Correlation Between Surface Hydrophilicity and Osteoblastic Differentiation on Microgrooved Titanium Substrata

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Surface microgrooves and acid etching on titanium (Ti) have been proposed to enhance various cell behaviors. In this study, surface hydrophilicity, protein adsorption, and alkaline phosphatase activity of osteoblasts were analyzed and compared between microgrooved Ti, Ti with microgrooves and further acid etching, smooth Ti, and acid-etched smooth Ti. Correlations between the results of each experiment were analyzed using Pearson's correlation analysis, and the influential factor on alkaline phosphatase activity was determined using multiple stepwise regression analysis. Among groups, the Ti substrata with microgrooves and subsequent acid etching showed significantly greater surface hydrophilicity and alkaline phosphatase activity compared with smooth Ti, whereas the Ti substrata with only microgrooves showed the greatest protein adsorption. Multiple stepwise regression analysis determined the surface hydrophilicity of Ti as the influential factor on alkaline phosphatase activity. This study indicates that surface microgrooves and acid etching on Ti substrata enhance surface hydrophilicity, leading to increased alkaline phosphatase activity.

Key Words: titanium, microgrooves, surface hydrophilicity, alkaline phosphatase activity

INTRODUCTION

To enhance cell responses to biomaterial surfaces, various methods of surface treatments have been introduced. One of these methods consisted of providing microstructural topographies to extensively alter cell shape,[1,2] which would further change cell adhesion and proliferation.[3,4] As to the osteoblasts cultured on biomaterial surfaces, cell number was verified to be

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increased on the surface microgrooves and discontinuous edges[5] or on the anodized hemispherical cavities[6] in micron scales. However, a synergistic effect of combined microtopography and nanotopography has been highlighted to play an important role in enhancing osteogenic activities on Ti surfaces. Intracellular mechanical signaling in the bone-forming unmineralized tissue, induced by various microtopographies on Ti surfaces, has not been elucidated.[7] Therefore, the requirement for both micron and submicron scale structures for synergistic responses of osteoblasts to Ti surfaces has been proposed.[8] Hydrofluoric acid (HF) treatment, depending on treatment time, has recently been reported to significantly enhance osteoblast responses, demonstrating its excellent use in submicron scale topography on Ti.[9]

Inspired by one of the previous studies emphasizing the ability of 30-μm-sized anodized hemispheric cavities to enable the cells to migrate in and actively proliferate,[6] investigators in this study designed surface microgrooves of 30 μm width and 5 μm depth and provided them on Ti surfaces to evaluate their effect on the alkaline phosphatase (ALP) activities of osteoblasts. Also, by slightly further etching the microgrooved surface with HF solution, we sought for another possibility—that the combination of micron and submicron scale topography would intensify the corresponding biological effect. Among known influential factors on osteogenic activities, the surface hydrophilicity of Ti[10] and its ability to attract plasma protein adsorption[11] were also evaluated in this study to enhance the validity of the results and to support the recently proven hypothesis.

Taken together, we hypothesized that the 30-μm-wide surface microgrooves or the microgrooves with further acid etching would alter surface characteristics on the Ti substrata and enhance the ALP activities of osteoblasts. The purpose of this study was to analyze the surface hydrophilicity, serum albumin adsorption, and ALP activities of MG63 human osteoblast-like cells on Ti substrata with surface microgrooves, and to evaluate the correlations between results to determine the influential factor on ALP activity.

Materials and Methods

Fabrication of titanium substrata

Grade 2 commercially pure Ti disks (TSM-TECH Co Ltd, Ulsan, Korea) were mechanically polished to attain a finish surface with Ra $\leq$ 0.1 μm. Ti substrata with surface microgrooves in uniform dimension of 30 μm width, 5 μm depth, and 20 μm bottom width (NE30/5) were fabricated using photolithography (MEMSware Inc, Kwangju, Gyeonggi, Korea) (Figure 1). Further acid etching was applied using 1% HF for 2 seconds to provide the combined surface of microgrooves and acid etching to Ti substrata (E30/5). Ti substrata with both polished surfaces and further acid etching were used as the control (NE0 and E0) (Table 1).

Scanning electron microscopy

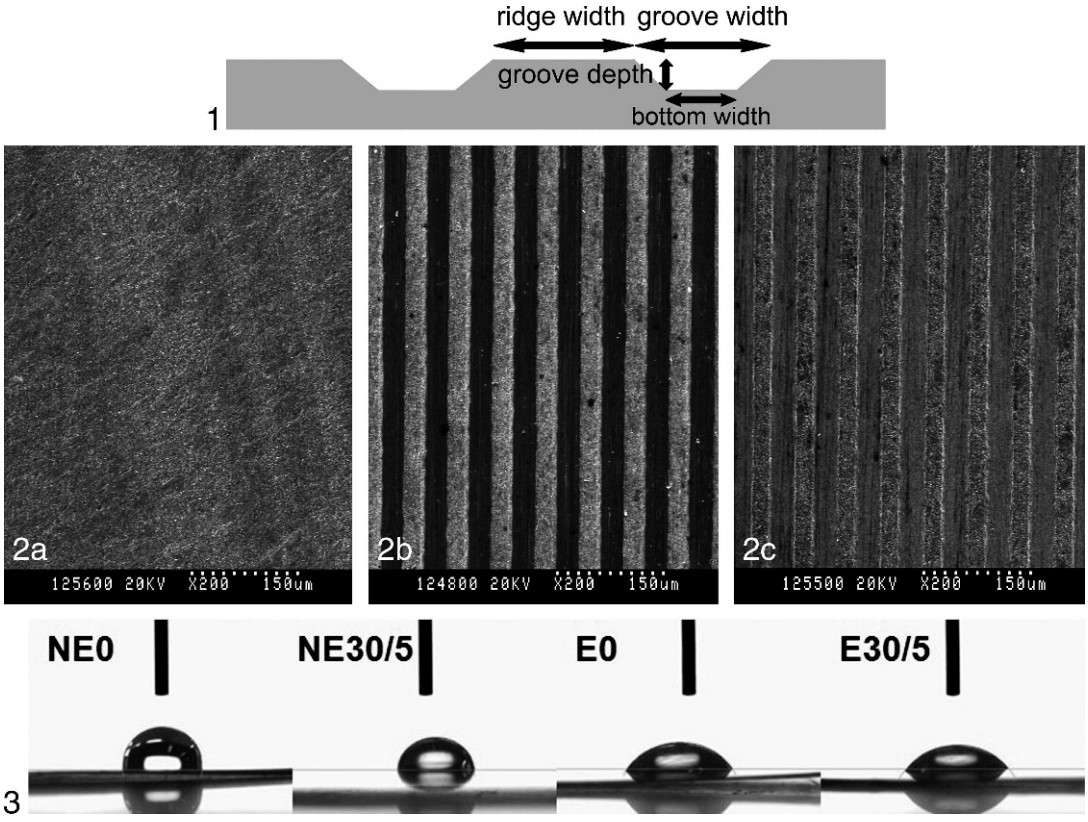
Surfaces of the fabricated Ti substrata were imaged under scanning electron microscopic observations (S-800 FE-SEM; Hitachi, Tokyo, Japan) (Figure 2).

Contact angle determination

Contact angles of the fabricated Ti substrata were measured with a contact angle–measuring instrument (EasyDrop; Krüss GmbH, Hamburg, Germany). Distilled water (6 μL per drop) was used as a probe for the contact angle calculation. Drop images were captured by a video camera in the direction perpendicular to surface microgrooves, and contact angles were determined by an image analysis system (Figure 3).

Bovine serum albumin adsorption

Bovine serum albumin (BSA) (Gibco BRL; Invitrogen Incorporated, Carlsbad, Calif) was



Figures 1–3. Figure 1. A schematic cross-sectional image and the structural nomenclature of microgrooved Ti substrata fabricated by photolithography. Note that ridge width and groove width were designed to be uniform in dimension. According to the isotropic principle, the bottom width inside the microgrooves with truncated V-shape can be calculated as (Groove width) – 2(Groove depth). **Figure 2.** Scanning electron microscopic images of (a) E0, (b) NE30/5, and (c) E30/5 (×200). See Table 1 for nomenclature and microstructural dimensions. **Figure 3.** The water-drop images on NE0, NE30/5, E0, and E30/5 captured by a video camera. Note that the images were captured in directions perpendicular to the surface microgrooves of NE30/5 and E30/5. See Table 1 for nomenclature.

used as the model protein. A total of 100 μg of the BSA solution (1 mg/mL protein/saline) was pipetted onto the Ti substrata. At 6 hours incubation, the nonadherent protein was removed, and the initial whole solution was mixed with Bradford agent (Bio-Rad, Hercules, Calif) at 37°C for 60 minutes. Protein concentrations were determined using the Bradford assay according to the manufacturer's instructions and were quantified using a microplate reader (Bio-Rad) at 595 nm.

Cell culture

MG63 human osteoblast-like cells (MG63 cells) were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in

Table 1
Titanium substrata with various surface topographies used in this study*

	NE0	NE30/5	E0	E30/5
Groove width, μm	0	30	0	30
Groove depth, μm	0	5	0	5
Bottom width, μm	0	20	0	20
Further acid etching	Non-etched	Non-etched	Acid etched	Acid etched

*E0 indicates smooth Ti with further acid etching; E30/5, Ti substrata with surface microgrooves of 30 μm width and 5 μm depth and with further acid etching; NE0, smooth Ti; NE30/5, Ti substrata with surface microgrooves of 30 μm width and 5 μm depth.

α-modified Eagle's medium (α-MEM; WelGene, Daegu, Korea) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Co, St Louis, Mo) and incubated at 37°C in a humidified atmosphere of 5% CO_2. To induce osteoblastic activities, cells were cultured in an osteogenic medium (Dulbecco's modified Eagle's medium [DMEM]; WelGene, Daegu, Korea) supplemented with 10% FBS, 50 μg/mL of α-ascorbic acid, 10 mM of β-glycerophosphate, 100 nM of dexamethasone, and antibiotics.

Alkaline phosphatase activity test

MG63 cells were seeded on the 12-well Ti substrata at a density of 5×10^4 cells/mL, cultured for 2 days for confluence, and incubated in an osteogenic medium (DMEM supplemented with 10% FBS, 50 μg/mL of α-ascorbic acid, 10 mM of β-glycerophosphate, 100 nM of dexamethasone, and antibiotics) at 37°C, 5% CO_2 for 1, 7, and 14 days. The cultured cells were washed with phosphate-buffered saline, removed by trypsin-ethylenediaminetetraacetic acid solution, lysated with 0.1% Triton X-100 buffer, and sonicated in ice. Aliquots of 50 μL were incubated with 100 μL of 1 M Tris-HCl (pH 9.0), 5 mM $MgCl_2$, and 20 μL of 5 mM *p*-nitrophenyl phosphate solution (Sigma-Aldrich) for 30 minutes at 37°C. The level of *p*-nitrophenol production in the presence of ALP was measured by measuring absorbance using a microplate reader (Bio-Rad) at 405 nm. Measurements were compared using *p*-nitrophenol standards and normalized by the total protein amounts.

Statistical analysis

Experiments were repeated independently 3 times. One-way analysis of variance (1-way ANOVA) in the Statistical Package for the Social Sciences 17.0 software program (SPSS Inc, Chicago, Ill) was used to compare mean values between groups of NE0, NE30/5, E0, and E30/5. Pearson's correlation analysis was used to analyze correlations between results from contact angle determination, BSA adsorption, and the ALP activity test. Multiple regression analysis was used to identify influential factors on ALP activities.

Results

Contact angle determination

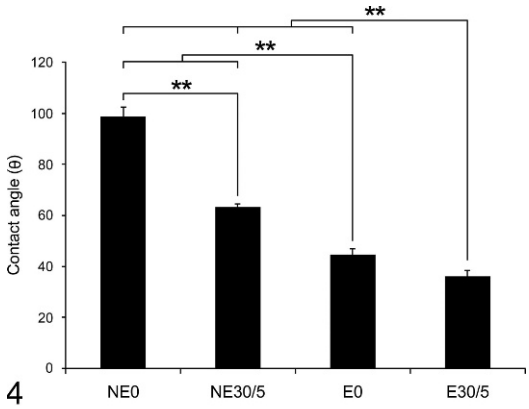
Multiple comparisons by 1-way ANOVA revealed that NE30/5 showed significantly smaller water contact angle (WCA) compared with that of NE0, and E0 showed a significantly smaller contact angle compared with those of NE0 or NE30/5 ($P < .01$). Also, E30/5 showed a significantly smaller contact angle compared with those of NE0, NE30/5, or E0 ($P < .01$), demonstrating that among NE0, NE30/5, E0, and E30/5, E30/5 shows the greatest surface hydrophilicity related to WCA (Figure 4).

Bovine serum albumin adsorption

Multiple comparisons by 1-way ANOVA revealed that NE30/5 showed significantly greater BSA adsorption compared with those of NE0, E0, or E30/5 ($P < .01$). Also, E30/5 showed significantly greater BSA adsorption compared with those of NE0 and E0 ($P < .01$) (Figure 5), demonstrating that, among NE0, NE30/5, E0, and E30/5, NE30/5 shows the greatest ability in attracting BSA adsorption at 6 hours incubation.

Alkaline phosphatase activity test

Multiple comparisons by 1-way ANOVA revealed that E30/5 showed significantly greater ALP activity compared with that of NE0 after 14 days of osteogenic culture ($P < .05$) (Figure 6), demonstrating that, among NE0, NE30/5, E0, and E30/5, E30/5 significantly enhances the ALP activities of MG63 cells at a relatively later phase of culture. No other comparisons between and within groups showed statistically significant differences ($P < .05$) (Figure 6).



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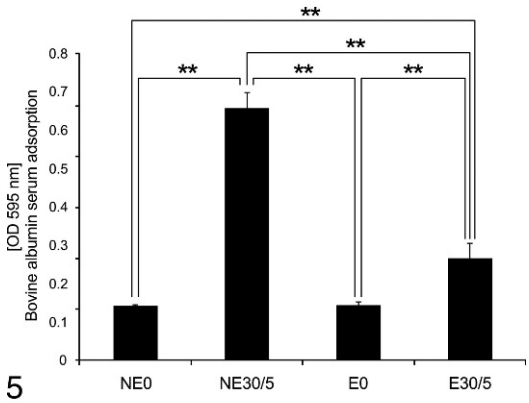

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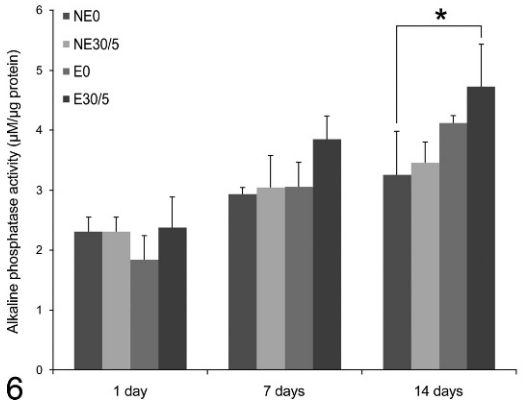

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Figures 4–6. Figure 4. Multiple-comparison results of contact angle determination on titanium substrata with various surface topographies. The contact angles on NE30/5 and E30/5 were measured in a direction perpendicular to the surface microgrooves. Statistical significances were tested among NE0, NE30/5, E0, and E30/5 using 1-way analysis of variance (ANOVA). ** indicates a significant difference ($P < .01$). See Table 1 for nomenclature. **Figure 5.** Multiple-comparison results of bovine serum albumin adsorption on titanium substrata with various surface topographies. Statistical significances were tested among NE0, NE30/5, E0, and E30/5 using 1-way ANOVA. ** indicates a significant difference ($P < .01$). See Table 1 for nomenclature. **Figure 6.** Multiple-comparison results of the alkaline phosphatase activity test of MG63 human osteoblast-like cells on Ti substrata with various surface topographies after 1, 7, and 14 days of osteogenic culture. Statistical significances were tested among NE0, NE15/3.5, NE60/10, E0, E15/3.5, and E60/10 using 1-way ANOVA. * indicates a significant difference ($P < .05$). See Table 1 for nomenclature.

Pearson's correlation analysis

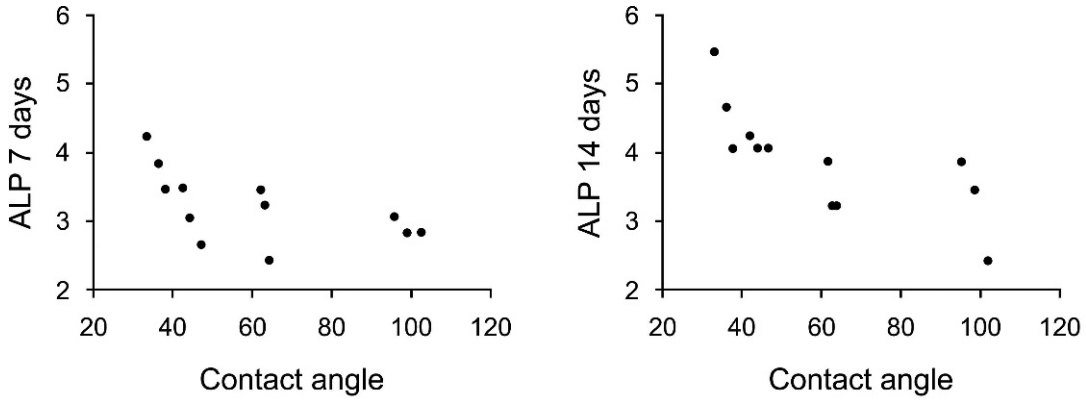
Results from contact angle determination (Contact Angle); BSA adsorption at 6 hours' incubation (BSA 6 h); and the ALP activity test of MG63 cells after 1, 7, and 14 days of osteogenic culture (ALP 1 day, ALP 7 days, and ALP 14 days) were used as the variables in Pearson's bivariate correlation analysis. As a result, significant correlations were noted between Contact Angle and ALP 14 days ($P < .01$) (Figure 7); ALP 1 day and ALP 7 days ($P < .05$); and ALP 7 days and ALP 14 days ($P < .01$) (Table 2).

Multiple stepwise regression analysis

Using ALP 14 days as the dependent variable, multiple stepwise regression analysis determined Contact Angle as the influential factor on ALP 14 days in the regression model (Figure 8).

Discussion

In this study, water contact angles on the microgrooved Ti substrata were measured in the perpendicular direction to the microgrooves. Setting both light source and corresponding camera direction perpendicular to the microgrooves enabled the measurement of water contact angles that were assumed to have been affected by the microgrooves. This was performed to apply the anisotropic wetting characteristics reported on the submicrometer-scale periodic grooved polymer surface by Zhao et al[12] to the present microgrooved Ti surface. Because the contact angles measured from the direction parallel to the grooves were larger than those measured from the perpendicular direction in the study by Zhao et al, we



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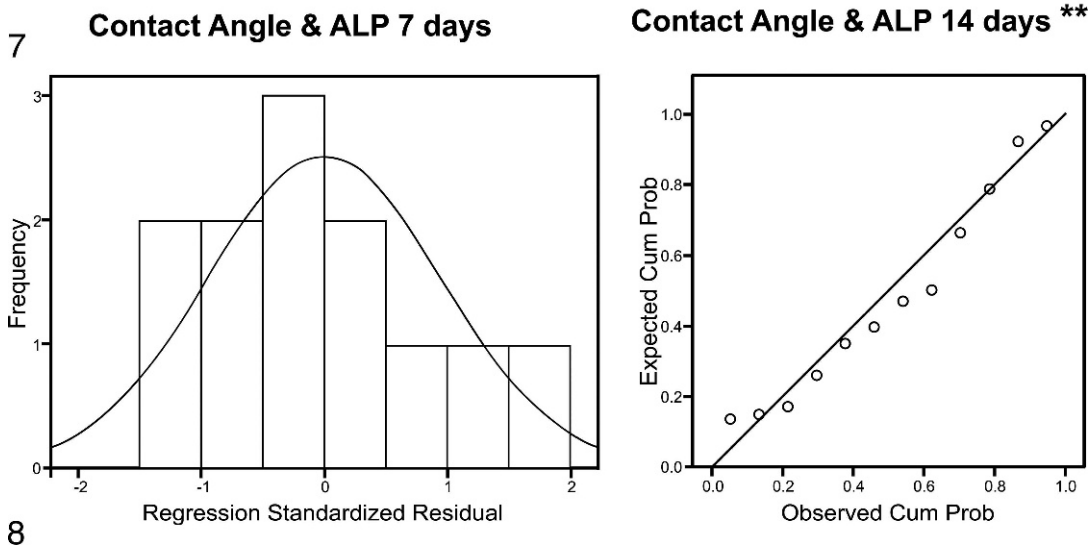

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Figures 7 and 8. Figure 7. Scatter-plot results from Pearson's correlation analysis between contact angles and alkaline phosphatase activities. Left, Contact Angle and ALP 7 days. Right, Contact Angle and ALP 14 days. Significant correlation was noted between Contact Angle and ALP 14 days ($P < .01$). ** indicates that correlation is significant at the .01 level (2-tailed). See Table 2 for nomenclature of the variables, correlation coefficients, and overall results. **Figure 8.** Histogram (left) and normal probability-probability (P-P) plot (right) results from multiple stepwise regression analysis using ALP 14 days as the dependent variable. Contact Angle and BSA 6 h were used as the requested independent variables. Contact Angle was determined to be the influential factor on ALP 14 days. See Table 2 for nomenclature of the variables. ALP 14 days = 5.266 − 0.023 · [Contact Angle]. $R = 0.749$. $R^2 = 0.561$.

Table 2

Pearson correlation coefficients between results of contact angle determination, bovine serum albumin adsorption, and alkaline phosphatase activity testing†‡

	Contact Angle	BSA 6 h	ALP 1 day	ALP 7 days
BSA 6 h	−0.092			
ALP 1 day	0.113	0.294		
ALP 7 days	−0.557	0.032	0.632*	
ALP 14 days	−0.749**	−0.151	0.320	0.763**

†N = 12.

‡ALP α day(s) indicates the results of alkaline phosphatase activity testing after α day(s) of osteogenic culture; BSA 6 h, the results of bovine albumin serum adsorption at 6 hours incubation; contact angle, the results of contact angle determination in the direction perpendicular to the microgrooves.

*Correlation is significant at the .05 level (2-tailed).

**Correlation is significant at the .01 level (2-tailed).

expected that a similar result would be obtained on the microgrooved surfaces to reflect the affected water drops. Indeed, the water contact angles of both NE30/5 and E30/5 were significantly smaller compared with that of NE0, suggesting a possibility that anisotropy occurred on our microgrooves as well. However, the polymer surface analyzed in the study by Zhao et al was extremely hydrophobic, which is inconsistent with the relatively hydrophilic microgrooved surfaces in this study. Further analysis of the size and shape of the water drops and the correlative water contact is strongly required. E0 showed greater hydrophilicity compared with NE0, suggesting that slight acid etching has an effect on enhancing the hydrophilicity to a moderate degree. The sand-blasted acid-etched Ti showed surface hydrophobicity,[13] and microstructured surfaces with acid-etched roughness were initially extremely hydrophobic with shift to total wettability once wetted[14]; these findings are contradictory to our hydrophilicity result. A possible explanation lies in the findings of a previous study suggesting that enhancement of hydrophilicity can be influenced by elimination of surface contamination using a specific treatment of Ti[10]; the slight HF etching in this study is considered to have induced the corresponding phenomenon. From the results of contact angle determination in this study, we suggest that microgrooves and further acid etching significantly enhance the surface hydrophilicity of Ti.

Studies reporting on the osteogenic activities of cells grown on microgrooved Ti substrata, not to mention studies using substrata with surface microgrooves and further acid etching, are scarce; however, we found 2 studies enabling comparison of their osteogenic activity results with ours. Upon comparing various in vitro osteoblast responses to microfabricated discontinuous-edge surface topographies on Ti with those of other topographies, including the microgrooves, Hamilton et al reported a lower degree of alkaline phosphatase staining of osteoblasts on the microgrooves at 1, 2, and 4 weeks of osteogenic culture compared with the flat surface.[5] Also in the work of Hamilton and Brunette, an increase in the nuclear translocation of the Runx2 transcription factor was not evident on the microgrooves compared with the flat surface, whereas osteocalcin was significantly deposited in nodules formed on the corresponding microgrooved surface at a later phase.[15]

Although the studies described in the previous paragraphs used epoxy resin replica with 30G45P grooves (V-shaped grooves of 30 μm depth and 45 μm pitch) as the culture substrata, which is the major difference from the substrata used in our study, results of the parameters for osteogenic activities in these studies were conflicting. Because E30/5 in this study showed a significant increase in ALP activity at 2 weeks compared with the smooth Ti, once again, the importance of acid treatment should be revisited. However, NE30/5 and E0 showed increases in ALP activity that were statistically nonsignificant compared with smooth Ti. Taken together, we suggest that a combined topography of microgrooves and further HF etching is necessary to enhance ALP activity. This corresponds with previous studies proposing the importance of combined micron and submicron topographies for a synergistic effect on enhancing osteogenic activities in vitro.[6,8,16]

The serum albumin adsorption result in this study showed a controversy related to the results of contact angle determination and ALP activity testing. A recent study using mirror surfaces with hexamethyldisiloxane coating and subsequent O_2-plasma treatment as the substrata reported that fibronectin showed greater adsorption on hydrophilic surfaces, whereas albumin showed

greater adsorption on hydrophobic surfaces.[17] However, NE30/5 in this study was not hydrophobic but showed greatest ability in attracting serum albumin, suggesting that other factors such as differences in the surface area of each Ti substratum in micron and/or submicron scales influenced the result, and that further investigation using Ti substrata with various dimensions of microgrooves is required.

Among numerous studies reporting on the effect of surface hydrophilicity of Ti on enhancing osteogenic activities, a canine study by Schwarz et al concluded that soft and hard tissue integration was influenced mainly by surface hydrophilicity rather than by microtopography.[18] However, the topography in combined micron and submicron scales in this study significantly enhanced both surface hydrophilicity and ALP activity on Ti; at the same time, results showed a strong correlation with definite influences on each other. Therefore, we suggest that microgrooves and further acid etching induce the enhancement of surface hydrophilicity of Ti and lead to increased ALP activities of osteoblasts.

This study indicates that Ti substrata with 30-μm-wide surface microgrooves and further acid etching could act as a promoter of ALP activity in osteoblasts. By supporting the recently proven hypothesis that increased surface hydrophilicity of Ti enhances osteogenic activity, we additionally verified the microgrooves with further acid etching as another factor promoting such activity. We demonstrated a strong correlation between the results of water contact angle determination and those of ALP activity testing influenced by the surface topography, from which, within the limits of this study, we conclude that enhanced surface hydrophilicity induced by microgrooves and further acid etching on Ti acts as a definite influential factor on the ALP activities of MG63 human osteoblast-like cells.

Abbreviations

ALP: alkaline phosphatase
BSA: bovine serum albumin
DMEM: Dulbecco's modified Eagle's medium
FBS: fetal bovine serum
Ti: titanium
WCA: water contact angle

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