BIOLOGICAL RESPONSES OF OSTEOBLAST-LIKE CELLS TO DIFFERENT TITANIUM SURFACE BY ANODIZING MODIFICATION

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Statement of problem. To improve a direct implant fixation to the bone, various strategies have been developed focusing on the surface of materials. The surface quality of the implant depends on the chemical, physical, mechanical and topographical properties of the surface. The different properties will interact with each other and a change in thickness of the oxide layer may also result in a change in surface energy, the surface topography and surface, chemical composition. However, there is limited the comprehensive study with regard to changed surface and biologic behavior of osteoblast by anodization.

Purpose of study. The aim of this study was to analyze the characteristics of an oxide layer formed and to evaluate the cellular biologic behaviors on titanium by anodic oxidation (anodization) by cellular proliferation, differentiation, ECM formation and gene expression. And the phospholipase activity was measured on the anodized surface as preliminary study to understand how surface properties of Ti implant are transduced into downstream cellular events.

Methods and Materials. The surface of a commercially pure titanium(Grade 2) was modified by anodic oxidation. The group 1 samples had a machined surface and other three experimental specimens were anodized under a constant voltage of 270 V(Group 2), 350 V(Group 3), and 450 V(Group 4). The specimen characteristics were inspected using the following five categories; the surface morphology, the surface roughness, the thickness of oxide layer, the crystallinity, and the chemical composition of the oxide layer. Cell numbers were taken as a marker for cell proliferation. While the expression of alkaline phosphatase and Runx2 (Cbfa1) was used as early differentiation marker for osteoblast. The type I collagen production was determined, which constitutes the main structural protein of the extracellular matrix. Phospholipase A_2 and D activity were detected.

Results. (1) The anodized titanium had a porous oxide layer, and there was increase in both the size and number of pores with increasing anodizing voltage. (2) With increasing voltage, the surface roughness and thickness of the oxide film increased significantly ($p<0.01$), the TiO_2 phase changed from anatase to rutile. During the anodic oxidation, Ca and P ions were more incorporated into the oxide layer. (3) The in vitro cell responses of the specimen were also dependant on the oxidation conditions. With increasing voltage, the ALP activity, type I collagen production, and Cbfa 1 gene expression increased significantly ($p<0.01$), while the cell proliferation decreased. (4) In preliminary study on the relation of surface property and phospholipase, PLD activity was increased but PLA_2 activity did not changed according to applied voltage.

Conclusion. The anodized titanium shows improved surface characteristics than the machined titanium. The surface properties acquired by anodization appear to give rise more mature osteoblast characteristics and might result in increased bone growth, and contribute to the achievement of a tight fixation. The precise mechanism of suface property signaling is not known, may be related to phospholipase D.

Key Words
Titanium, Anodization, Biologic response, Gene expression, Phospholipase

P ure titanium (Ti) and titanium alloys are frequently used as dental and orthopedic implant materials because of their excellent mechanical strength, chemical stability, and biocompatibility.[1] Biocompatibility of titanium is closely related to the properties of the surface oxide layer, in terms of its structure, morphology and composition. Various physical and chemical treatments of the Ti surface have been proposed with a view to obtaining the most biocompatible implant surface. Among the techniques, which have been found to be beneficial to the biological performance of the implants, are increasing the surface roughness and the oxidation of Ti to form a TiO_2 layer on the surface. The method used in this study for preparing porous oxide surface was anodic oxidation, an electrochemical method. In anodic oxidation (anodization), the ionic current and the processes at the electrode lead to the growth of an eventually oxide film (anodic oxide). The rough and porous oxide layer can be formed by anodization. The chemical composition, crystallinity, roughness, and topography of the implant surface are changed after the anodizing procedure.[2] The response of cell and tissue to implant is affected by not only the chemical properties but also the surface topography or roughness of the implant surface. Therefore, we investigated TiO_2 layers with different thickness and roughness on the Ti surface, by controlling the applied voltage used in this study, The crystal structure, composition and morphology of the oxide layer were monitored with respect to the applied voltage for anodization. To identify the effect of anodization, the biological properties of the layers were evaluated by in vitro tests, in terms of the proliferation, differentiation, ECM formation, and gene expression. The preliminary study was also carried out to confirm the involvement of phospholipases during signaling of surface property modified by anodic oxidation treatment of Ti surface in MG63 cells.

MATERIALS AND METHODS

1. Ti disc preparation using anodic oxidation

Commercially available pure Ti (grade 2) machined into disks with dimensions of 23mm diameter and 1mm thickness. Prior to use, degreasing and acid prepickling of all discs were done. The pretreated discs were further processed, as described below, to produce surface of varying roughness.

Group 1: Machined, used as control.
Group 2: Anodized under constant voltage, 270V.
Group 3: Anodized under constant voltage, 350V.
Group 4: Anodized under constant voltage, 450V.

All discs were ultrasonically cleaned in trichloroethylene for 15min followed by soaking in 70% ethanol for 10 min three times and distilled water overnight. The samples were autoclaved and dried prior to cell culture experiments.

2. Characterization of oxide layer

The crystal structure and microstructure of the specimens were evaluated by X-ray diffraction (XRD; M18XHF-SRA, Mac Science Co., Yokohama, Japan) analysis and scanning electron microscopy (SEM; JSM-5600, JEOL, Tokyo, Japan), respectively. The composition of the surface layer was analyzed with EDS(INCA, Oxford, England). The surface roughness was measured by means of an optical interferometer (Accura 2000®,

Intekplus Co., Seoul, Korea). The area of measurement was $80\mu m \times 80\mu m$. Three parameters, the average roughness (R_a), 10 point average roughness (R_z) and Peak-to-Valley height (R_{max}) were used to characterize the roughness of the specimens. The oxide thickness was estimated by disc sectioning and the sectioned surface was examined with SEM. These images were also analyzed by image program (Image access, Bildanalys system AB, Sweden) for the measurement of oxide's thickness.

3. Biological properties

1) Cell culture

For experiments rat calvarial osteoblast-like cells and human osteosarcoma MG63 cells were used. Cells were isolated from calvaria of 1-2 day old rats and collected and cultured in α-minimal essential medium (α- MEM : Gibco, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotics (Gibco, USA). MG63 cells were obtained from the American Type Culture Collection (Rockvill, MD, USA). Cells were sedded of 2×10^5 cells in RPMI1640 medium (Gibsco BRL, Grand Island, NY, USA) 10% heat inactivated FBS and 10U/ml penicilline/streptomycin. Control group was cultured on the plastic culture surfaces. Experimental groups were cells cultured on the titanium surfaces in 6 well culture plates. For the RT-PCR and phospholipase detection experiments, cells were cultured on 8 discs of each group placed in 100 mm dishes (Corning, IL, USA). The media were changed every second day throughout the experiment. After reaching visual confluence, growth medium were suctioned, extra cells and growth medium were washed with PBS (phosphate buffered saline) solution. The discs of each group were transferred to new dishes and cells were har-

vested with a hand scrapper or trypsin-EDTA solutions.

2) Cell number

At visual confluence, the MG63 cells were washed twice with PBS and then transferred discs and released from the cultured surface by addition of 0.25% trypsin-EDTA. Cell suspensions from both trysinizations centrifuged at 500 $\times$ g for 10min. Cell number was determined using a Coulter counter (Hialeach, FL, USA). Cells harvested in this manner exhibited more than 95% viability based on trypan blue exclusion.

3) Alkaline phosphatase activity

The ALP activity was determined colorimetrically using an ALP kit as described by the manufactures (Sigma, USA). At visual confluence of rat calvarial osteoblast cells, alkaline phosphatase activity was assayed as the release of ρ-nitrophenol from ρ-itrophenylphosphate at pH 10.4. The reaction was stopped after 30 min using 2N sodium hydroxide and the absorbance read was performed at 405nm using ELISA reader (Bio-tek, USA). The specific activity was calculated referring to protein concentration of lysates. Experiments were performed in 6 replicates, and the activity was averaged from four independent experiments.

4) Type I collagen measurement

The type I collagen production was determined in rat calvarial osteoblast. Collagen assay was based on the binding of a dye, Sirius Red F3BA (Chroma, Stuttgart, Germany), to the triple helical collagen fibril. At visual confluence of rat calvarial osteoblastlike cells grown on each Ti surface groups, 50 μl/well of cell lysate was dispersed into 24-well plates, incubated at 37℃ for 16 hours in humidified atmosphere and then for 24 hours in dried atmosphere. The staining was performed

with 100 µl/well of 0.1% Sirius Red F3BA in saturated picric acid for 1 hour at room temperature. The plates were rinsed five times with 200 µl of 10mM HCl, then washed with 0.1M NaOH and absorbance was read at 540 nm. The optical density values were compared to the standard curve of type I collagen samples.

5) RT-PCR

MG63 cells were cultured (1 × 10⁶cells) on to titanium surfaces for 1, 3 and 1 day after visual confluence (6~7days). Total cellular RNA was extracted using the RNeasy Protect kit (Qiagen, Germany), DNAse 1-treated then quantified by measuring absorbance at λ_{260} nm on a UV160U spectrophotometer. For the first strand cDNA synthesis, an initial RT mixture was treated and incubated at 65℃ for 5 minutes, then quenched on ice. 10µl of diluted cDNA was transferred into a 10µl PCR reaction mixture that contained 5pmol/µl of sense and antisense oligonucleotide primers, 1 × PCR buffer(10mM Tris-HCl, 50mM KCl, 1.5mM MgCl₂), 0.1mM dNTPs, and 0.5U TaqDNA polymerase. The forward primer (5-TCTG-GCCTTCCACTCTCAG T-3) and reverse primer (5-TATGGAGTGCTGCTGGTCTG-3) of Runx2 were synthesized based on the Runx2 mRNA sequence. Amplification reactions for the Runx-2 cDNA and the house keeping gene glyceraldehydes phosphate dehydrogenase (GAPDH) were carried out. Amplifications were performed using a thermal cycler (Eppendorf, Germany), with the temperature cycling being set as follows: 94 ℃ for 60s, 58℃ for 30s, 72℃ for 60s: 5cycles, 94℃ for 30s 56℃ for 30s, 72℃ for 60s: 5cycles, 94℃ for 30s, 54℃ for 30s, 72℃ for 60s: 25cycles, followed by a final extension at 72 ℃ for 10minutes. PCR products were analyzed by 1% agarose gel electrophoresis containing 0.01% ethidium bromide. Visualized PCR product bands were sliced from the gel and fluorescence within the gel was detected using a Lumi-imager F1 Workstation (Roche Molecular Biochemicals, Indianapolis, IN). The relative intensity of each band was determined and analyzed as the relative abundance of Runx2(Cbfa1)/ GAPDH.

6) Phospholipase assay

(1) PLA₂ assay

PLA₂ activity was assayed as described elsewhere,[3] except that, for separation of [³H]arachidonic acid (AA), authentic AA was added and a heptane/diethylether/acetic acid (60:40:2) solvent system was used. MG63 cells in RPMI containing FBS were plated on different surface of Ti disc and tissue culture plastic to a density of 2 × 10⁵ cells per dish and were cultured for visual confluence of 80% at 37℃. Then, 2 mCi/mL of [³H]AA were added to each well, incubated for 6 h washed at least twice with PBS containing 10% FBS. The [³H] AA released into the medium measured using liguid scintillation counter. At the end of the experiments, the cells were solubilized with 0.5 mL of methanol to determine the intracellular total incorporation. Counts were corrected for intracellular pools of AA.

(2) PLD Assay

PLD activity in intact cells was determined by measuring PBt, the unique transphosphatidylation product of PLD in the presence of n-butanol, as described previously.[4] After culturing on tissue culture plate and anodized Ti surface at 37 ℃ to visual confluence of 80~90%, cells were labeled to [³H]myristic acid and incubated for 3 hours and were resuspended in the assay medium supplemented with 0.3 % n-butanol. [³H]PBt produced was separated on a TLC plate using a solvent system of ethylacetate/isooctane/acetic acid/water (13/2/3/10) and counted in a liquid scintillation counter (Wallac, Finland).

RESULTS

1. Surface analysis of Ti modified by anodization

1) Surface morphology

The surface morphologies of the Ti after anodization using different treatment conditions are shown in Fig. 1. Before the oxidation treatment, only the machining grooves were observed on the surface. When a pulsed DC field of 270V was applied, a porous oxide layer became uniformly porous. The resulting layer was actually composed of small craters with holes at the center. When the voltage was increased to 350V, the size of these craters became so large that they were connected together, and the presence of tiny cracks was observed. When the voltage attained 450V, the size of the holes, as well as that of the craters, became much larger. Table I shows the average pore size of the experimental specimens.

2) Surface Roughness

Table II is showed the optical interferometer analysis results. The anodized discs showed a rougher surface than the machined discs. The Ra value of the anodic groups was significantly higher than that of machined group (p<0.05). As the applied voltage increased, the values of R_a, R_z and R_{max} increased.

3) Oxide thickness of anodized Ti discs

The oxide layer which was anodized at the higher voltage, had a thicker oxide layer. Table III
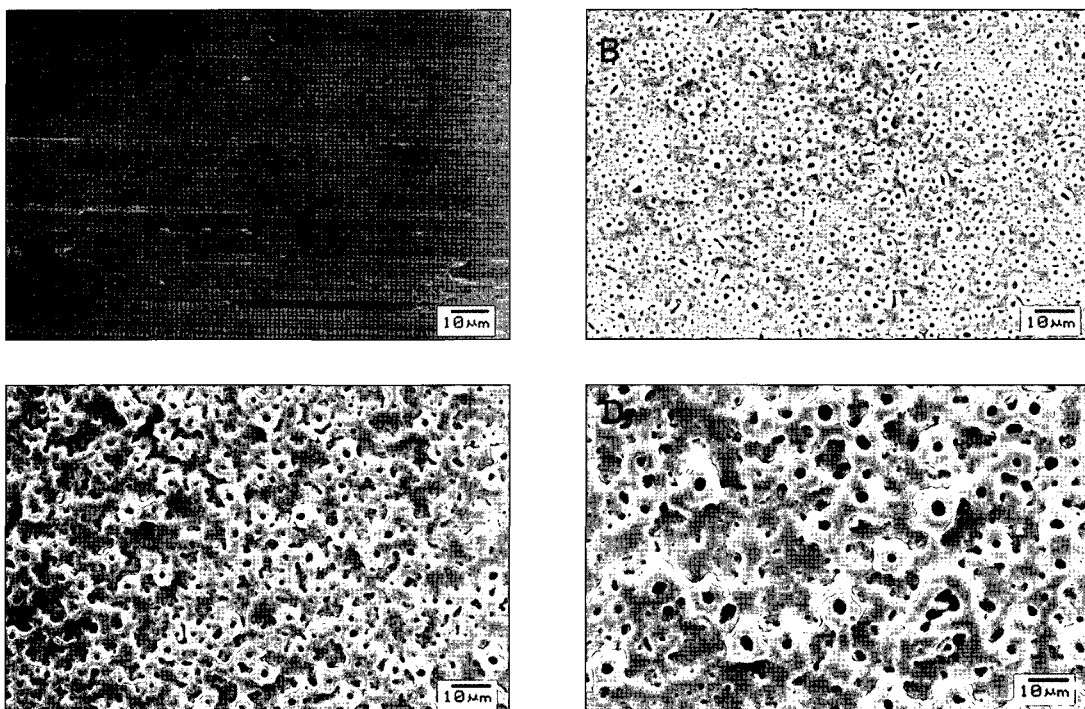

Fig. 1. SEM surface morphologies of Ti surfaces treated with anodization at different voltages: (A) machined (B) 270V, (C) 350V and (D) 450V. Magnification × 1000

Table I. The pore size of the experimental group specimens

	A 270 V	A 350 V	A 450 V
Mean ± SD (μm)	0.79 ± 0.27[a]	1.87 ± 0.45[b]	2.63 ± 0.62[c]

Data were expressed as mean values and standard deviation (S.D.).
Statistical significance were tested by ANOVA (P<0.01).
The matching letters indicate significant difference between groups based on Scheffe's multiple comparison test.

Table II. The surface roughness(Ra) of the titanium discs

Groups	average roughness (Ra, μm)	10 point roughness (Rq, μm)	Peak-to-Valley height (Rmax or Rt μm)
Machined (M)	0.608 ± 0.107[a]	0.619 ± 0.095[a]	4.468 ± 1.003[a]
Anodized (A270V)	0.897 ± 0.121[b]	1.112 ± 0.139[b]	9.105 ± 1.024[b]
Anodized (A350V)	1.719 ± 0.197[c]	2.549 ± 0.304[c]	19.783 ± 1.248[c]
Anodized (A450V)	2.543 ± 0.318[d]	3.425 ± 0.478[d]	24.569 ± 2.451[d]

Data were expressed as mean values and standard deviation (S.D.).
Statistical significance were tested by ANOVA (P<0.01).
The matching letters indicate significant difference between groups based on Scheffe's multiple comparison test.

Table III. The average thickness of the oxide layer of each specimen

	A 270 V	A 350 V	A 450 V
Mean ± SD (μm)	2.89 ± 0.42*	7.07 ± 1.02*	10.19 ± 2.45*

Data were expressed as mean values and standard deviation (S.D.).
Statistical significance were tested by Kruskall-Wallis test.
* indicates significant difference on machined group (P<0.01).

is showed the average thickness of the oxide layer of each specimen. With increasing voltage, the layer became significantly thicker.

4) Phase and structure of oxide layer

The phase of the oxide layers formed by the anodic oxidation process was characterized by XRD analysis, as shown in Fig. 2. Group 1 specimens showed peaks of titanium substrate on X-ray diffraction pattern. They did not show features of the crystal structure of the oxide film. The Group 2 specimens showed many anatase peaks (Fig. 2).

After further increasing the voltage, the intensity of the anatase and rutile phase increased. In conclusion, the Group 1 specimens have an amorphous oxide layer. The Group 2 specimens have an anatase structure while Group 3 and 4 specimens have an anatase and rutile structure in the oxide layer.

5) Chemical Composition

The chemical composition of the surface layer formed by the anodic oxidization process was determined by EDS analysis, as shown in Table

IV. Oxygen and titanium were the major elements in the oxide layers. The concentrations of Ca, P and O increased with increasing voltage, while that of Ti decreased steadily.
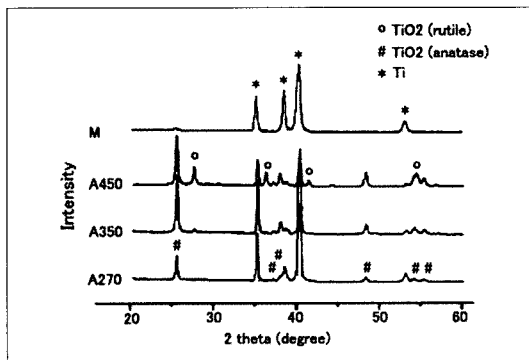

Fig. 2. XRD patterns of machined and anodized specimens.

2. Biologic response of osteoblast-like cells grown on anodized Ti

1) Cell numbers in MG63 cells

The number of cells was counted after culturing them for 7 days (1day after visual confluence). In all cases, the cells proliferated at least 10 times compared to the originally plated cells. When the Ti was anodized at 270V, the proliferation rate was the highest but significantly low than machined Ti surface. As the voltage used for the oxidation process increased, the number of cells decreased steadily (Table V).

2) The ALP activity and type I collagen production in rat calvarial osteoblast cells

The differentiation behavior of the cells was quite different from the proliferation behavior. The ALP activities of the rat calvarial osteoblast-

Table IV. The chemical composition of oxide layer of experimental group

Element	Machined	A 270 V	A 350 V	A 450 V
Carbon(%)	8.46±1.47	6.05±1.49	5.52±2.15	4.95±1.49
Oxygen(%)	20.86±2.40	61.60±1.61	63.51±1.67	64.12±1.71
Phosphorus(%)	not detected	2.35±0.15	2.67±0.18	3.26±0.26
Calcium(%)	not detected	1.11±0.26	2.48±0.13	5.15±0.25
Titanium(%)	70.68±2.64	27.60±0.56	25.92±0.71	22.52±0.56

Data were expressed as mean values and standard deviation (S.D.).
All anodic groups were significantly different on machined group based on Kruskall-Wallis test ($p<0.01$)

Table V. Number of MG63 cells after culturing for visual confluence

Groups	Cell number (10^6 cells)	% of cell number
Control (tissue culture plate)	1.885 ± 0.098	100 ± 5.1[a]
Machined (M)	1.603 ± 0.096	86.4 ± 5.1[b]
Anodized (A 270V)	1.541 ± 0.077	81.8 ± 4.1[b]
Anodized (A 350V)	1.246 ± 0.092	66.1 ± 4.9[c]
Anodized (A 450V)	1.046 ± 0.085	55.5 ± 4.5[d]

Data were expressed as mean values and standard deviation (S.D.).
Statistical significance were tested by ANOVA ($P<0.01$).
The same letters indicate non-significant difference between groups based on Scheffe's multiple comparison tests.
Each set of tests was performed in 6 replicate with 4 independent experiments.

Table VI. ALP activity and type I collagen of rat calvarial osteoblast-like cells

Groups	total protein (mg / ml)	ALP activity (sigma U/10μg)	type I collagen (μg / ml)
Machined (M)	1.062 ± 0.015^a	3.148 ± 0.153^a	0.418 ± 0.015^a
Anodized (A 270V)	1.018 ± 0.010^b	3.396 ± 0.163^b	0.467 ± 0.022^b
Anodized (A 350V)	0.901 ± 0.019^c	3.963 ± 0.149^c	0.498 ± 0.024^c
Anodized (A 450V)	0.879 ± 0.014^c	4.318 ± 0.198^d	0.541 ± 0.022^d

Data were expressed as mean values and standard deviation (S.D.).
Statistical significance were tested by ANOVA ($P<0.01$).
The same letters indicate non-significant difference between groups based on Scheffe's multiple comparison tests.

like cells cultured for 1day after visual confluence (6~7days) on various specimens are shown in Table VI. The ALP activity was not much affected by the aodization process when the applied voltage was 270V than machined Ti surface. However, when the voltage was 350 and 450, the activity of the cells significantly increased rapidly. Table VI also showed the amounts of type I collagen produced by primary rat calvarial osteoblast-like cells at 1day after confluent cultures. The type I collagen productions of anodized Ti were higher those on the MA surface ($P<0.01$).

3) Cbfa 1 (Runx 2) gene expression in MG63 cells
To investigate the potential influences of anodized treatments on the osteogenic key marker Runx2 gene expression, MG63 cells were incubated on different discs for 1 days, 3 days, and 1day after visual confluence. The expression of Runx2 gene was examined using Reverse transcription PCR and the electrophoresis result is shown in Fig. 3. The Runx2 gene expression increased consistantly from 1 day to 1day after visual confluence in all groups. The Runx2 mRNA from cells cultured showed highest level expression in 450 V anodized surface discs. The time course results demonstrate more rapid Runx2 expression in cells grown on 450 V anodized surface at day 1 of

culture, on 350 V anodized surface at day 3 of culture, on machined surface at day 5 ~ 6 (1 day after visual confluence) of culture. The patterns of the gene expression with anodized surface treatment were noted along the time table. These results were identified with relative intensities measured from transilluminated, ethidium bromide stained gels (Table VII).

4) The phospholipase activity in MG63 cells
In order to examine the role of phospholipase in mediating cell signaling by surface property of Ti, the PLA2 and PLD activity was measured after culturing on anodized surface of Ti response with respect to proliferation or differentiation of osteoblast. Phospholipase A2 (PLA2) hydrolyzes the acyl group of glycerophospholipids, generating free fatty acids and lysophospholipids. The products of the PLA2-catalyzed reaction can potentially act as second messengers, or be further metabolized to eicosanoids, platelet-activating factor, and lysophosphatidic acid. Neither machined Ti surface nor anodized Ti surface activated the release of arachidonic acid in a significant manner (Fig. 4). Furthermore, we did not observe any significant increase in PLA2 activity for rougher sandblasted and electric discharge machined specimens (data not shown).
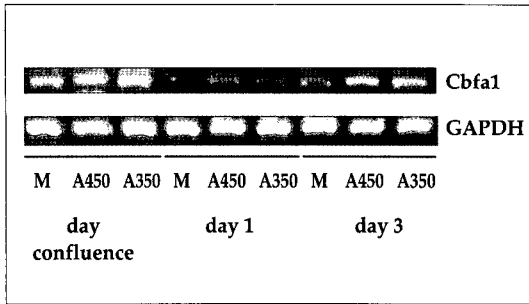
Fig. 3. Cbfa1 (Runx2) mRNA expression in MG63 cells grown on machined and anodized surface.

Under physiological conditions, PLD is the primary enzyme for the hydrolysis of phosphatidylcholine, which leads to the generation of choline and phosphatidic acid. We next examined PLD activity in response to modified surface by measuring PBt formation in the presence of 1-butanol. The MG63 cells grown on A450V surface resulted in a fourfold increase of PLD activity (Fig. 7), in contrast, there was no significant activation of PLA2. As increase of surface roughness and oxide layer by anodization, PLD activity was stimulated with surface-dependent pattern.

Table VII. The calculated Runx2/GAPDH relative intensities(%)

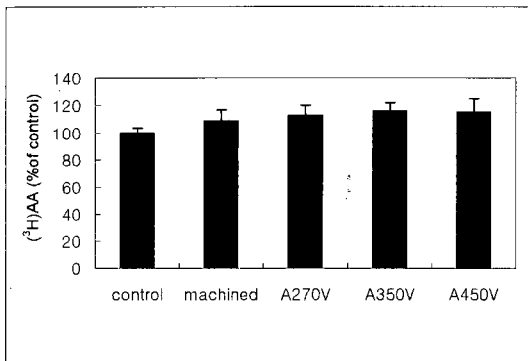| Culture period	Intensities	Machined	Anodized(A450V)	Anodized(A350V)
1 Day	Runx2/GAPDH	0.29	0.46	0.39
3 Day	Runx2/GAPDH	0.40	0.62	0.57
	Δ(increase)	0.11	0.16	0.18
1 day after	Runx2/GAPDH	0.58	0.79	0.69
visual confluence	Δ(increase)	0.18	0.17	0.12

Fig. 4. PLA2 activity in MG63 cells grown on modified Ti surface.
The results are plotted as a percentage of the PLD activity in control cells and represent the means±SD of four independent experiments with 6 replicates. Statistical significance were tested by ANOVA (P>0.05).
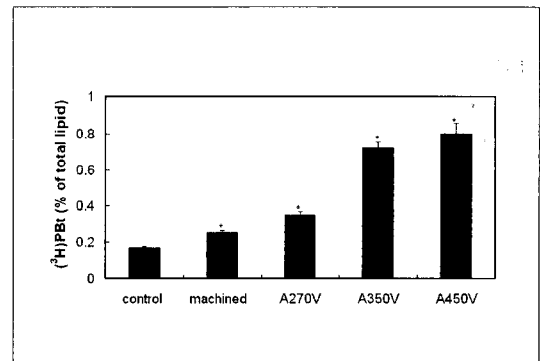

Fig. 5. Stimulation of PLD activity by anodized surface in MG63 cells.
The results are plotted specific PLD activity as a the percentage of [³H]PBt in total lipid and represent the means ± SD of four independent experiments with 6 replicates. Statistical significance were tested by ANOVA. The asterica indicate significant difference between groups (P<0.01).

DISCUSSION

The properties of the oxide layer, such as its thickness, microstructure, roughness, and concentrations of Ca and P are easily controllable by adjusting the voltage, current, processing time, and the concentration of the electrolyte during the anodic oxidization.[5-8] The microstructural change of the oxide layer was found to be closely related to the voltage used for the anodization. Increasing the voltage resulted in an increase in both the roughness and pore size, as well as in the thickness of the oxide layer (Table I and Table II). The phase and composition changes were also closely related to the processes involved in the anodization. With increasing voltage, rutile TiO_2 was formed along with the anatase (Fig. 2). Analogous behavior was observed during the thermal oxidation of Ti; at low temperature, anatase is formed, and at higher temperatures, the rutile phase becomes more stable than the anatase phase.[1] During the anodic oxidation, the energy supplied to the system is proportional to the applied voltage. Therefore, the formation of rutile TiO_2 at higher voltage is a reasonable phenomenon. The concentrations of Ca or P ions in the oxide layer are strongly dependent on the applied voltage (Table IV). When the applied voltage was increased, more Ca or P ions present in the electrolyte were incorporated into the oxide layer.

The changes in chemical composition, oxide layer and roughness of the Ti surface played crucial roles in the biocompatibility of the implant. Cell numbers were taken as a marker for cell proliferation. While the expression of alkaline phosphatase and Runx2 (Cbfa1) was used as early differentiation marker for osteoblast. The type I collagen production was determined, which constitutes the main structural protein of the extracellular matrix and thus may be a reliable indicator of bone matrix synthesis. The prolifera-

tion rate was highest when the specimen was oxidized at the relatively low voltage of 270V, and it decreased steadily with increasing voltage (Table V). Even though there was some variation depending on the applied voltage, the number of cells increased more than 10 times compared to the originally plated cells. These proliferation results simply indicate that all of the specimens offered a biologically favorable environment. In contrast to the proliferation behavior, the ALP activity of the cells increased when the applied voltage was increased (Table VI). Generally, surface roughness affects cell proliferation and differentiation: increasing surface roughness decreases the cell proliferation rate, but increases ALP activity.[9,10] Our result shows that the roughness and the amount of Ca and P ions incorporated into the titanium oxide layer strongly affect the cell response. Especially, the ALP activity significantly increased at higher voltages, which is deemed to be closely related to the increase in surface roughness and the increased amount of Ca and P contained in the oxide layer. There were controversies about the relationship of oxide thickness and cellular responses. Ellingen and Videm[11] showed that there was no significant correlation between oxide thickness of titanium implants and the bony responses. They explained the reason why the outermost molecular layer of the biomaterials is the most important part for the bone response, because the only surface of oxide layer is exposed to the tissue. But, Li et al.[12] reported that the level of ALP activity was higher in the anodized group with higher roughness and thicker oxide layer than in MA groups and described that the anodized surface was more favorable than other surface in osteoblast differentiation. In this study, thicker oxide film between aodized groups showed higher alkaline phosphatase activity. Our results suggested that rougher surface and surface with thick oxide film were more

favorable than other surfaces in osteoblast differentiation. In present study, collagen production on anodized surface was significantly high level. It seems that collagen synthesis was clearly influenced by oxide layer and surface roughness. Previous studies that have supported similar responses have already been reported for osteoblast-like cells.[13-15] The cellular growth and type I collagen production was influenced either by the chemical nature of the substratum or by the configuration of its surface.

The transcription factor Cbfa1 (Core binding factor a 1, Runx2) regulates osteoblast differentiation and expression of genes necessary for the development of a mineralized phenotype.[16,17] Runx2 plays an essential role in steering multipotent mesenchymal precursor cells toward an osteoblastic lineage[18] and promotes osteoblast differentiation at an ealry stage, but inhibits osteoblast differentiation at a late stage.[19] Shui et al. demonstrated that human BMSC (bone marrow stromal cell) constitutively express Cbfa1 mRNA and protein and the activity of Cbfa1 binding to DNA was markedly increased in a time-dependent manner.[20] We studied the effects of anodized treatments to a titanium surface on the expression of Cbfa1 in MG63 cells. Human osteosarcoma MG63 cells are committed osteoblasts that are more mature compared to a preosteoblast or bone marrow stromal cell but more immature than other osteoblasts. Therefore, early expression and activity of Runx2 during that time in MG63 cells could be more meaningful. The amounts and patterns of Cbfa1 gene expressions differ with time according to applied voltage (Fig. 4). In cells on machined surface, the lowest expression level was noted. The expression level was highest in the 1 day sample and was maintained to 5 days in cells on A450V disc. More rapid expression of Cbfa1 in cells grown on A450V surface at 1 day culture, on A350V surface at 3 days culture, on machined sur-

face at 5 days culture, were noted (Table VII). Roughness and oxide layer were increased M, A350V and A450V in turn. From this result, we hypothesize the rougher implant surface, the sooner the Cbfa1 gene expresses. The early expression of Cbfa1 in MG63 cells on Ti disc of increased surface roughness or topographic complexity is congruent with in vivo observations regarding the extent of osteogenesis on implants of increasing surface roughness. Bowers et al. reported that the increased Cbfa1 gene expression was noted on rougher surfaces.[21] Another paper reported that differentiation of preosteoblasts is affected by implant surface microtopographies.[22] Among the surface characteristics, only roughness differences could not explain our results. We should consider the effect and interaction of not only roughness but also chemical composition and the characteristics of surface microstructure on Cbfa1 expression. Therefore, further studies that focus on the different effects of chemical composition, microstructure and oxide layer with same roughness on cell behaviors will be required.

Boyan et al. reported upon the relationship between phospholipid metabolism and osteoblast differentiation in MG63 cells.[23] The main objective of present preliminary study was to evaluate the potential and differential activation of phospholipases, such as PLA2 or PLD, by modified surface property of Ti in MG63 cells based on the our experimental results of lipid composition analysis (Data are not shown). These enzymes are considered to play an important role as effector enzymes in membrane lipid mediated signal transduction. Boyan et al. suggested that protein kinase A and PLA2 is involved in surface roughness-induced osteogenic differentiation in MG63 cells. Sung et al. reported the activation of phospholipase C and D may contribute to neuronal differentiation by neurogenic PDGF in the HiB5 cells.[24]

Although a previous report upon the relationship between phospholipid metabolism and osteoblast differentiation in MG63 cells implied that PLA2 is involved in surface roughness-induced osteogenic differentiation, our current report shows that the activation of PLA2 does not occur during the surface roughness-dependent differentiation on of MG63 cells. The results obtained from Boyan et al. suggested that surface roughness of Ti is mediated with accumulated PGE2 though PLA2 activation via integrin receptor.[25] The increase of PLA2 activity was deduced from the accumulated PGE2 compared with our study. Otherwise PLD activity was increased during the surface properties-dependent differentiation on of MG63 cells. In a variety of cell lines phosphatidic acid serves as an important second messenger. Important regulatory proteins whose activities are known to be activated directly by PA in vitro include Raf-1 kinase, PKC , protein tyrosine phosphatase 1C and PLCγ.[26-27] The present study suggests that the activation of phospholipases D may play an important role in the transduction of signals by modified surface property (increased roughness and/or oxide layer of surface) in MG63 cells. Further analysis of downstream signaling pathways activated by differential phospholipases may give deeper insights into the mechanism of different biological outcomes induced by surface roughness of Ti.

CONCLUSIONS

The present study demonstrated that the anodized surface treatment could affect the surface properties and the surface roughness, oxide thickness and surface chemical composition have an effect on osteoblast-like cell proliferation, differentiation and matrix production. Within the limitation of this study, the following conclusions could be drown.

1. The anodized titanium had a porous oxide layer, and there was increase in both the size and number of pores with increasing anodizing voltage.
2. With increasing voltage, the surface roughness and thickness of the oxide film increased significantly (p<0.01), the TiO_2 phase changed from anatase to rutile. During the anodic oxidization, Ca and P ions were more incorporated into the oxide layer.
3. The in vitro cell responses of the specimen were also dependant on the oxidation conditions. With increasing voltage, the ALP activity, type I collagen production, and Cbfa 1 gene expression increased, while the cell proliferation decreased.
4. In preliminary study on the relation of surface property and phospholipase, PLD activity was increased, but PLA2 activity did not changed according to applied voltage.

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