseointegration. However, the details of such an interaction remain elusive. Here, we demonstrated that nanotopography on the Ti surface is a crucial factor for modulating intracellular signal transduction in bone marrow-derived macrophages (BMMs). To define this, intracellular Ca²⁺ and ROS were simultaneously measured in BMMs that were seeded on polished Ti and TiO₂ nanotubes. We found that UV photocatalysis of TiO₂ immediately elicits intracellular calcium concentration ([Ca²⁺]_i) increase and intracellular reactive oxygen species concentration ([ROS]_i) reduction in cells on TiO₂ nanotubes. UV photocatalysis-mediated [Ca²⁺]_i increase is dependent on extracellular and intracellular ROS generation. Furthermore, extracellular Ca²⁺ influx through voltage-gated calcium channels (VGCCs) is critical for the UV photocatalysis-mediated [Ca²⁺]_i increase, while phospholipase C (PLC) activation is not required. Considering the physiological roles of Ca²⁺ signaling in BMMs and osteoclastogenesis, nanotopography on the Ti surface should be considered an important factor that can influence successful dental implantation.

1. Introduction

Titanium (Ti) and its alloys are well known to be one of primary metallic biomaterials used in dental and orthopedic implants requiring load-bearing capacity and feature excellent chemical resistance and considerable strength. However, due to the strong chemical stability of Ti and Ti alloys resulting in excellent biocompatibility, they have limited chemical and biological responses, which can react directly with bone forming related cells and is required for rapid osseointegration and strong fixation in the patient [1, 2]. Many researchers have sought to develop various surface treatment of Ti implant in order to create an excellent chemical and biological reactivity to the surface of Ti [3–6].

Osseointegration is determined by numerous factors linked to the host (bone remodeling) and to the implant materials (surface properties). The former is mainly regulated by cell-to-cell interactions between osteoblasts, which deposit bone matrix, and osteoclasts, which resorb bone tissue [7]. In particular, modified osteoclastogenesis or activities of mature osteoclasts cause severe bone disorders and result in poor osseointegration [7]. In the latter case, the surface topography of the implant plays a critical role in the clinical success of bone-anchored implants [8]. Surface physicochemical treatments modifying implant surface chemistry and topography are commonly employed to improve osseointegration of the implant [9–11]. Many studies about biochemical surface modification of Ti report enhanced osseointegration of the Ti

surface, depending on surface roughness, bioactive coating, and varied mixture methods. Particularly, many researchers have analyzed that micro surface roughness and morphology were related to the bone contact, primary stability, and intermittent load bearing *in vitro* and *in vivo* [12–18].

Nanotopography, as well as microstructures, has been of great interest in the implant field due to the high surface-to-volume ratio, excellent bone cell behavior, and osseointegration capabilities [19–21]. In the field of *in vitro* molecular biology, it was reported that cellular behavior and functionality were affected by the size of topographical environment [22–25]. Titania (TiO₂) nanotubes have been widely studied in the fields of photocatalysis/photoelectrolysis [26–30], water purification [31, 32], solar cells [33–37], and biomedical engineering [38–42]. In particular, the surface structure on vertically aligned TiO₂ nanotubes had an important effect on improving the *in vitro* proliferation and mineralization of osteoblasts [43–45], reducing immune response [46], and upregulating *in vivo* osseointegration [20, 43]. In this study, we demonstrate that altered UV photocatalytic activity by surface modification of Ti resulted in the transmission of intracellular signals by mobilizing secondary messengers such as Ca²⁺ and ROS in BMMs.

2. Materials and Methods

2.1. Cell Culture and Reagents. Primary bone marrow-derived macrophages (BMMs) were cultured in alpha-modified minimum essential medium (α -MEM; Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS) and M-CSF (30 ng/mL). Soluble recombinant mouse receptor activator of nuclear factor kappa-beta ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were purchased from KOMA Biotech (Seoul, Korea). N-Acetyl-L-cysteine (NAC), U73122, nicardipine, Fura-2-acetoxymethyl ester (Fura-2/AM) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, and acetyl ester (CM-H₂DCFDA) were purchased from Sigma Aldrich (MO, USA).

2.2. Fabrication of TiO₂ Nanotubes. TiO₂ nanotubes were prepared by anodization, as described previously [47]. Briefly, a machined Ti sheet was electropolished under perchloric acid (Sigma, MO, USA) solution mixed with butoxy ethylene glycol (Junsei Co., Japan) and methanol (Sigma, MO, USA) at –40°C for 30 min. The nanotubes were formed on an electropolished Ti sheet (Alfa-Aesar; 0.25 mm thick, 99.5%) by using a mixture of 0.5 wt% hydrofluoric acid (EM Science; 48%) and acetic acid (Fisher; 98%, volumetric ratio = 7:1) at 15 V for 30 min. A platinum electrode (Alfa-Aesar; 99.9%) served as the cathode. The specimen was rinsed with deionized water, dried at 80°C, and heat treated at 500°C for 2 h to transform the as-anodized amorphous TiO₂ nanotubes into the crystalline phase. The specimens (1.27 × 1.27 cm² area) used for all experiments were sterilized by autoclaving before use. An identically sized flat Ti sample was used as a control after being cleaned with acetone and isopropyl alcohol, dried, and autoclaved.

2.3. Scanning Electron Microscopy (SEM). Machined, polished, and fabricated TiO₂ nanotubes were sputter-coated with very thin gold for examination by scanning electron microscopy (SEM). The morphology of the TiO₂ nanotubes was observed using SEM (XL30, FEI Corporation).

2.4. Simultaneous Measurement of [Ca²⁺]_i and [ROS]_i. [Ca²⁺]_i and [ROS]_i levels were determined as previously described by using the Ca²⁺-sensitive fluorescent dye Fura-2/AM or the ROS-sensitive fluorescent dye CM-H₂DCFDA, respectively [48]. Briefly, isolated BMMs were seeded on the designated plate (Ti sheet or cover glass) at approximately 80% confluence (6 × 10⁵ cells/35-mm dish) and cultured in α MEM medium supplemented with 10% FBS and M-CSF (30 ng/mL). The following day, cells were loaded with Fura-2/AM and CM-H₂DCFDA for 50 min at room temperature. The plate containing cells was placed in a perfusion chamber and then connected to a perfusion system. Cells were briefly washed out with regular HEPES buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 10 mmol/L glucose, adjusted to pH 7.4 and 310 mOsm). Each of the indicated compounds was diluted in regular HEPES buffer or Ca²⁺ free HEPES buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L EGTA, and 10 mmol/L glucose, adjusted to pH 7.4 and 310 mOsm) and perfused for a designated length of time. Under continuous perfusion with regular HEPES buffer (37°C), titanium plates containing BMMs were sequentially exposed to specific wavelengths of light (340, 380, and 488 nm), and emitted fluorescence (510 nm) was captured using a CCD camera. Captured images were digitized and analyzed using MetaFluor software. [Ca²⁺]_i data were expressed as ratio of fluorescence intensities (F_{340}/F_{380}), and intensity of ROS (F_{488}) was normalized and expressed as the relative value of initial intensity.

2.5. Statistical Analysis. Results were analyzed using Student's two-tailed *t*-test and the data are presented as mean ± SEM of the stated number of observations obtained from the indicated number of independent experiments. *P* values less than 0.05 were considered statistically significant ***P* < 0.01.

3. Results and Discussion

3.1. UV Exposure of TiO₂ Nanotubes Mediates [ROS]_i Reduction and [Ca²⁺]_i Increase in BMMs. We previously reported that modification of the Ti surface, such as by fabrication of nanotubes, dictates cellular fate [49], and aligned TiO₂ nanotubes significantly accelerate the growth of osteoblasts [47]. This is a critical factor in determining osseointegration. In the process of bone remodeling, the osteoclast is also responsible for enhancing osseointegration by resorbing bone on the border between the implant and bone tissue, which triggers the deposition of bone matrix [50]. This evidence raised a question as to whether or not topographical modification of Ti can affect the cellular response of osteoclasts.

Free Ca²⁺ ions act as secondary messengers that mediate diverse cellular responses such as differentiation, motility,

FIGURE 1: SEM micrographs of self-aligned TiO₂ nanotubes and simultaneous measurement of intracellular Ca²⁺ and ROS levels. (a) The self-assembly layers were generated by anodizing Ti sheets (scale bar, 70 nm). Machined and polished Ti sheets were presented as negative control (scale bar, 100 μm). (b) As control experiment, isolated BMMs were seeded on the cover glass and maintained for 24 h. H₂O₂ (1 mM) diluted in regular HEPES buffer was acutely treated and [Ca²⁺]_i and [ROS]_i levels in the same cell were simultaneously measured. [Ca²⁺]_i and [ROS]_i levels were normalized and presented as a ratio (F₃₄₀/F₃₈₀; black line) and a relative value (F₄₈₈; red line) compared to initial intensity.

and apoptosis [51]. Importantly, our previous report indicates that stimulation of BMMs (the precursors of osteoclasts) with RANKL induces ROS generation, which is essential for differentiation of BMMs into osteoclasts [48]. Considering that Ti is immediately oxidized upon exposure to air, forming titanium dioxide (TiO₂, titania), and TiO₂ generates ROS under UV light exposure, characterizing the correlation between intracellular Ca²⁺ signaling in BMMs and TiO₂-originated ROS is crucial for understanding the interaction between osteoclasts and implant materials, especially Ti. This led us to examine how UV photocatalysis of TiO₂ nanotubes affects intracellular Ca²⁺ responses in BMMs.

As shown in Figure 1(a), self-aligned TiO₂ nanotubes were synthesized by anodization. The nanotubes were fabricated with an electropolished Ti sheet in order to remove unwanted foreign materials deposited on the Ti sheets and to improve the uniformity of the nanotubes. We subsequently measured [Ca²⁺]_i and [ROS]_i in cells seeded on a cover glass as a pilot experiment and confirmed whether [Ca²⁺]_i and [ROS]_i levels can be measured in the same cell. Cells were then exposed to 340 nm, 380 nm, and 488 nm wavelength lights, in sequence, to measure [Ca²⁺]_i and [ROS]_i levels simultaneously. Each emitted fluorescence signal was collected at 510 nm and presented as described in Section 2. H₂O₂ treatment of macrophage cells is known to elicit an acute [Ca²⁺]_i increase [52]. As expected, [Ca²⁺]_i and [ROS]_i increased in response to H₂O₂ (Figure 1(b)).

Next, BMMs seeded on polished Ti and TiO₂ nanotubes were loaded with both fluorescent dyes and [Ca²⁺]_i and [ROS]_i levels were measured simultaneously. Interestingly, cells on polished Ti showed no change in [Ca²⁺]_i levels and a small reduction was observed in [ROS]_i levels, whereas cells on TiO₂ nanotubes showed an acute and large [Ca²⁺]_i increase and significant [ROS]_i reduction in response to UV exposure (Figures 2(a) and 2(b)). To define whether [Ca²⁺]_i increase in cells on TiO₂ nanotubes results from ROS generation by the Ti surface, UV-mediated [Ca²⁺]_i increase was measured in the presence of NAC (10 mM). Figures 2(d) and 2(e) clearly show that removal of extracellular and intracellular ROS abolishes [Ca²⁺]_i increase in cells on TiO₂ nanotubes. This suggests that ROS generated from TiO₂ nanotubes are responsible for UV-mediated [Ca²⁺]_i increase in cells grown on TiO₂.

3.2. Nicardipine Significantly Attenuates UV Photocatalysis-Mediated [Ca²⁺]_i Increase but Does Not Attenuate [ROS]_i Reduction. Considering these results, we next aimed to determine how UV photocatalysis of TiO₂ nanotubes elicits a [Ca²⁺]_i increase in BMMs. We first noted that UV photocatalysis of TiO₂ unexpectedly reduces [ROS]_i even though UV photocatalysis is known to generate ROS on the surface of TiO₂. We also noted that ROS scavenging by NAC abolished UV photocatalysis-mediated [Ca²⁺]_i

FIGURE 2: UV-mediated photocatalysis of TiO₂ nanotubes elicits an increase in the concentration of cytosolic Ca²⁺ ($[Ca^{2+}]_i$) and a decrease in the concentration of cytosolic reactive oxygen species ($[ROS]_i$) in BMMs, both of which are abolished by N-acetyl-L-cysteine (NAC) treatment. (a, b) Under continuous perfusion with HEPES buffer, cells seeded on the (a) polished Ti and (b) TiO₂ nanotubes were, respectively, exposed to UV light (wavelength = 340 nm and 380 nm). Following UV exposure, $[ROS]_i$ (red line) and $[Ca^{2+}]_i$ (black line) levels were simultaneously measured and presented as described in “Section 2”. (c) The columns show the percentage of $[ROS]_i$ decrement compared to the initial intensity. (d) $[Ca^{2+}]_i$ response in cells seeded on TiO₂ nanotubes was measured in the presence of 10 mM of NAC. NAC diluted in regular HEPES buffer was treated for the indicated time and washed out with regular HEPES buffer. (e) The columns show $[Ca^{2+}]_i$ increment (F_{340}/F_{380}).

increase. Based on these key observations, we assumed that loss of $[ROS]_i$ might change membrane potential and activate voltage-gated Ca²⁺ channels (VGCCs). A previous report indicates that it is possible that UV photocatalysis of TiO₂ turns Ti into a semiconductor, allowing electrons-transfer reactions to occur [53]. To confirm this suspicion, we treated cells with nifedipine, an inhibitor of voltage-gated Ca²⁺ channels, and measured UV photocatalysis-mediated $[Ca^{2+}]_i$ increase and $[ROS]_i$ reduction. In Figures 3(a)–3(c), UV photocatalysis-mediated $[Ca^{2+}]_i$ increase was significantly attenuated by inhibition of VGCCs. However, nifedipine did not affect $[ROS]_i$. These results support our hypothesis that UV photocatalysis activates VGCCs and elicits a $[Ca^{2+}]_i$ increase and that $[ROS]_i$ reduction by UV photocatalysis may be involved in VGCC activation and $[Ca^{2+}]_i$ increase. Our previous study demonstrated that the Ca_v1A and Ca_v1D subunits, which are constituents of VGCCs, are the most highly expressed subunits. Further studies are necessary to determine how these molecules are involved in the effects observed after UV photocatalysis of TiO₂.

3.3. Phospholipase C (PLC) Activity Is Not Involved in UV Photocatalysis-Mediated $[Ca^{2+}]_i$ Increase and $[ROS]_i$ Reduction. ROS are highly reactive and can nonspecifically activate molecules in the plasma membrane or inside the cell. Diverse extracellular stimuli including hormones, neurotransmitters, and exogenous ROS function through PLC to mobilize Ca²⁺ from internal Ca²⁺ stores [54]. To determine whether UV photocatalysis-mediated $[Ca^{2+}]_i$ increase is mediated by PLC activation, cells on TiO₂ nanotubes were treated with U73122 to inhibit PLCs. When the cells on TiO₂ nanotubes were exposed to UV in the presence of U73122 (10 μM), $[Ca^{2+}]_i$ was not significantly increased compared to that in cells treated with HEPES buffer (Figures 4(a) and 4(b)). Moreover, inhibition of PLCs by U73122 did not affect UV photocatalysis-mediated $[ROS]_i$ reduction compared to that observed in a control treated with HEPES buffer (Figure 4(c)). These results demonstrate that UV photocatalysis-mediated $[Ca^{2+}]_i$ increase and $[ROS]_i$ reduction are not related to PLC activation. Considering previous results that showed that UV photocatalysis of TiO₂ mediates $[ROS]_i$ reduction and had no

FIGURE 3: Nicardipine, an inhibitor of voltage-gated Ca^{2+} channel, attenuates UV photocatalysis-mediated $[Ca^{2+}]_i$ increase but not $[ROS]_i$ reduction. Isolated BMMs were seeded on polished Ti and TiO_2 nanotubes and loaded with Fura-2/AM and CM-H2DCFDA. (a) UV photocatalysis-mediated $[Ca^{2+}]_i$ increase in cells on TiO_2 nanotubes was measured in the presence of nicardipine ($10 \mu M$) diluted in HEPES buffer. (b, c) The columns indicate $[Ca^{2+}]_i$ increment and $[ROS]_i$ decrement in BMMs.

FIGURE 4: U73122, an inhibitor of Phospholipase C, has no effects on UV photocatalysis-mediated $[Ca^{2+}]_i$ increase and $[ROS]_i$ reduction. Isolated BMMs were seeded on polished Ti and TiO_2 nanotubes and loaded with Fura-2/AM and CM-H2DCFDA. (a) UV photocatalysis-mediated $[Ca^{2+}]_i$ increase in cells on TiO_2 nanotubes was measured in the presence of U73122 ($10 \mu M$) diluted in HEPES buffer. (b, c) The columns indicate $[Ca^{2+}]_i$ increment and $[ROS]_i$ decrement in BMMs.

effects on PLC activity, we suggest that ROS generated by UV photocatalysis on TiO_2 have no permeability.

4. Conclusions

In summary, our study demonstrates that UV photocatalysis of TiO_2 immediately elicits $[Ca^{2+}]_i$ increase and $[ROS]_i$ reduction in cells growing on TiO_2 nanotubes. UV photocatalysis-mediated $[Ca^{2+}]_i$ increase is dependent on extracellular and intracellular ROS generation. Furthermore, extracellular Ca^{2+} influx thorough VGCCs is critical for UV photocatalysis-mediated $[Ca^{2+}]_i$ increase, while PLC activation is not. Considering the physiological roles of Ca^{2+} signaling in BMMs and osteoclastogenesis, nanotopography on the Ti surface should be considered an important factor that can influence successful dental implantation.

Conflict of Interests

The authors state that they have no conflict of interests.

Authors' Contribution

Seunghan Oh and Eun-Joo Choi contributed equally to this work.

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