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치의학박사학위논문

Osteoclastic responses on the titanium surfaces immersed in modified simulated body fluid

Modified simulated body fluid에 침전한 타이타늄 표면에서의 파골세포 반응 연구

2014년 2월

서울대학교 대학원
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지도교수 곽 재 영

이 논문을 치의학박사 학위논문으로 제출함

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김 문 형

김문형의 치의학박사 학위논문을 인준함

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위 원 장 (인)

부위원장 (인)

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위 원 (인)

위 원 (인)

-ABSTRACT-

Osteoclastic responses on the titanium surfaces immersed in modified simulated body fluid

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*(Directed by Professor **Jai-Young Koak**, D.D.S., M.S.D.,Ph.D.)*

Osseointegration is achieved by bone remodeling around the implant titanium (Ti) surfaces. This bone remodeling is a harmonious coupling of bone formation and bone resorption and requires a coordinated activity of osteoblasts and osteoclasts. Biomimetic deposition using modified simulated body fluid (SBF) substantially improved the surface wettability and surface energy of the Ti surfaces and also increased osteoblast responses on anodized Ti surfaces. We hypothesized that thin calcium phosphate (CaP) deposition onto Ti surfaces using biomimetic deposition led to not only improve osteogenesis but also suppress osteoclastogenesis in terms of high surface hydrophilicity. To understand bone remodeling on Ti surfaces with thin CaP coatings by means of immersion in modified SBF, we investigated the effect of biomimetic deposition on osteoclast differentiation activity on Ti surfaces.

Ti discs with two different surface topographies were prepared: machined and anodized surfaces. The specimens of two different surfaces were immersed in modified SBF solution for two weeks at physiologic condition of 37℃, initial pH 7.4, and p(CO_2) = 0.05 atm. The control groups were dry Ti (machined and anodized) discs not immersed in any solution. In this study we used murine RAW 264.7 cells as osteoclast precursor cells. To evaluate osteoclast differentiation activity on Ti surfaces, Tartrate-resistant acid phosphatase (TRAP) activity assay was performed. After 11 days of culture, cells on Ti surfaces were observed using a field emission-scanning electron microscope (FE-SEM) and confocal laser scanning microscopy (CLSM) to investigate osteoclast differentiation activity visually. The expression of nuclear factor of activated T cells 1 (NFATc1) and c-Fos, two critical transcriptional factors involved in osteoclastogenesis, were also assessed in terms of mRNA and protein levels by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot, respectively. The results were as follows:

1. TRAP activities on both machined and anodized Ti surfaces immersed in modified SBF were significantly lower compared to non - immersed ones.

2. FE-SEM and CLSM observation showed that the number of differentiated osteoclasts was lower on anodized surfaces immersed in modified SBF compared to Ti surfaces not immersed.

3. mRNA and protein expression of two osteoclastogenesis marker, NFATc1 and c-Fos,

were significantly decreased in anodized Ti surfaces immersed in modified SBF than in anodized dry surfaces.

4. The effects of immersion of Ti discs in modified SBF on osteoclastogenesis were higher in anodized surfaces than in machined surfaces.

These results indicate that osteoclastogenesis was inhibited by biomimetic deposition using modified SBF especially on anodized Ti surfaces.

Keywords: Anodic oxidation, Surface energy, Simulated Body Fluid (SBF), Biomimetic deposition, Osteoclast, Osteoclastogenesis

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CONTENTS

I. INTRODUCTION

The clinical success of dental implants is determined by a variety of factors such as biocompatibility of the implant material, macroscopic and microscopic nature of implant surfaces, the status of the implant bed, the surgical technique per se, the undisturbed healing phase, the subsequent prosthetic design and long-term loading phase.[1] Among these factors, implant surface characteristics are critical in the initial healing phase for successful osseointegration because the success of osseointegration is the formation of a hybrid function between titanium (Ti) and living hard and soft tissues[2] and the physical and chemical features of implant surfaces influence the interaction between implant surfaces and adjacent biosystem and consequently affect the rate, quantity and quality of osseointegration.[3] Surface chemical composition, hydrophilicity and roughness are important surface properties in implant–tissue interaction and osseointegration.[3,4]

Therefore, various attempts have been made to achieve rapid healing and early loading

by means of implant surface modification. Implant surface modifications have been performed in terms of surface microtopography or surface chemistry. There are numerous *in vitro* and *in vivo* studies that demonstrate that implants with moderate surface roughness have many clinical benefits, such as early fixation and long-term stability.[3,4,5,6] Consequently, dental implants with micro-level roughness are now generally accepted in the dental field. It has also been known that the surface energy and hydrophilicity of implant surfaces may be important during initial conditioning by proteins and during initial cell adhesion.[7] Sufficient wettability increases initial conditioning of the surface by blood components and affects subsequent cellular responses.[8] Therefore, hydrophilic surfaces with higher surface energy has been accepted to be desirable for bone implants.[9] As an example of the attempt to increase surface wettability and surface energy of Ti implants, a SLActive surface processed under nitrogen protection to prevent exposure to air and storing them in sealed tubes containing an isotonic NaCl solution was introduced.[7] The ultraviolet (UV) treatment of Ti surface is another attempt to increase surface wettability and surface energy of Ti implants.[10]

As a method for surface chemistry modification, biomimetic deposition was introduced to deposit thin calcium phosphate (CaP) coatings onto Ti implant surface.[11] Biomimetic deposition is a method in which a biologically active bone-like apatite layer is formed on a substrate surface by immersion of the substrate in simulated body fluid (SBF) under physiological conditions of temperature (37℃) and pH (7.4).[12,13] SBF is a solution with ion concentrations nearly equal to those of human blood plasma. Both serum and SBF are supersaturated towards apatite crystals and thermodynamically metastable. SBF was first introduced by Kokubo[12] as an *in vitro* test solution to predict the *in vivo* bonding ability of a material. Since its introduction, a small number of compositions of SBF solution have been developed and used in the study of biomaterials.[13] A previous study using modified SBF demonstrated that immersion of anodized Ti surfaces in modified SBF substantially improved the surface wettability and surface energy of the Ti surfaces and also increased osteoblast responses on anodized Ti surfaces, such as cell attachment capacity, cell proliferation rate, and initial cell differentiation.[14]

By the way, osseointegration which is defined as a direct structural and functional connection between living bone and the surface of a load – carrying implant[15], the successful healing of implants around bone, is achieved by bone remodeling around the implant surfaces.[11,16] This bone remodeling after implant placement is a harmonious coupling of bone formation and bone resorption and requires a coordinated activity of osteoblasts and osteoclasts.[17] It has been known that this osteoblast-osteoclast coupling is regulated by a complex signaling network mediated through cytokines, cell surface receptors, and various signal transducers.[18] Various studies using different animal models and different implant systems reported that early tissue response after implant placement proceeds as such[14,16,19,20,21,22]: After two to three days after Ti implants were inserted into bone, a

densely organized fibrin network with a mixture of red blood cells and bone fragments apposed the implant. At seventh day, the amorphous matrix and fibrin clot are replaced by a loose connective tissue stroma, and resorption lacunae with large osteoclasts are observed on the surface of the cortex closest to the marrow cavity. On the fourteenth day, intracortical remodeling of the old bone occurs within 1mm of the implant surface. Bone remodeling proceeds during the twenty-eight-day period and the new bone is remodeled and replaced by lamellar bone, which was completed six weeks to three months after insertion.[19,23] It has been generally accepted that osteoclasts and macrophages prepare or prime bone surfaces for the deposition of osteoid by osteoblasts.[24] Furthermore, bone is a dynamic tissue and the bone-implant interface is continuously remodeled to maintain the structural integrity of the osseointegrated implant.[16] Osteoclast activities near the surface of the implant and bone deposition over the resorbed surface were observed in the studies of loaded and unloaded Ti implants.[25,26] This late tissue response to Ti implants is the interfacial bone remodeling around the implant surface resulting from cellular activity initiated by the osteoclast. It is obvious that the osteoclasts may play an important role, not only in the early tissue response after implant placement but also in the late tissue response while in function.[16]

Osteoclasts are multinucleate giant cells which are responsible for the resorption of mineralized tissue and derived from the monocyte/macrophage haematopoietic lineage. Osteoclastogenesis is dependent upon a permissive microenvironment comprised of both cell-associated and soluble factors provided by stromal cells in the bone marrow or specialized osteoblasts lining the bone surface.[27] It is now known that two haematopoietic factors that are necessary for ostaoclastogenesis are the tumor mecrosis factor (TNF) - related cytokine receptor activator of nuclear factor kappa B ligand (RANKL) and the polypeptide growth factor colony-stimulating factor-1 (CSF-1) and subsequent activation of receptor activator of nuclear factor kappa B (RANK) on the surface of haematopoietic precursor cells should be done for osteoclast differentiation.[28] The mature multinucleated osteoclast is activated by signals, which leads to initiation of bone remodeling. Mature osteoclast is a highly motile cell that can migrate along bone surfaces, locate and attach areas to be resorbed, establish a sealing zone and form a highly invaginated ruffled membrane specialized for the secretion of protons and hydrolytic enzymes which resorb the mineral and organic constituents of bone, forming a resorption lacuna.[16]

Recent studies reported that a microrough implant surface topography increases osteogenesis by reducing osteoclast formation and activity[29] and that a modified SLA surface (SLActive) with improved wettability and hydrophilicity features promoted further osteogenesis at an *in vitro* level.[30] In another study about osteoblastic and osteoclastic response on SLActive surface, it has been known that hydrophilic SLActive surface promotes osteogenic effect and prevents osteoclastic differentiation.[31] These findings from recent studies demonstrated that osteoclastic activity be also significant in the bone remodeling

process surrounding implant surfaces and be investigated in addition to osteoblastic resposnes to characterize implant surfaces.

To achieve maximal and prompt peri-implant bone formation in the early healing phase, it is more advantageous for implant surfaces to have high osteogenic and low bone resorption activity. It is also required minimal peri-implant bone resorption for long term stability maintaining the marginal bone level of implants during the maintenance phase. Many studies on the osseointegration of implant surfaces have primarily focused on the bone forming osteoblasts, however, the role of bone resorbing osteoclasts during osseointegration and long-term maintenance has less investigated relatively. Likewise, the effect of biomimetic deposition on osteogenesis was previously elucidated but the effect of biomimetic deposition on osteoclastogenesis, an axis of bone remodeling, has not yet been described. To understand bone remodeling on Ti surfaces with thin CaP coatings by means of immersion in modified SBF, it is also important to elucidate not only osteoblastic responses but also osteoclastic responses as previously mentioned. Therefore, it is necessary to investigate the effect of biomimetic deposition on osteoclast differentiation and activity on Ti surfaces. We hypothesized that thin CaP deposition onto Ti surfaces using biomimetic deposition led to not only improve osteogenesis but also suppress osteoclastogenesis in terms of high surface hydrophilicity.

The present study investigated osteoclasts differentiation activity quantitatively and visually and assessed several important markers for osteoclastogenesis in mRNA and protein level especially on Ti surfaces immersed in modified SBF.

II. MATERIALS AND METHODS

1. Titanium disc preparation

All Ti discs were manufactured by cutting from Ti rods (commercially pure Ti grade III) and had uniform dimensions of 1mm in thickness and a diameter of 25mm (Warantec, Seoul, South Korea). To prepare machined surfaces, no further polishing procedure was performed on the surfaces of discs. Machined Ti discs were ultrasonically degreased for 20 minutes, cleaned in 99% ethanol for two cycles of 20 minutes, and then soaked in distilled water overnight. Anodized surfaces were prepared as follows. Anodic oxidation was performed with the use of a regulated DC power supply at a constant current mode of 70 A/m^2 and final voltage of 300 V at room temperature for 3 minutes. The electrolytes used for anodization were 0.15 M calcium acetate monohydrate and 0.02 M calcium glycerophosphate. After anodization, the specimens were rinsed with distilled water and absolute alcohol, dried. All the specimens (machined and anodized surfaces) were sterilized by ethylene oxide (EO) gas. The optical interferometer (Accura 2000, INTEK PLUS, Daejon, Korea) analysis was performed to measure for the surface roughness. The average surface roughness (Ra) was 0.541 ± 0.112 μm for machined surfaces and 0.873 ± 0.162 μm for anodized surfaces.

2. Modified simulated body fluid (SBF) preparation

Modified SBF in this study was prepared by the dissolution of several salts in distilled and deionized water, according to Bohner and Lemaitre's suggestion[32], in order to obtain reproducible results (Table I).

Table I. Dual solution preparation of modified SBF (amounts to a final mixture of 2L)

Starting materials			Weights of starting materials (g/L)	
			Modified SBF	
Formula	MW[g/mol]	purity[-]	Sol. A	Sol. B
NaCl	58.44	99.5%	6.213	6.213
$NaHCO_3$	84.01	99.5%	5.948	
KCl	74.55	99.5%	0.450	
$K_2HPO_4 \cdot 3H_2O$	228.22	99.0%	0.462	
$MgCl_2 \cdot 6H_2O$	203.31	98.0%	0.622	
$CaCl_2$	110.99	95.0%		0.584
Na_2SO_4	142.04	99.0%	0.144	
Volumes of HCl solution(ml/L)				
HCl 1.00M	Aq. Sol.	[mL/L]	0.850	0.850

In the present study, original Kokubo's SBF was modified as such: (1) Two thermodynamically stable stock solutions (Sol. A and Sol. B of Table I) were respectively prepared to prevent premature CaP precipitation and mixed just before use with a 1:1 volume ratio. (2) The solutions were filtered immediately after preparation (75mm, NALGENE, Rochester, NY, USA) and ultrafiltered during mixture (0.2 μm, NALGENE). (3) Instead of using buffer Tris(hydroxymethyl) amino methane (TRIS), a 5% CO_2 atmosphere was maintained to control the carbonate content of the SBF solution.

3. Immersion of Ti discs in modified SBF

The specimens of the two different Ti surfaces (machined and anodized) were immersed in modified SBF solution for two weeks at physiological conditions of 37℃, initial pH of 7.4, and $p(CO_2)$ of 0.05 atm. The specimens were kept in a vertical position inside polypropylene tubes containing 10 mL immersing solution (mixed amount of 5 mL of Sol. A and 5 mL of Sol. B), as previous studies suggested.[13, 33] Each tube contained one Ti disc. The control groups were dry Ti (machined and anodized) discs not immersed in any solution. The ionic composition of modified SBF is shown in Table II, together with the composition of human blood plasma. The specimens were divided into four groups according to surface topography and existence of immersing solution (modified SBF).

Group I: machined and dry
Group II: machined and immersed in modified SBF
Group III: anodized and dry
Group IV: anodized and immersed in modified SBF

After immersion, all the specimens were rinsed three times with distilled and deionized water and dried in a dessicator for two hours.

Table II. Ionic composition of modified SBF and blood plasma

Ion (mM)	Na	Cl^-	HCO^{3-}	K^+	Mg^{2+}	Ca^{2+}	HPO_4^{2-}	SO_4^{2-}
Modified SBF	142.0	117.6	35.2	5.0	1.5	2.5	1.0	0.5
Blood plasma	142.0	103.0	27.0	5.0	1.5	2.5	1.0	0.5

4. Cell culture

Murine RAW 264.7 macrophage/monocyte cells (TIB-71; ATCC) were cultured on the surfaces of Ti specimens placed at the bottom of the 6-well plates at 37℃ in a humidified atmosphere of

5 % CO_2 in Minimum Essential Medium Alpha (α-MEM, Gibco, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at a density of 0.5×10^4 cells/well and from the next day seeding 100 ng/ml of mouse RANKL was supplied. Medium and RANKL were exchanged every 2 days. Cells were cultured for 11 days at most. RANKL was purchased from Peprotech (Rocky Hill, NJ, USA).

5. Tartrate-resistant acid phosphatase (TRAP) activity assay

RAW 264.7 cells were seeded onto each Ti disc at a density of 5×10^4 cells/cm^2. They were cultured in α–MEM containing 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin.

After three and six days of culture, TRAP activity was measured using the TRACP assay kit (Takara, Kyoto, Japan) according to the protocol. The TRAP activity of the cultured cells was examined by a colorimetry-based assay utilizing the conversion of colorless p-nitrophenol phosphate (pNPP, Takara) to colored p-nitrophenol. The absorbance of the samples was measured spectrometrically at 405nm (Bio-Rad, Hercules, CA, USA). Measurements were performed in triplicate and repeated in six cultures (n= 6).

6. Field emission-scanning electron microscopy (FE-SEM)

Cells on each Ti disc were fixed with 2% glutaraldehyde in phosphate buffered saline (PBS) for 10 min at room temperature (RT) after 11 days of culture, following by staining in 2% osmium tetroxide in PBS for 20 min and dehydrated in an ascending ethanol series. After critical point drying, samples were sputter-coated with 6 nm platinum and then examined in the Field emission-scanning electron microscope (FE-SEM, Hitachi S-4700, Tokyo, Japan).

Ti surfaces were observed using a FE-SEM with an accelerating voltage of 15 kV at low and high magnification (× 100, × 2000). FE-SEM observation was performed at five different areas.

7. Confocal laser scanning microscopy (CLSM)

After 11 days of culture, cells were washed twice in PBS, fixed in 4% formaldehyde (Sigma-Aldrich, BUchs, Switzerland) for 10 min at RT, washed three times with PBS, permeabilized in PBST (0.25 % Triton X-100 in PBS) for 10 min, washed three times with PBS and blocked with 1 % BSA in PBS for 1 h at RT. The actin cytoskeleton was stained with phalloidin 1: 100 in PBS for 1h, and cell nuclei were stained with DAPI 1:1000 in PBS , for 15 min. Using CLSM, samples were imaged at three different spots each. 100 × magnification was used to observe overall differentiation activity of cells into osteoclasts on each Ti surface.

8. Real time reverse transcriptase-polymerase chain reaction (Real time RT-PCR)

Real time RT-PCR was performed to determine the gene mRNA expression levels in osteoclast cells for one reference gene: HPRT, and two target genes.

A. c-Fos (1 day after RANKL supplement)
B. NFATc1 (3 day after RANKL supplement)

Total RNA was isolated from cell samples after 1 and 3 days of RANKL supplement. Cells were lysed by adding 1 mL of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) per 5×10^6 cells. The homogenized samples were incubated for 5 minutes at RT to permit the complete dissociation of nucleoprotein complexes. A 0.2 mL of chloroform was added to each sample and the samples were mixed vigorously and then incubated at RT for 3 minutes. The samples were centrifuged at 12000g for 20 min at 4℃. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was precipitated from the aqueous phase by mixing with 0.5 mL of isopropyl alcohol. The samples were incubated at RT for 20 minutes and centrifuged at 12000g for 15 minutes at 4℃. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube. After removing the supernate, the RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 12000g for 10 minutes at 4℃. At the end of the procedure, the RNA pellet was dried for 10 minutes. RNA was dissolved in RNase‐free water and incubated at 55℃ for 10 minutes. Total RNA was quantifed using a Nanodrop Spectrophotometer (ThermoScientific Nanodrop Technologies, Wilmington, DE, USA).

1 μg of total RNA was reverse transcribed to cDNA at 42℃ for 50 min using the Superscript II reverse transcriptase (Invitrogen) that contains both oligo (dT), 2.5 mM dNTP, 5×FS buffer, 0.1M DTT, SSII enzyme, and RNase out.

The following mouse specific primers were used: HPRT [(F) 5′-CCACAGGGACTAGAACACCTGCTAA-3′, (R) 5′-CTTGTGGACTGTGTGACT-3′]; c-Fos [(F) 5′-CTGGTGCAGCCCACTCTGGTC-3′, (R) 5′-CTTTCA GCAGATTGGCAATCTC-3′], and NFATc1 [(F) 5′-CGGCTGCCTTCCGTCTCA TAG-3′, (R) 5′-CGGCTGCCTTCCGTCTCATAG-3′].

Real-time RT-PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) using SYBR green (Invitrogen) detection. Each reaction contained 5 μl of cDNA, 0.4 μl of the forward and reverse specific primers, 0.4 μl of Rox in a final volume of 20 μl. The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (3 min 95℃), followed by 40 cycles consisting of a denaturation step (15 s 95℃), an annealing step (15 s 60℃), and an extension step (30 s 72℃). Samples were run in triplicate. Relative levels of c-Fos and NFAcT1 were normalized to HPRT.

9. Western blot

Western blot for two proteins was performed from RAW 264.7 cells grown up on Ti discs after 0, 1, 2, and 3 days of RANKL supplement. β-actin was used as a loading control.

A. c- Fos
B. NFATc1

After washing with PBS, total cell lysates were harvested by lysing the cells with chilled RIPA buffer (10 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1% Triton X-100, 0.1% SDS and 1% deoxycholate). Total cell lysates were incubated for 20 min and centrifuged at 14,000 rpm for 20 min at 4℃. Harvested proteins were separated by 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was probed with specific antibodies and reactivity of immune complexes was detected by using enhanced chemiluminescence (ECL) reagents.

10. Statistical analysis

All data were analyzed by one – way analysis of variance and the *post hoc* t test (SPSS 21, IBM SPSS, USA). P-values less than 0.05 were considered statistically significant.

III. RESULTS

1. TRAP activity assay

TRAP enzyme activities on Ti surfaces were measured to evaluate osteoclast differentiation activity quantitatively. There were significant differences between the TRAP activities of cells grown on Ti surfaces as the result of modified SBF immersion on culture day 6. However, on culture day 3, there was no significant difference between groups (Figs. 1(a) and 1(b)). TRAP activity at 6 day was significantly lower for cells grown on anodized Ti surfaces immersed in modified SBF (Group IV) compared to non-immersed anodized Ti surfaces (Group III) ($p < 0.05$). Similarly, TRAP activity of cells on machined Ti surfaces immersed in modified SBF (group II) at 6 day was significantly lower compared to dry machined surfaces (group I) ($p < 0.05$). On the one hand, there was no significant difference of TRAP activity between anodized and machined surfaces.

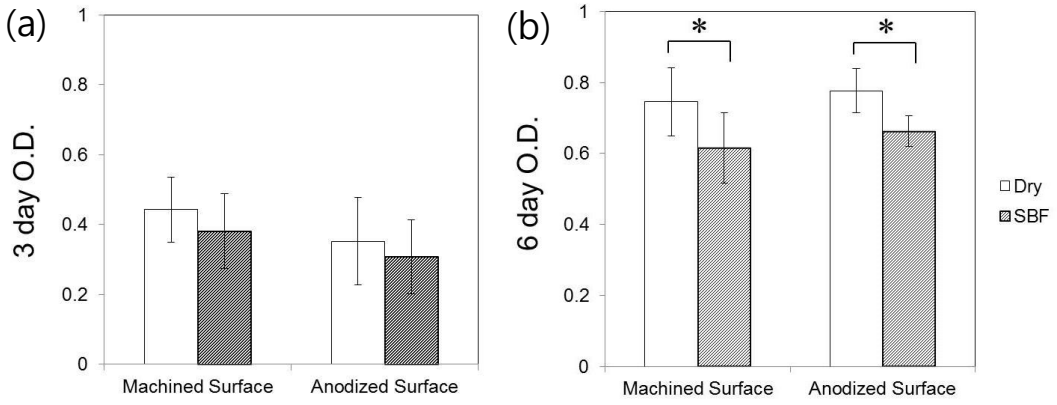

Fig. 1. TRAP activitis on Ti surfaces. The expression of TRAP was monitored among the group I – IV. (a) At 3 day, there was no significant statistical difference between groups. (b) Group II (Machined Surface, shaded bar) and group IV (Anodized Surface, shaded bar) showed lower value compared with group I and group III, respectively at 6 day. *$p < 0.05$

2. FE-SEM

FE-SEM was used to visualize and compare the distribution of differentiated osteoclasts on Ti surfaces. Fewer differentiated osteoclasts were observed on both anodized and machined Ti surfaces immersed in modified SBF compared to respective non-immersed surfaces (× 100) (Figs. 2 (a), 2(b), 2(c) and 2(d)). High magnification FE-SEM image (× 2,000) confirmed characteristic morphology of differentiated osteoclasts on group I and III (Figs. 2(e) and 2(f)).

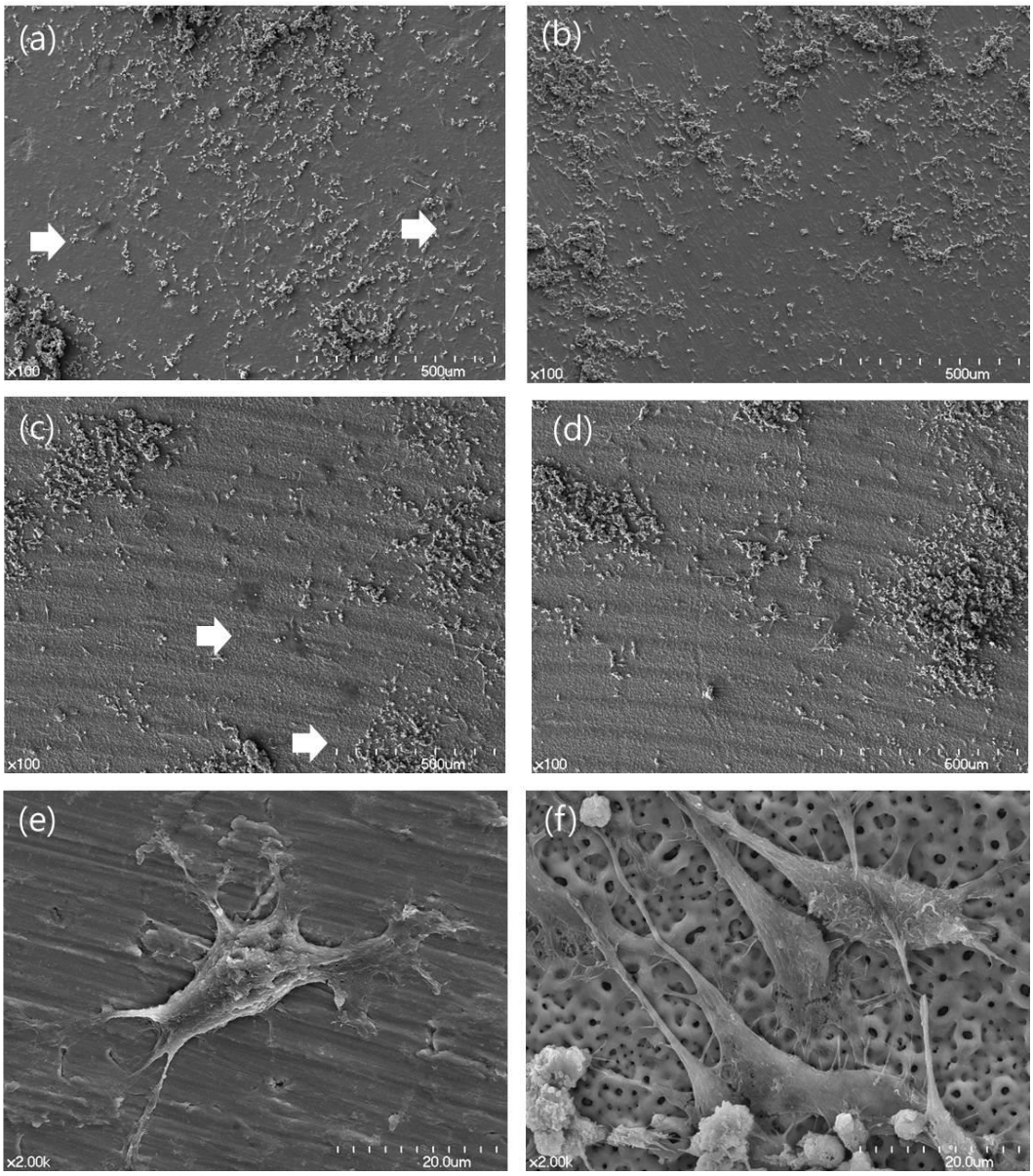

Fig. 2. FE-SEM photographs of RAW 264.7 cells cultured on Ti surfaces (a) machined and dry surface (magnification × 100), (b) machined surface immersed in modified SBF (× 100), (c) anodized and dry surface (× 100), (d) anodized surface immersed in modified SBF (× 100), (e) differentiated osteoclast on machined and dry surface (× 2,000) and (f) differentiated osteoclast on anodized and dry surface (× 2,000). Arrows in Fig. 2(a) and 2 (c) shows differentiated osteoclasts.

3. CLSM analysis

To examine the adhesion and morphology of differentiated osteoclasts on different Ti surfaces, cultured RAW 264.7 cells on Ti surfaces were immunostained for F-actin (cytoskeleton) and DNA (cell nuclei) and observed by CLSM.

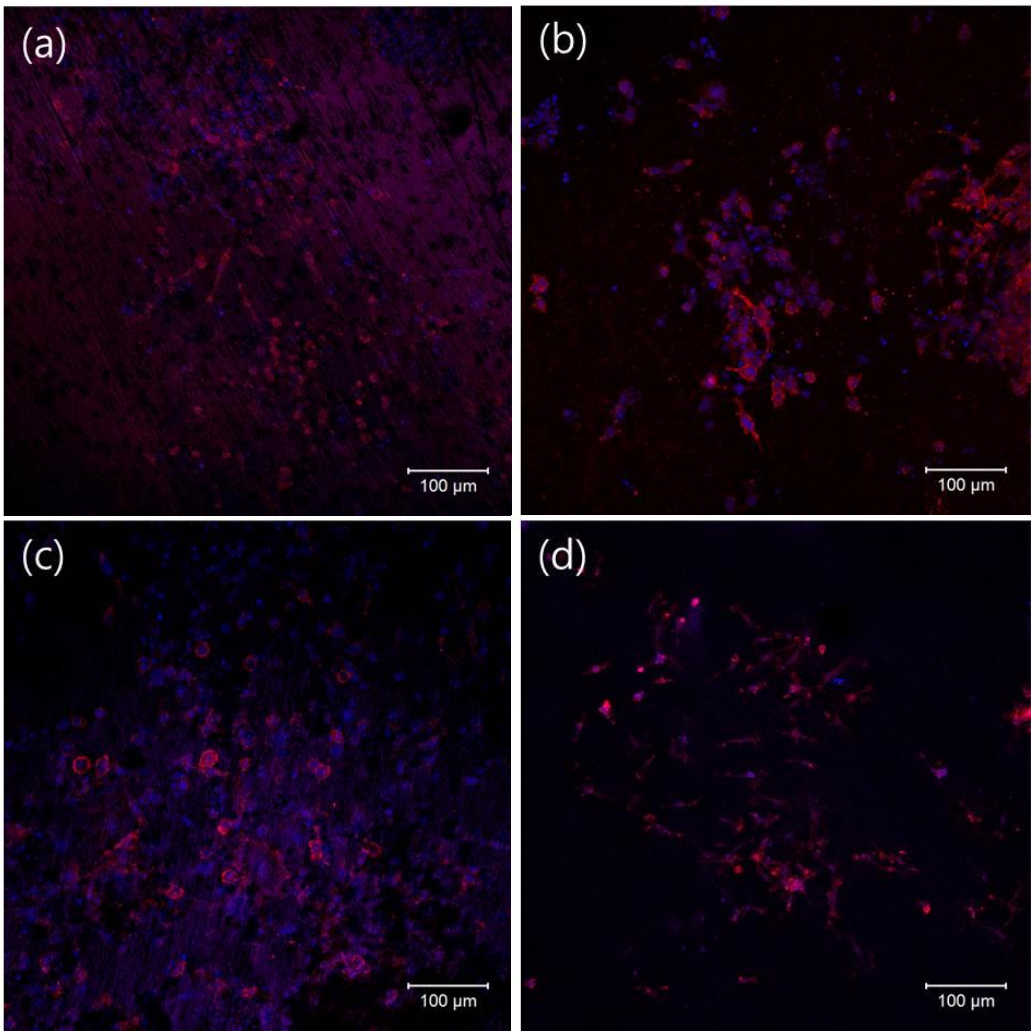

Fig. 3. Confocal photographs of RAW 264.7 cells cultured and immunostained for F- actin (red) and cell nuclei (blue) on Ti surfaces (a) machined and dry surface, (b) machined surface immersed in modified SBF, (c) anodized and dry surface, and (d) anodized surface immersed in modified SBF (magnification × 100).

No significant difference was observed between CLSM images of group I and group II (× 100) (Figs. 3(a) and 3(b)). Cells on both machined surfaces showed a similar mixed distribution of fusiform and round shaped cells. Fully or partially differentiated osteoclasts with podosome, actin ring, and cell nuclei more than three were uncommonly seen on the images of both groups. Whereas, fewer differentiated osteoclasts were found on anodized surfaces immersed in modified SBF (group IV) compared to dry anodized surfaces (group III) (× 100) (Figs. 3(c) and 3(d)). In case of group III, differentiated osteoclasts were observed. Cells with podosomes, which is found in premature osteoclasts, were found in anodized surfaces not immersed (group III). Actin rings which can be seen in activated osteoclasts and cells with nuclei more than three were observed on this surface. Furthermore, the number of attached cells to anodized surface immersed in modified SBF was much lower than that of non-immersed anodized surfaces.

4. Osteoclast differentiation marker analysis

The expression of NFATc1 and c-Fos, two critical transcriptional factors involved in osteoclastogenesis, were assessed in terms of mRNA and protein levels by real-time RT-PCR and western blot respectively.

4.1 mRNA expression assessment by real- time RT-PCR

To compare c-Fos mRNA level, which is expressed at the early phase of differentiation, real time RT-PCR of c-Fos was performed at the first day after RANKL supplement and that of NFATc1, triggered by c-Fos, at the third day in this study. c-Fos mRNA was significantly lower ($p < 0.001$) in cells grown on anodized Ti surfaces immersed in modified SBF compared to non-immersed anodized surfaces. However, there was no statistically significant difference between machined surfaces (Fig. 4(a)).

NFATc1 mRNA was also significantly lower ($p < 0.001$) in cells grown on anodized Ti surfaces immersed in modified SBF compared to non-immersed anodized surfaces. There was a similar tendency in case of machined surfaces ($p < 0.001$) (Fig. 4(b)).

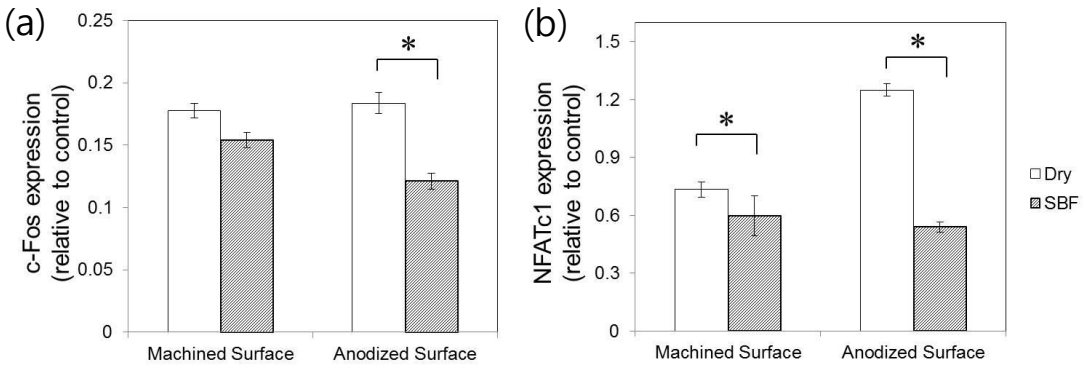

Fig. 4. The expression level of osteoclastogenic transcriptional factors. (a) c-Fos mRNA levels at 1 day. Only on anodized surfaces, c-Fos mRNA expression was significantly lower when immersed in modified SBF. (b) NFATc1 mRNA levels at 3 day. There were significant differences between the immersion and non-immersion groups. *$p < 0.001$.

4.2 Protein expression assessment by Western blot

c-Fos protein level reached a peak at 1 day after RANKL supplement and decreased with time, followed by NFATc1 induction. NFATc1 protein level reached a peak at 3 day after RANKL supplement. This tendency was observed in all experimental groups (Fig. 5).

No significant difference in c-Fos protein was observed on the machined surfaces according to the immersion in modified SBF. However in case of the anodized surfaces the treatment of immersion in modified SBF decreased c-Fos protein expression compared to the non-immersion group. In addition, NFATc1 protein at 3 day significantly decreased by the immersion in modified SBF on both surfaces. β-actin was used as a loading control.

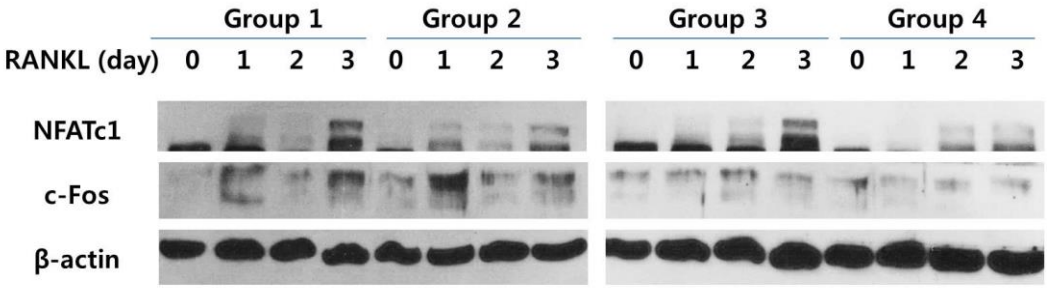

Fig. 5. Western blots of NFATc1, c-Fos and β-actin. Both machined and anodized surfaces showed significant differences of NFATc1 protein expression level according to the immersion in modified SBF, whereas in case of c-Fos protein there was significant difference only between anodized surfaces.

IV. DISCUSSION

The aim of the present study was to evaluate osteoclast differentiation activity on Ti surfaces with higher surface energy and improved wettability resulting by biomimetic deposition using modified SBF. It has been previously reported that osteoblastic responses to same Ti surfaces were upregulated, implying that biomimetic deposition using modified SBF has the positive osteogenic effect.[14]
The murine bone marrow macrophage cell line, RAW 264.7, differentiated by the addition of RANKL, is a widely accepted model and has been generally used for *in vitro* studies of osteoclastogenesis.[34] Therefore we used RAW 264.7 cells in this study as osteoclast precursor cells.

A prominent characteristic of the osteoclasts, which is most often used as an aid to its identification in tissue sections, is the presence of high amounts of the phosphohydrolase, tartrate-resistant acid phosphatase (TRAP).[16] TRAP is one of the lysosomal enzymes that osteoclasts secrete to perform their specialized resorption process. Acid phosphatase is an enzyme which hydrolyzes aliphatic and aromatic phosphate esters resulting in the release phosphates in the acid pHs. Potent acid phosphatase activity is found in the osteoclasts[35] and the acid phosphatase activity of osteoclasts was shown to be of the type that retains phosphatase activity in the presence of tartrate. Activated osteoclasts express the enzyme TRAP, which is also an important marker molecule for osteoblast activation.[16, 34]
TRAP enzyme activities were significantly lower on Ti surfaces immersed in modified SBF than on non-immersed Ti surfaces in both machined and anodized groups at 6 day after RANKL supplement. As such, both machined and anodized surfaces immersed in modified SBF showed lower TRAP activity than the control (dry) group at 3 day, but these were not significantly different. These results suggest that the differentiation activity of RAW 264.7 cells into osteoclasts may be significantly prevented by biomimetic deposition procedures using modified SBF. Low TRAP activity is related to the low osteoclast resorption activity and this may mean that on Ti surfaces immersed in modified SBF, less bone resorption results. Therefore, the negative influence of biomimetic deposition on osteoclast differentiation could be advantageous for the initial bone formation near Ti implant surfaces because of reducing the stability dip during the second and fourth week after implant placement.[31] On the one hand, there was no significant difference of TRAP activity between anodized and machined surfaces at 6 day compared to the case at 3day. This result has been associated with cell saturation according to cell culture period.

FE - SEM observation, as such, revealed that there were fewer differentiated osteoclasts on both machined and anodized Ti surfaces immersed in modified SBF compared to control group and confirmed the anti-osteoclastogenic effect of biomimetic deposition by modified SBF visually. Furthermore we also found that the number of attached cells was fewer on anodized surfaces than that on machined surfaces but a large number of differentiated osteoclasts were found on ano-

dized surfaces compared to that found on machined surfaces. The cells attached on anodized surfaces existed in scattered pattern.

CLSM image also showed a remarkable difference between cells grown on anodized surfaces. Cells grown on anodized surfaces immersed in modified SBF showed a relative low number of differentiated osteoclasts compared to Ti surfaces not immersed. Most cells on anodized surfaces immersed in modified SBF had an elongated fusiform appearance and formation of actin rings was rarely observed. Differentiated osteoclasts with more than three nuclei were also scarcely found. In case of group III, differentiated osteoclasts were observed. Cells with podosomes, which is found in premature osteoclasts, were found in anodized surfaces not immersed (group III). Actin rings which can be seen in activated osteoclasts and cells with nuclei more than three were observed on this surface. However, no significant difference between immersed and non-immersed machined surfaces was observed.

Osteoclasts have a well – developed cytoskeleton consisting of the cytoplasmic proteins actin, myosin, fimbrin, α-actinin, gelsosin, vinculin, and talin, which are organized, at the inner face of the cell membrane, to form specialized podosomal complexes.[16, 36] Activated mature osteoclasts are polarized multinucleated cells that form specialized adhesive structures, podosomes, formed by F-actin, which essentially mature into the sealing zone and bind the cell tightly to the bone surface. Sealing zones are microspically visible as ring-like actin structures near the interface of the cell to the substrate surface and its complete assembly is essential for bone resorption.[37] Therefore, CLSM image in this study repeatedly suggest that bone resorption may be suppressed on anodized surfaces immersed in modified SBF.

It can be thought that the low osteoclast differentiation activity on anodized surfaces immersed in modified SBF is associated with the low cell attachment capacity to these surfaces. A pilot study about the attachment of RAW 264.7 cells onto Ti surfaces was performed and that study showed that attachment capacity of RAW 264.7 cells decreased in anodized surfaces immersed in modified SBF compared to non-immersed ones.

To elucidate how biomimetic deposition influence on osteoclast differentiation activity in molecular level, the impact of biomimetic deposition to gene expression of two key transcriptional factors, NFATc1 and c-Fos, related to osteoclastogenesis was also assessed. For this assessment, mRNA and mature protein expression level of two transcription factors were investigated by real-time RT-PCR and western blot respectively.

Osteoclasts are derived from bone marrow monocyte/macrophage lineage precursor cells (BMMs) and two important cytokines are essential in the differentiation of precursor cells to osteoclasts: Macrophage colony-stimulating factor (M-CSF) and RANKL.[38,39,40] RANKL is expressed mainly as a membrane-bound form on osteoblasts and induces the signaling essential for precursor cells to differentiate into osteoclasts, whereas M-CSF, secreted by osteoblasts, is responsible for the survival signal in the cell proliferation and osteoclastogenesis.[38,39,40]

RANKL signaling (the binding of RANKL to its receptor RANK expressed on BMMs) activates

TRAF-6, c-Fos, and calcium signaling pathways, all of which are indispensable for the induction and activation of NFATc1, the master transcription factor for osteoclstogenesis[38,40] (Fig. 6). NFATc1 is the most strongly induced transcription factor gene following RANKL stimulation and autoamplifies its own gene by binding to its own promoter and its induction is dependent on c-Fos.[38,41,42] It is believed that the autoamplification of the NFATc1 gene is the most specific event in osteoclast differntiaion.[38] These signaling pathways triggered by RANKL also induce and activate two transcription factors such as activator protein 1(AP-1) through c-Fos, and nuclear factor kappa B (NF-kB) through TRAF-6. AP-1 transcription factors composed of c-Fos, Jun and ATF family members are critical for the induction of NFATc1 at the early phase of differentiation and important transcriptional partners of NFATc1. At the final stage of differentiation NFATc1 cooperates with AP-1 to induce osteoclast-specific genes such as TRAP, calcitonin receptor, and cathepsin K.[38, 41]

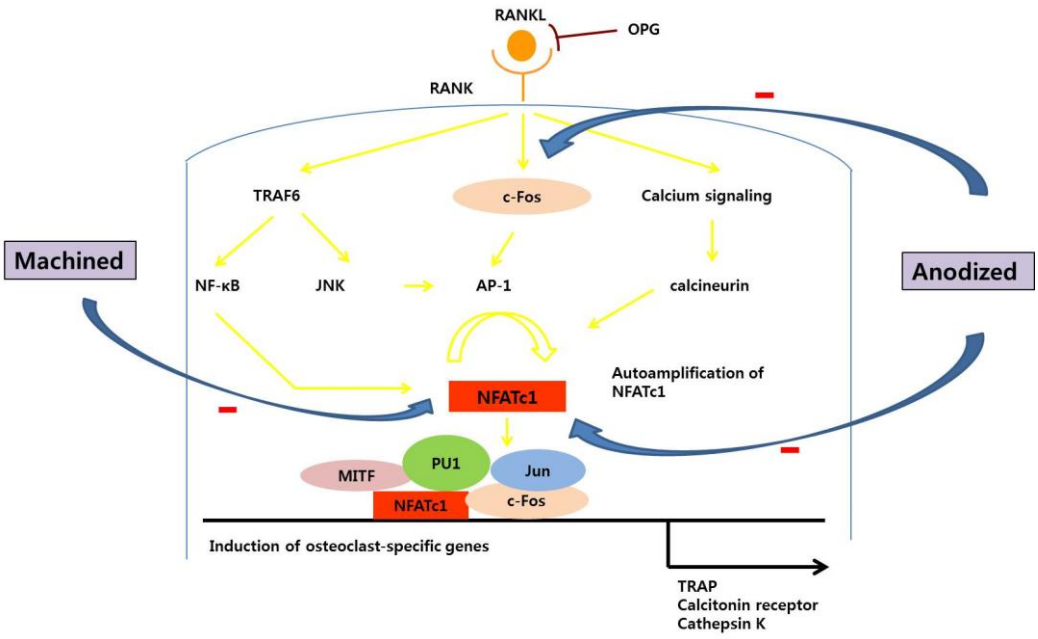

Fig. 6. RANKL-mediated osteoclastogenesis and effect of biomimetic deposition on the expression of two transcriptional factors.

NFATc1 mRNA level was found to be significantly lower in RAW 264.7 cells cultured on both anodized and machined Ti surfaces immersed in modified SBF, compared with corresponding Ti

surfaces not immersed. c-Fos mRNA level was also significantly lower on anodized surfaces immersed in modified SBF than control group. However, there was no significant difference between machined surfaces according to the immersion in modified SBF.

Similarly, NFATc1 protein expression level were significantly lower in RAW 264.7 cells cultured on both machined and anodized Ti surfaces immersed in modified SBF, compared with the Ti surfaces not immersed. However, likewise mRNA expression, c-Fos protein expression level decreased on anodized surfaces when immersed in modified SBF but this phenomenon didn't apply on machined surfaces.

In this way, mRNA and protein expression of two transcriptional factors, NFATc1 and c-Fos, has shown a consistent tendency depending on the immersion and the surfaces. While the expression of NFATc1 was downregulated on both machined and anodized surfaces when immersed in modified SBF, the expression of c-Fos was downregulated only on anodized surfaces under identical condition.

These results indicated that biomimetic deposition using modified SBF has the anti-osteoclastogenic effect on Ti surfaces via the RANK signaling network in the gene level and this effect is particularly noticeable in anodized Ti surfaces. From these results, it could be speculated that the difference of inhibitory effect between machined and anodized surfaces may be involved in calcium signaling pathway. NFATc1 can be induced directly via calcium signaling bypass, not by way of c-Fos signaling pathway (Fig. 6). Therefore, it can be thought that the immersion in modified SBF might inhibit calcium signaling pathway on machined surfaces unlike on anodized surfaces. However, to elucidate the concrete mechanism of the difference of inhibitory effect between machined and anodized surfaces immersed in modified SBF, further studies are needed.

All results (TRAP activity, SEM and CLSM observation, osteoclastogenesis-related gene and protein expression) have consistently shown that osteoclast differentiation activity was suppressed on Ti surfaces immersed in modified SBF. These phenomena were more remarkable on anodized surfaces than machined surfaces. From the findings of the present study, it could be suggested that osteoclastogenesis was inhibited by biomimetic deposition using modified SBF especially on anodized Ti surfaces. These surfaces have higher surface energy and improved wettability as it has been earlier reported.[14] The inhibitory effect of hydrophilic implant surfaces on osteoclast differentiation agreed with the findings of earlier studies.[31, 43] The anti-osteoclastic effect of hydrophilic implant surfaces was investigated in the SLActive surfaces.[43] In this study the osteoclastogenesis was regulated by modulating RANKL-RANK-OPG axis. Osteoclastic differentiation depends on activation of RANK (receptor of RANKL) present on osteoclast precursors. Also, it is controlled by relative level of sRANKL and osteoprotegrin (OPG: the osteoclastogenesis inhibitory factor), which is also a product of osteoblasts and prevents the binding of RANKL to its receptor RANK[39, 40, 44] (Fig. 6). Hence alterations in RANKL/OPG ratio are also critical to de novo bone formation and may control the process of osteogenesis.[43, 44] Previous studies revealed that the alteration in sRANKL/OPG ratio was also regulated by the implant sur-

face and consequently implant surface affected osteoclast formation and proliferation indirectly.[29, 31, 43]

Additionally, it should be considered that biomaterial particles as such CaP may elicit a heavy macrophage response which has been associated with inflammative bone resorption surrounding implant surfaces.[45] Therefore, there is an important significance in the present study that Ti surfaces with higher surface energy and improved wettability resulting by biomimetic deposition using modified SBF may not show inflammatory tissue response but have an inhibited osteoclast differentiation activity. However, the inhibitory mechanism of biomimetic deposition using modified SBF was not clearly elucidated in this study. Future studies are needed to unveil the anti-osteoclastic mechanism whether due to the alterations in RANKL/OPG ratio or due to the direct action on the genes of osteoclast precursor cells.

Although in vivo animal studies are also needed to identify the outcomes in this study, this study suggests that biomimetic deposition using effective SBF has anti-osteoclastogenic effect. Furthermore, it has previously reported that osteogenesis on anodized Ti surfaces was promoted by identical biomimetic deposition using same modified SBF.[14] This means that this surface has on the other hand positive- osteogenic effect. This difference between osteoblastic and osteoclastic responses on biomimetic deposited Ti surfaces may be involved in the difference of integrins expressed by two cell types. Integrins are transmembrane molecules composed of α chains and β chains that assemble noncovalently as heterodimers and are involved in cell-cell and cell-surface adhesion.[46, 47] Some integrins have been established as important mediators of bone cell function. In osteoclasts, $\alpha_v\beta_3$ integrin is essential for cell attachment to bone matrix proteins and for the formation of actin rings and cell spreading necessary for the bone resorbing activity of osteoclasts.[48] One of integrins expressed by osteoblasts and involved in bone formation is $\alpha_5\beta_1$, a cell-surface receptor for fibronectin implicated in cell spreading, proliferation, differentiation and survival.[48] It is regarded that this difference of integrins adhesion signals between cell types can provoke different cell responses to specific Ti surfaces.
Therefore, it is interpreted that anodized Ti surfaces immersed in modified SBF, with high surface energy and hydrophilicity, are more favorable in peri-implant bone formation clinically due to the stable and accelerated bone healing and osseointegration.

V. CONCLUSION

Based on findings from the present study, anodized Ti implant surfaces with high surface energy and improved wettability resulting by biomimetic deposition using modified SBF may be useful to achieve maximal and prompt peri-implant bone formation in the early healing phase and minimal peri-implant bone resorption during the maintenance phase.

TRAP activities on both machined and anodized Ti surfaces immersed in modified SBF were significantly lower compared to non - immersed ones. FE-SEM and CLSM observation showed that the number of differentiated osteoclasts was lower on machined and anodized surfaces immersed in modified SBF compared to Ti surfaces not immersed. Both mRNA and protein expression of two osteoclastogenesis marker, NFATc1 and c-Fos, were significantly decreased in anodized Ti surfaces immersed in modified SBF than in anodized dry surfaces. While the expression of NFATc1 was downregulated on both machined and anodized surfaces when immersed in modified SBF, the expression of c-Fos was downregulated not on machined but only on anodized surfaces under identical condition. The effects of immersion of Ti discs in modified SBF on osteoclastogenesis were higher in anodized surfaces than in machined surfaces.

These results indicate that osteoclastogenesis was inhibited by biomimetic deposition using modified SBF especially on anodized Ti surfaces.

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국문초록

Modified simulated body fluid에 침전한 타이타늄 표면에서의 파골세포 반응 연구

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임플란트 표면에서의 치유, 즉 골조직에서의 임플란트의 성공적인 결합인 osseointegration은 임플란트 주위에서 일어나는 골 리모델링에 의해 이루어진다. 이 골 리모델링 과정은 골 생성과 골 흡수가 조화를 이루는 과정이며 이를 위해 파골세포와 조골세포의 활성화의 균형이 이루어져야 한다. modified simulated body fluid (SBF)를 사용한 biomimetic deposition에 대한 기존 연구에 의하면 modified SBF에 침전된 타이타늄 디스크에서 친수성 및 표면에너지가 매우 증가하였다. 또한 modified SBF에 침전한 양극산화 타이타늄 표면에서 조골세포의 반응, 즉 조골세포의 부착, 증식, 분화가 매우 활발히 이루어짐이 보고되었다. 그러나 골 리모델링의 한 축을 이루는 osteoclastogenesis에 대한 이 표면의 효과는 아직 연구되지 않았다. modified SBF에 침전한 결과로 얇게 calcium phosphate coating 처리된 타이타늄 표면 주위에서의 골 반응을 이해하기 위해서는 이 표면에서의 조골세포 반응과 함께 파골세포의 반응을 연구하는 것 또한 중요한 의미를 지닌다. 따라서 biomimetic deposition을 통한 타이타늄 표면처리가 파골세포 분화와 활성에 어떤 영향을 주는 지 확인해볼 필요가 있다. 우리는 biomimetic deposition에 의해 타이타늄 표면에 thin calcium phosphate이 침전되어 표면 친수성이 증가되었을 경우 osteogenesis가 증가할 뿐만 아니라 osteoclastogenesis도 변화할 것이라고 가정하였다. 이 연구의 목적은 타이타늄 표면을 modified SBF로 biomimetic deposition 처리하여 표면에너지와 친수성을 변화시켰을 때 타이타늄 표면에서의 ostoeclastogenesis가 어떻게 달라지는지 평가하는 것이다.

두 종류의 다른 표면 특성을 가진 타이타늄 디스크를 준비하였다. 기계 가공과 양극산화 디스크를 2주 동안 37℃, 초기 pH 7.4의 생리적인 조건하에서 modified SBF에 침전하였으며 대조군으로 건조된 타이타늄 디스크를 사용하였다. 파골세포 전구세포로는 murine RAW 264.7 cell을 사용하였다. 타이타늄 표면에서의 파골세포 분화활성을 평가하기 위해 TRAP (Tartrate-resistant acid phosphatase) activity assay를 시행하였다. 타이타늄 표면에서의 세포 분포 및 분화 양상은 FE-SEM (Field emission- scanning elec-

tron microscopy)과 CLSM (Confocal laser scanning microscopy)를 이용하여 비교하였다. 또한 osteoclastogenesis에서 결정적인 두 가지 전사인자인 NFATc1과 c-Fos의 mRNA 및 단백질 발현 수준을 조사하기 위해 real time RT-PCR 과 western blot을 각각 실시하였다. 본 연구 결과는 다음과 같다.

1. TRAP activity는 기계 가공 표면과 양극 산화 표면 모두에서 modified SBF에 침전했을 경우 유의성있게 감소하였다.

2. FE-SEM과 CLSM 촬영 결과 양극 산화 표면에서 modified SBF에 침전한 경우에 분화된 파골세포의 수가 유의성있게 적었다.

3. 두 가지 osteoclastogenesis marker인 c-Fos와 NFATc1 의 mRNA와 단백질 발현은 양극 산화 표면에서 modified SBF에 침전했을 때 유의성있게 감소하였다.

4. osteoclastogenesis에 대한 modified SBF의 억제 효과는 기계가공 표면보다 양극 산화 표면에서 더 컸다.

이는 modified SBF를 이용한 biomimetic deposition이 양극산화 타이타늄 표면에서 osteoclastogenesis를 억제하는 효과를 가짐을 보여준다.

주요어: 양극 산화, 표면에너지, Simulated body fluid(SBF), 파골세포, Osteoclastogenesis
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