

Cellular activities of osteoblast-like cells on alkali-treated titanium surface

Jin-Woo Park¹, Deog-Hye Lee¹, Shin-Il Yeo¹,
Kwang-Bum Park², Seok-Kyu Choi², Jo-Young Suh¹

1. Department of Periodontology, College of Dentistry, Kyungpook National University.

2. Megagen co., Ltd.

I. INTRODUCTION

Titanium is one of the most commonly used implant materials, particularly for dental and orthopedic applications and its uses are continuously increasing. This extensive use is based on the properties of material, including a combination of corrosion resistance and biocompatibility with satisfactory mechanical performance¹. This high degree of biocompatibility is thought to be the result, in part, from the protective and stable oxide layer, typically 4 nm thick film of amorphous or poorly crystallized and non-stoichiometric titanium oxide that presumably aids in the bonding of the extracellular matrix at the implant-tissue interface. The char-

acteristic composition and structure of the oxide layer often differ depending on the technique used to prepare the surface of the metal. It has been shown that methods of implant surface preparation can significantly affect the resultant properties of the surface and subsequently the biological responses that occur at the surface². Successful long-term stability of the biomaterial in bone tissue relies on several factors; among them is the important connection between the implant and bone tissue.

However, titanium is generally considered to be bio-inert and not likely to form direct bonding with bone, and it must be fixed clinically to bone by mechanical interlocking. However, mechanically fixed implants can

* This work was supported by the Korea Science and Engineering Foundation(KOSEF) grant funded by the Korea government(MOST)(No. M10646020002-06N4602-00210)

* Correspondence: Jo-Young Suh, D.D.S. Ph.D, Department of Periodontology, college of Dentistry, Kyungpook National University, Jung-Gu, , Daegu, 702-412, KOREA (E-Mail: jysuh@knu.ac.kr)

loosen over long periods of use. Thus, various surface treatments have been added to titanium to enhance bone formation around the implant to overcome this problem. The surface structure, morphology, and chemical and biochemical properties of biomaterial are important for establishing binding between the bone tissue and biomaterial.

To improve osseointegration at the bone-to-implant interface, several studies have been carried out to modify titanium surface. In these methods, there are sandblasting method, acid etching method, combination of sandblasting and acid etching method, plasma spraying method, deposition of calcium-phosphate, and anodizing method. Such surface modifications can be divided into two classes, one aiming on optimized three-dimensional physical microarchitecture of the surface, and the other focusing on the biochemical properties of surface coatings and impregnations. Blasting titanium implants with titanium dioxide particle is categorized into physical and mechanical method of increasing surface roughness and enlarging surface area³⁾. A number of animal model have demonstrated that a roughened implant surface will lead to an elevated level of bone contact^{4,5)}. Moreover, osteoblast-like cells appear to be more differentiated on rougher surfaces with respect to morphology, extracellular matrix synthesis, alkaline phosphatase specific activity, and osteocalcin production^{6,7)}. Synthesis of extracellular matrix and subsequent mineralization were both substantially enhanced on rough textured and

porous coated titanium in vitro⁸⁾. It is known that osteoblast cells initially respond in a different manner to titanium surface roughness. Higher levels of cellular attachment have been found on rough surfaces of titanium with irregular morphologies in vitro^{2,9)}. The primary goal of producing rough surfaces is mainly to create the micro-mechanical interlocking between in-growth bone and implants not by chemical bonding.

As a method of chemical bonding, the metals are often coated with bioactive ceramics to provide materials with bone-bonding ability. One of the most acceptable and commercialized bioactive coating materials is plasma sprayed hydroxyapatite¹⁰⁾. Although good short-term clinical results have been reported using this method, there have been several problems with its porosity, low-fatigue strength, degradation and delamination during long-term implantation¹¹⁾.

Recently, it has been suggested that the reactivity of titanium implants can also be enhanced by anatase surface structure of TiO₂ layer, because of their capability to induce calcium phosphate formation in vitro and in vivo. To improve the bioactivity of titanium implant by anatase structure of TiO₂ layer, several methods such as anodic oxidation, alkali treatment¹²⁾, and sol-gel coating¹³⁾ have been studied. In vitro studies have been demonstrated that titanium with an anatase surface structure are most effective for apatite nucleation¹⁴⁾.

However, regarding the effect of roughened surface by physical and mechanical

method including acid etching and blasting, most studies carried out on the reactions of cells to micrometric topography, that is micro-scaled roughness especially Ra, and cellular features have been related to the surface micrometric topography. Little work has been preformed on the reaction of cells to nanotopography. Titanium implant surface with TiO₂ layer composed of anatase structure has shown good bone response in vivo¹⁵). However, few studies have analysed the effect that anatase structure has on differentiation of cells into osteoblasts.

The purpose of this study was to determine if the surface topography with sub-micro-scale isotropic pattern of implant surfaces contributed to the response of osteoblasts by observing the cell attachment and cellular viability, and by analysing the gene expression of osteoblastic phenotype using ROS 17/2.8 cell lines. And the other purpose of this study was to investigate whether the effect of this designed surface on bone cell response over-ride the one of the surface with micro-scale topography produced by conventional methods.

II. MATERIALS AND METHODS

1. Cell culture

Rat Osteosarcoma (ROS) 17/2.8 cell lines were used for these experiments. ROS 17/2.8 cell lines were derived from a rat osteosarcoma and plated in Dulbecco's Modified Eagles Medium (DMEM, Gibco Co., USA)

containing 10% fetal bovine serum (FBS, Gibco Co., USA) and 500 unit/ml penicillin (Keunhwa Pharm. Co., Korea), 500 µl/ml streptomycin (Donga Pharm. Co., Korea), and cultured in an atmosphere of 100% humidity, 5% CO₂ and 37°C. Media were changed every other day until the cells reached to confluence. When the cells reached confluence, the cells were passaged with 0.05% trypsin/0.02% EDTA.

2. Sample preparation and surface treatment procedures

Grade 2 commercially pure titanium (Ti) plate with a thickness of 0.5 mm and three different dimensions were used in this study. Rectangular shaped Ti samples (10×10×0.5 mm) were used for surface characterization and for the experiment to observe attachment and proliferation of osteoblast-like cells with scanning electron microscope. For the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay to evaluate cell viability, Ti disks with 6 mm diameter were used. Square shaped samples sized of 55×55×0.5 mm³ were used for Northern blot analysis. As a pretreatment procedure, samples were wet abraded with 1200-grits using SiC abrasive paper and rinsed with acetone in an ultrasonic cleaner. After this, all the samples were chemically washed in a solution consisting of hydrofluoric acid and nitric acid (HF/HNO₃, 1 : 3 molar ratio) in order to remove the oxide formed on the surface. The samples were then thoroughly

rinsed with ultrapure water and dried at room temperature. The samples without further treatment were used as the control in this study. The other samples were subject to further surface treatment including blasting or alkaline treatment. To fabricate sub-micro-scale porous structure with TiO₂ layer composed of anatase structure, surface treatment of specimens was done according to the method described elsewhere¹⁶⁾. Briefly, titanium plate was immersed in 5 M NaOH aqueous solution at 60°C for 24 hr, gently washed with ultra-pure water, and then soaked in ultra-pure water at 80°C for 24 hr. After drying, the plates were heat-treated at 600°C for 1 hr in the furnace. The experimental groups were divided into four types described as follows.

Control : machined surface (No further treatment)

Group 1: machined followed by alkali treatment with 5M NaOH (60°C, 24 hr) immersion in ultra-pure water (80°C, 24 hr), and heat treatment (600°C, 1 hr) (Alkali treated surface).

Group 2: machined and blasted with 100 μ m particles of hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂) (Blasted surface).

3. Surface characteristics: morphology and roughness

Before and after surface treatment, the surfaces of Ti samples were analyzed as follows. The morphology of the surface was

observed using the scanning electron microscope (S-4200, Hitachi, Tokyo, Japan) and surface roughness were measured by profilometry using a Taylor Hobson profilometer (London, UK). The crystalline phase of the surface TiO₂ layer was characterized using X-ray diffractometry (XRD, X'Pert-APD, Philips, Netherlands).

4. Attachment of osteoblast-like cell

The cultured cells were seeded at a seeding density of 2.0×10^4 cells/cm² on each well which contains each specimen of the 24 well plate and cultured in an atmosphere of 100% humidity, 5% CO₂, and 37°C for 1, 3, and 24 hr. The specimens with attached cells were prefixed in 0.5% glutaraldehyde for 1 hr, and postfixed in 1% osmium tetroxide for 1 hr and critical point-dried (Freeze dryer, Model ES 2300, Hitachi, Japan) and sputter coated with Pt-Pd (Ion Sputter, Model E-1030, Hitachi, Japan). The cells attached on specimens were examined using the scanning electron microscope (S-4200, Hitachi, Tokyo, Japan)

5. MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay¹⁹⁾ is transformed by mitochondrial dehydrogenases into formazan, enabling mitochondrial activity and cell viability to be assessed. The cultured cells were seeded at a seeding density of 1.5×10^4 cells/cm² on each well which

contains each specimen in the 96 well plate. Three round shaped specimens ($\varnothing = 6$ mm) per each group were used. The cells were cultured in an atmosphere of 100% humidity, 5% CO₂, and 37°C for 1, 4 and 7 days, and then 50 μ l of MTT solution pre-warmed to 37°C was added in each well and cultured for 3 hr under the same condition. The 200 μ l of dimethyl sulfoxide(DMSO) and 50 μ l of glycin buffer were added and the solution was transferred to other set of wells which does not contain the specimens. The optical density was measured at a wavelength of 570 nm by ELISA reader (Precision Microplate Reader, Molecular Devices, USA).

6. Northern blot analysis

The cultured cells were seeded at a seeding density of 1.1×10^5 cells/well which contains each specimen in the $\varnothing = 100$ mm petri dishes. Two square shaped specimens ($55 \times 55 \times 0.5$ mm³) per each group were used. The cells were cultured in an atmosphere of 100% humidity, 5% CO₂, and 37°C during 7 days for an $\alpha 1(I)$ collagen mRNA, alkaline phosphatase mRNA and osteopontin mRNA.

Total RNA was extracted from cultured cells by using a modified acid phenol method. Briefly, the growth medium was removed and the cells were lysed with Trizol (Life Technologies, USA). The lysate was cleared and extracted with 1/10 volume of 1-bromo-3-chloropropane. The aqueous layer was collected in a new tube and precipitated

with isopropanol. After 75% ethanol washing, the pellet was air-dried and resuspended in DEPC-treated water, and quantified by A260/A280 measurement by using UV spectrometer (DU530, Beckman, USA). Ten μ g of total RNAs were heated to 65°C for 15 min in 50% formamide, 0.02% formaldehyde, 40 mM MOPS (3-[-N-morpholino] propanesulfonic acid), 10 mM sodium acetate, 1 mM EDTA, and 0.1 mg/ml ethidium bromide prior to gel electrophoresis on 1% agarose, 55% formaldehyde, 40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA. The RNA was blotted onto a zeta-probe blotting membranes in 20 $\times$ SSC. The RNA was air-dried and then cross-linked by exposure to ultraviolet light. The probes were labelled with [α -P³²]-dCTP by Megaprime DNA labelling system kit. Prehybridization and hybridization were performed by using the Express Hyb solution. After hybridization, the membrane was washed in 2 $\times$ SSC- 0.1% SDS at room temperature and then in 0.1 $\times$ SSC /0.1% SDS at 55°C, and exposed to Agfa X-ray film at -70°C with intensifying screens.

7. Statistical analysis

Analysis of variance(ANOVA) was used to evaluate the differences of surface roughness and cell viability between the groups.

III. RESULTS

1. Surface characteristics

1) Surface morphology

In scanning electron micrographs, machined, the alkali treated, and the blasted surfaces demonstrated microscopic differences in the surface topography. The specimens of the control and machined surfaces of titanium showed relatively smooth surface characteristics with abrasive marks. The specimens of group 1, which was alkali-treated with NaOH followed by water treatment (80°C, 24 hr) and subsequent heat treatment (600°C, 1hr), had a submicro-scaled porous surface, with pore size about

200 nm. The specimens of group 2, which was blasted with hydroxyapatite particles, showed irregularity in morphology with small (<10 μm) depression and indentations among flatter-appearing areas of various sizes.

2) surface roughness

Based on profilometry (Table I) the machined surface (control) and group 1 had similar Ra values of 0.31 and 0.33 μm , respectively. The surface of group 2 was the roughest and had an Ra of 1.93 μm . Blasted surfaces (group 2) were significantly rougher than the machined (control) and the alkali treated surfaces (group 1)($p<0.01$). Before and after alkali treatment, the surface roughness was almost identical.

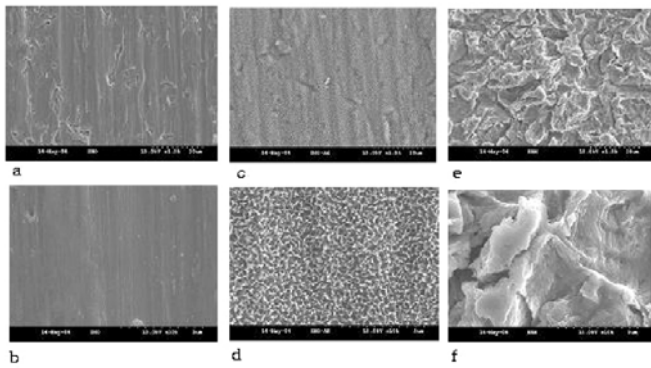


Figure 1. Scanning electron micrographs of three differently treated titanium surfaces. (a) Machined surface ($\times 1500$), (b) Machined surface ($\times 10000$), (c) Alkali treated surface (Group 1, $\times 1500$), (d) Alkali treated surface (Group 1, $\times 10000$), (e) Blasted surface (Group 2, $\times 1500$), (f) Blasted surface (Group 2, $\times 10000$).

Table 1. Surface roughness in each group

Group	Ra (μm)	Rz(μm)	Rt (μm)	Rq (μm)
Control	0.31 ± 0.07	2.03 ± 0.50	2.32 ± 0.71	0.40 ± 0.09
Group 1	0.33 ± 0.05	2.56 ± 0.57	2.96 ± 0.70	0.43 ± 0.06
Group 2	1.93 ± 0.19	13.35 ± 1.77	14.85 ± 2.08	2.46 ± 0.23

The values are the mean $\pm$ standard deviations. ($p<0.01$)

Ra : Arithmetic average deviation of the absolute values of all points

Rz: Average peak-to-valley height of the profile

Rt : Maximum peak to valley height of the entire magnitude trace of the profile

Rq : Root mean square of the values of all points of the profile

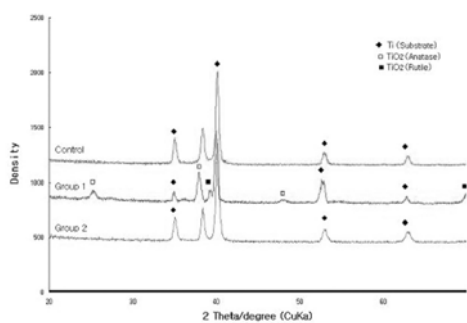


Figure 2. Thin-film X-ray diffraction patterns of differently treated titanium surfaces. The sodium-free structure was also confirmed in EDS and XPS analysis (data not shown here).

3) X-ray diffraction

XRD analyses of the prepared surfaces showed the Ti (substrate) peaks in the all diffractograms and the peak of hydroxypatite did not appear. TiO_2 (anatase) was observed on the alkali treated surface, whereas crystalline phases (anatase and rutile) of TiO_2 were not observed on machined and blasted surfaces. In the XRD pattern, the peak ascribed to sodium titanate were not observed on alkali treated samples, which means TiO_2 layer of treated surface was composed of sodium-free anatase structure.

2. Attachment and spreading of osteoblast-like cell

After 1 hr incubation, scanning electron microscopy showed that the cells were in the process of adhesion and spreading on the all prepared surfaces (Figure 3). The morphologic appearance of the cells on blasted and alkali treated surfaces was similar to that on machined surface, and the cells appeared to be round shaped and were slightly spreading. The cells had been the cytoplasm with numerous microvilli. Filopodia were often observed extending along the titanium surface on the machined surface, but filopodia or the formation of 'footpads' which may mediate cell-substrate adhesion were often observed attaching on the peak of irregular surface on the blasted and alkali treated surfaces. And the bulge of cell nucleus was visible on the all prepared surfaces.

After 3 hr incubation, cells on all prepared surfaces continued to spread radially from the center and had a flattened and round shape and it was observed filopodia as well as larger cytoplasmic extensions, la-

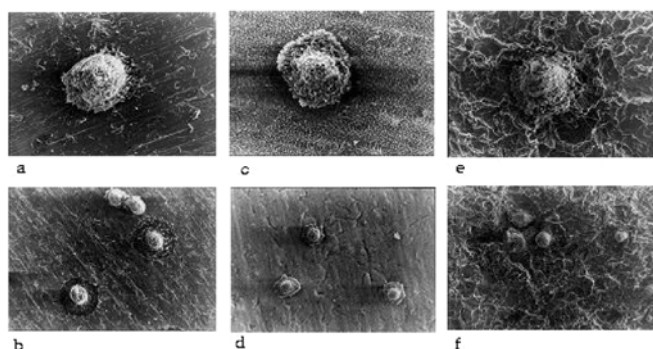


Figure 3. Scanning electron micrographs of cells after 1 hr culturing on (a) Machined surface(×1100), (b) Machined surface (×400), (c) Alkali treated surface (Group 1, ×1100), (d) Alkali treated surface (Group 1, ×400), (e) Blasted surface (Group 2, ×1100), (f) Blasted surface (Group 2, ×400).

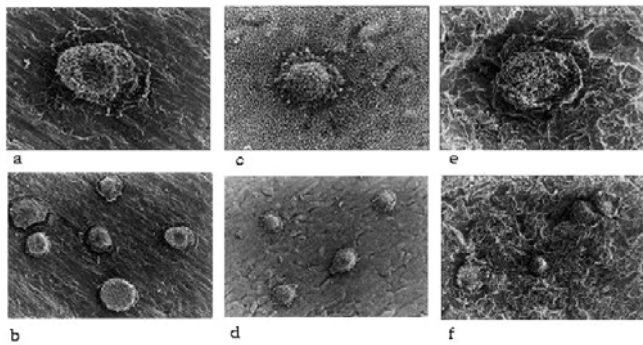


Figure 4. Scanning electron micrographs of cells after 3 hr culturing on (a) Machined surface ($\times 1100$), (b) Machined surface ($\times 400$), (c) Alkali treated surface (Group 1, $\times 1100$), (d) Alkali treated surface (Group 1, $\times 400$), (e) Blasted surface (Group 2, $\times 1100$), (f) Blasted surface (Group 2, $\times 400$).

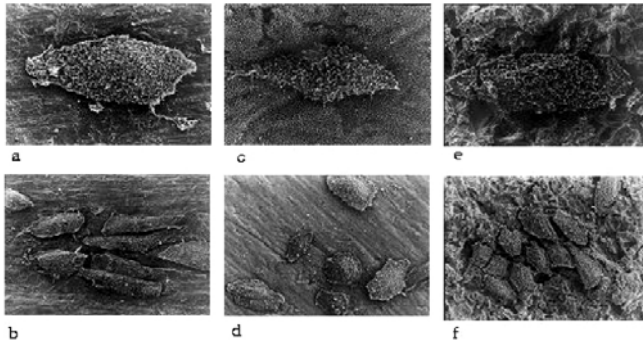


Figure 5. Scanning electron micrographs of cells after 24 hr culturing on (a) Machined surface ($\times 1100$), (b) Machined surface ($\times 400$), (c) Alkali treated surface (Group 1, $\times 1100$), (d) Alkali treated surface (Group 1, $\times 400$), (e) Blasted surface (Group 2, $\times 1100$), (f) Blasted surface (Group 2, $\times 400$).

mellipodia on the cells (Figure 4). On the machined surfaces, the cells were more intimately attached to the surfaces, with further areas of contact between lamellipodia and the surfaces. However, on the blasted and alkali treated surfaces, the spreading cells exhibited slightly irregular shapes and some gaps or spaces were seen where there was no area of contact for the cytoplasmic extensions.

After 24 hrs incubation, most cells of all the groups had a flattened, polygonal shape, however, the cells were more spread on the machined surfaces than on the blasted and alkali treated surfaces (Figure 5).

3. MTT assay

The MTT assay was carried out to evaluate the cellular viability on the machined, blasted and alkali treated surfaces at 1, 4 and 7 days. As shown in Figure 2, the MTT assay indicated the increase as following order: on machined, alkali treated and blasted surfaces according to the time. It was considered that alkali treatment has little cytotoxicity because the MTT assay was increased according with time. The group 1 and 2 were significantly increased in optical density compared with the control at 1 day ($p < 0.01$), however, there was no significant difference in optical density among the groups at 4 and 7 days ($p > 0.05$).

4. Gene expression of the bone matrix protein

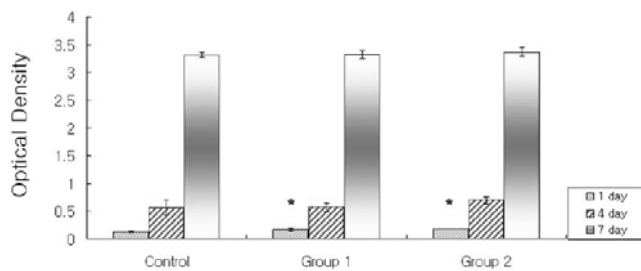


Figure 6. The optical density measured after culture for 1, 4, and 7 days at a wavelength of 570 nm by ELISA reader (n=3 in each group). The group 1 and 2 were significantly increased in optical density compared to control at 1 day ($p < 0.01$), but there was no significant difference in optical density among the groups at 4 and 7 days ($p > 0.05$).

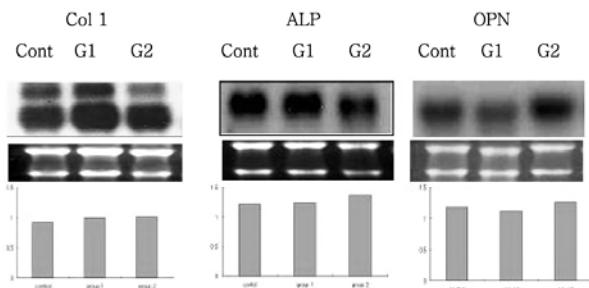


Figure 7. Northern blot analysis of the mRNAs of ROS 17/2.8 cell on three differently treated titanium surfaces at day 7. The mRNA expression level of $\alpha 1(I)$ collagen (Col 1), alkaline phosphatase (ALP) and osteopontin (OPN) of the osteoblast-like cells showed a tendency to be higher on blasted and alkali treated surfaces than on the machined surfaces.

To prove that bone formation occurs in osteoblast-like cell cultures, we examined the expression of genes implicated in osteoblastic differentiation. Therefore, we examined the expression of $\alpha 1(I)$ collagen mRNA and alkaline phosphatase mRNA, osteopontin mRNA in osteoblast-like cell culture during 7 days in culture on the machined, the blasted and the alkali treated surfaces. The results from this experiment are shown in Figure 3. The mRNA expression of alkaline phosphatase and $\alpha 1(I)$ collagen were slightly high on blasted and alkali treated surfaces compared with machined surface, and the mRNA expression of osteopontin was the highest on the blasted surfaces. These results showed mRNA expression level of $\alpha 1(I)$ collagen, alkaline phosphatase and osteopontin of the osteoblast-like cells showed a tendency to be

higher on blasted and alkali treated surfaces than on the machined surfaces, although no significant difference in the mRNA expression level of $\alpha 1(I)$ collagen, alkaline phosphatase and osteopontin was observed among all groups.

IV. DISCUSSION

Interactions between implants and adjacent tissues are dependent, in part, on surface properties of implant materials¹⁷⁾. The initial interactions occurring at the bone-implant interface outcome at this site is dependent not only on successful wound healing, but also on successful bone formation⁴⁾. Mineralizing tissues have demonstrated the ability to bond directly to titanium and that the overall success of cellular attachment, whether occurring

in vitro or in vivo, is dependent on many parameters including surface roughness, oxide composition and thickness, and other properties¹⁸⁾.

In an attempt to improve the quantity and quality of bone-implant interface, numerous surface modifications have been used. There are basically two ways to modify the implant surface to improve osseointegration. The one way is to fabricate an optimized three-dimensional physical micro-architecture of the surface, sand blasting and/or acid etching, TiO₂ plasma spraying methods are included in this approach. The development and use of these surface modifications have been based on the theory that improved osseointegration can be achieved by increasing surface roughness and surface area of the implant surface¹⁹⁾. It has been suggested that bone bonding to titanium implant surface is achieved by micromechanical interdigitation of surrounding bone with the material surface²⁰⁾. Several studies reported a good correlation between increased Ra value and stronger bone anchorage^{21,22)}. The other way is the method focusing on the change of biochemical properties to titanium surface. Surface coatings, hydroxyapatite plasma spraying, anodic oxidation, sol-gel coating of titania and simple chemical methods are included in this category. It has been suggested that these methods produce the anatase surface structures, which enhances reactivity of titanium implants, and the anatase crystal structure, the porous Ti-OH network, and the negative surface charge den-

sity of the titania may be responsible for the apatite nucleation²³⁾.

In this study, to fabricate bioactive 1 μm thick TiO₂ layer composed of mainly anatase with submicron-scaled porous structure, surface treatment was done according to the method of Uchida et al.¹⁶⁾ To fabricate rough surface with micron-scaled structure, the surface was blasted with 100 μm particles of hydroxyapatite

In this study, we observed different topographies of pure titanium by different treatments in all groups and these topographies were similar to topographies which reported previously on machined, blasted and alkali treated surface in SEM observation. Alkali treatment alone had a little effect on the surface roughness (micron-scaled roughness, Ra=0.33 μm) but overall surface morphology at nanometer scale (pore size of about 200 nm) was apparently different after alkali treatment. Blasted surfaces were significantly rougher (Ra=1.93 μm) than the machined and the alkali treated surfaces (p<0.01) and showed irregularities in morphology with small (<10 μm) depression and indentations among flatter-appearing areas of various sizes. The differences in roughness resulting from blasted surfaces were statistically significant in comparison with machined and alkali treated surfaces (p<0.01). Many researches have done in order to explore the limits of roughness values that cells are able to detect^{2,10,24)}. Wojciak-Stothard et al.²⁵⁾ have discovered that macrophage-like cells react down to dimension at least as small as 44

nm, whereas endothelia respond to the steps of 100 nm height or greater²⁶⁾. Deligianni and coworkers³⁵⁾ reported that osteogenic cells can sense changes in roughness of the range of 0.6 μm and other cells did “ignore” the surface topography on 0.5 μm grooved surface. Matsuzaka and coworkers²⁸⁾ concluded that microgrooves are able to influence bone cell behavior by determining the alignment of cells and cellular extensions, altering the formation and placement of cell focal adhesions, and altering ECM production. Therefore, microgrooved surfaces seem to be interesting to be applied on bone-anchored implants. Most studies were done on effect of the surface with grooved pattern, ie, anisotropic surface and with dimensions of 0.5 μm and greater. Little work has been performed on the reaction of bone cells to isotropic implant surface with nanotopography, submicro-scaled structures. It has been suggested that topographic cue over-ride the chemical one. In the work of Britland and colleagues²⁹⁾, they used a chemical cue oriented at right angles to a topographic one. When the grooves were 500 nm deep or less, the BHK fibroblasts reacted chiefly to the chemical cue, on deeper groove the topographic cue over-ride the chemical one and at 5 μm depth the topographic effect oriented about 80% of the cells and the chemical one, 7%. The surface characteristics used by Britland and colleagues²⁹⁾, however, was different from the one used in this study. We fabricated isotropic surface pattern to compare the effect of de-

signed surfaces. The other purpose of this study was to determine whether submicro-scaled surface topography(nanotopography) with anatase structure over-rides the effect of micrometric roughness produced by blasting. There is no work to compare the effect of crystalline oxide films such as anatase structure on bone cell response with the one of surface with micrometric roughness directly. Nishiguchi et al³⁰⁾ reported that alkali treated titanium has a submicron-level trabecular and irregular structure on its surface. This surface topology may play a role in the bone-bonding strength of this material. However, the average roughness is less than 1 μm and only weak mechanical interlocking occurs if at all. Thus, it is supposed that chemical bonding between alkali treated titanium metal and bone via an apatite layer plays a major role in bone-bonding behavior. By blasting with hydroxyapatite particles, we obtained similar topography to blasted surface with Al_2O_3 particles and could prevent the Al contamination on the titanium surfaces in this study. We also could not find the hydroxyapatite on the X-ray diffraction analyses. On the X-ray diffraction analyses, we observed the presence of anatase and rutile phases of TiO_2 from the alkali treated surface in NaOH solution, whereas it was not observed on machined and blasted surfaces. It has been known that apatite nucleation is effectively induced from anatase structures in Ti-OH groups. Once apatite nuclei are formed, they grow spontaneously by consuming the calcium and phosphate ions

from the surrounding fluid³¹⁾. The formation of a thin layer of apatite on the implant surface believed to be the first step of bone bonding process for the bioactive implant including titania³²⁾.

The process of adhesion of cells in suspension to substrate involves the following steps: (1) adsorption of serum proteins on to the substrate: (2) contact of rounded cells with the substrate: (3) attachment of the cells to the substrate: and (4) spreading of the cells on the substrate³³⁾. In scanning electron micrographs observation, the morphologic appearance of the cells on the blasted and alkali treated surfaces was similar to that of machined surface. After 1hr and 3hr incubation, most of cells showed focal cytoplasmic extensions or circumferential spreading. However the spreading cells on roughened surface exhibited irregular shapes, with gaps or spaces where there was no area of contact for the cytoplasmic extensions, because the cells spanned over the surface and creating spaces or gaps underneath³⁴⁾. After 24 hours incubation, most cells of the all groups had a flattened, polygonal shape, however the cells spread more on the machined surfaces than on the blasted and alkali treated surfaces. It has been suggested that smooth surface is better to spread the cell than submicro-scaled and micro-scaled surface.

Using the MTT assay, cellular viability has increased on machined, alkali treated and blasted surfaces according to time. It has been considered that alkali treatment has

little cytotoxicity because the MTT assay was showed increased optical density according to time. The blasted and alkali treated groups were significantly increased in optical density compared to machined surface at 1 day($p < 0.01$), however there was no significant difference in optical density among the groups at 4 and 7 days ($p > 0.05$). Deligianni et al.³⁵⁾ showed that there is a time-dependent increase in cell attachment on materials, what was observed in this study, as more cells attached after 24 hr than 4 hr. And Zinger et al.³⁶⁾ showed that cell attachment depends on cavity spacing. It was considered that initial increase of cellular viability on submicro-scaled as well as micro-scaled surface topography is due to increase of the protein adsorption onto titanium surface and different cavity spacing.

To prove that bone formation occurs in osteoblast-like cell cultures, we examined the expression of several genes implicated in osteoblastic differentiation at day 7 after incubation. To date, there have been a little reports regarding the analysis of osteogenic cells on micro-scaled and submicro-scaled surfaces. This study chose the mRNA expression level of $\alpha 1(I)$ collagen, alkaline phosphatase and osteopontin, which are established markers of osteogenic differentiation^{37,38)}. The results from this experiment show that the mRNA expression of alkaline phosphatase and $\alpha 1(I)$ collagen was slightly higher on blasted and alkali treated surfaces compared to machined surface, and the mRNA expression of osteopontin was

the highest on the blasted surfaces. These results were similar to the report by Matsuzaka et al.³⁹⁾ that mRNA expression of osteopontin and osteocalcin of the osteoblast-like cells showed a tendency to be higher on multigrooved surfaces than on smooth surfaces. Nishio et al.⁴⁰⁾ reported that mRNA expression of osteopontin and $\alpha 1(I)$ collagen showed no difference between smooth surfaces and alkali treated surfaces at day 7 bone marrow cell culture. The results of this study showed that the mRNA expression level of $\alpha 1(I)$ collagen, alkaline phosphatase and osteopontin of the osteoblast-like cells showed a tendency to be higher on blasted and alkali treated surfaces than on the machined surfaces, although no significant difference in the mRNA expression level of $\alpha 1(I)$ collagen, alkaline phosphatase and osteopontin was observed among all groups. It means that the micro-scaled and submicro-scaled surfaces could accelerate the osteogenic cell function.

In conclusion, we suggest that submicro-scaled surfaces on osteoblast-like cell response do not over-ride the one of the surface with micro-scale topography produced by blasting method, although the micro-scaled and submicro-scaled surfaces can accelerate osteogenic cell attachment and function in comparison with the machined surfaces. Also we could not observe the synergistic effect by TiO_2 layer composed of anatase, although it is not clear as the design of this study. Then further studies are required to assess the difference of the os-

teogenic cell response between the submicro-scaled surfaces and surface composed of anatase, and the effect of submicro-scaled surfaces on the bone formation in vivo.

V. SUMMARY

To improve osseointegration at the bone-to-implant interface, several studies have been carried out to modify titanium surface. Variations in surface texture or microtopography may affect the cellular response to an implant. Osteoblast-like cells attach more readily to a rougher titanium surface, and synthesis of extracellular matrix and subsequent mineralization were found to be enhanced on rough or porous coated titanium. However, regarding the effect of roughened surface by physical and mechanical methods, most studies carried out on the reactions of cells to micrometric topography, little work has been performed on the reaction of cells to nanotopography.

The purpose of this study was to examine the response of osteoblast-like cell cultured on blasted surfaces and alkali treated surfaces, and to evaluate the influence of surface texture or submicro-scaled surface topography on the cell attachment, cell proliferation and the gene expression of osteoblastic phenotype using ROS 17/2.8 cell lines.

In scanning electron micrographs, the blasted, alkali treated and machined surfaces demonstrated microscopic differences in the surface topography. The specimens of alkali treatment had a submicro-scaled porous sur-

face with pore size about 200 nm. The blasted surfaces showed irregularities in morphology with small (<10 μm) depression and indentation among flatter-appearing areas of various sizes. Based on profilometry, the blasted surfaces was significantly rougher than the machined and the alkali treated surfaces ($p < 0.01$). On the x-ray diffraction analysis, anatase and rutile (TiO_2) were observed on alkali treated surfaces, whereas not observed on machined and blasted surfaces.

The attachment morphology of cells according to time was observed by the scanning electron microscope. After 1 hour incubation, the cells were in the process of adhesion and spreading on the prepared surfaces. After 3 hours, the cells on all prepared surfaces were further spreaded and flattened, however on the blasted and alkali treated surfaces, the cells exhibited slightly irregular shapes and some gaps or spaces were seen. After 24 hours incubation, most cells of the all groups had a flattened and polygonal shape, but the cells were more spreaded on the machined surfaces than the blasted and alkali treated surfaces.

The MTT assay indicated the increase on machined, alkali treated and blasted surfaces according to time, and the alkali treated and blasted surfaces showed significantly increased in optical density comparing with machined surfaces at 1 day ($p < 0.01$).

Gene expression study showed that mRNA expression level of $\alpha 1(\text{I})$ collagen, alkaline phosphatase and osteopontin of the osteoblast-like cells showed a tendency to be

higher on blasted and alkali treated surfaces than on the machined surfaces, although no significant difference in the mRNA expression level of $\alpha 1(\text{I})$ collagen, alkaline phosphatase and osteopontin was observed among all groups.

In conclusion, we suggest that submicro-scaled surfaces on osteoblast-like cell response do not over-ride the one of the surface with micro-scaled topography produced by blasting method, although the micro-scaled and submicro-scaled surfaces can accelerate osteogenic cell attachment and function compared with the machined surfaces.

VI. REFERENCES

1. Ito A, Okazaki Y, Tateishi T, Ito Y. In vitro biocompatibility, mechanical properties and corrosion resistance of Ti-Zr-Nb-Ta-Pd and Ti-Sn-Nb-Ta-Pd alloys. *J Biomed Res* 1995;29:893-900
2. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast response in vitro. *Int J. Oral. Maxillofac. Implants* 1992;7:302-303
3. Wennerberg A., Albrektsson T., Andersson B. Bone tissue response to commercially pure titanium implants blasted with fine and coarse particles of aluminum oxide. *Int J Oral Maxillofac Implants* 1996;11: 38-45
4. Cochran DL, Schenk RK, Lussi A, Higginbottom FL, Buser D. Bone response to unloaded and loaded titanium implants with a sandblasted and acid-etched surface : A histometric study in the canine mandible. *J Biomed Mater Res*

- 1998;40:1-11
5. Lazzara RJ, Testori T, Trisi P, Porter SS, Weinstein RL. A human histologic analysis of Osseotite and machined surfaces using implants with 2 opposing surfaces. *Int J Periodont Res Dent* 1999;19:3-16
6. Boyan BD, Batzer R, Kieswetter K, Liu Y, Cochran DL, Szmukler-Moncler S, Dean DD, Schwartz Z. Titanium surface roughness alters responsiveness of MG63 osteoblastic-like cells to $1\alpha,25-(OH)_2D_3$. *J Biomed Mater Res* 1998;39:77-85
7. Hanato K, Inoue H, Kojo T, Matsunaga T, Tsujisawa T, Uchiyama C & Uchida Y. Effect of surface roughness on proliferation and alkaline phosphatase expression of rat calvarial cells cultured on polystyrene. *Bone* 1999;25:439-445
8. Groessner-Schreiber B, Tuan RS. Enhanced extracellular matrix production and mineralization by osteoblasts cultured on titanium surfaces in vitro. *J Cell Sci* 1992;101(Pt 1):209-17
9. Grook K, Geesink R, Klein C. P. A. T., Selektion P. Plasma sprayed coating of hydroxyapatite. *J Biomed Mater Res* 1987;21:1375-1381
10. Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford Jr J, Dean DD, Cochran DL, Boyan DB. Effect of titanium surface roughness on proliferation, differentiation and protein synthesis of human osteoblast-like cells (MG63). *J Biomed Mater Res* 1995;29(3):389-401
11. Wang BC, Chang E, Lee TM, Yang CY. Changes in phases and crystallinity of plasma-sprayed Hydroxyapatite coating under heat treatment. : a quantitative study. *J Biomed Mater Res* 1995;29:1483-1492
12. Kim HM, Miyaji F, Kokubo T, Nishiguchi S, Nakamura T. Graded surface structure of bioactive titanium prepared by chemical treatment. *J Biomed Mater Res* 1999;45:100-107
13. Peltora T, Patsi M, Rahiala H, Kongsasniemi I Yli-Urpo A. Calcium phosphate induction by sol-gel-derived titania coatings on titanium substrates in vitro. *J Biomed Mater Res* 1998;41:504-510
14. Wei M, Uchida M, Kim HM, Kokubo T, Nakamura T. Apatite forming ability of CaO-containing titania. *Biomaterials* 2002;23(1):167-72
15. Sul Y-T, Johansson CB, Roser K, Albrektsson T. Qualitative and quantitative observation of bone tissue reactions to anodized implants. *Biomaterials* 2002;23:1809-17
16. Uchida M, KIM H-M, Kokubo T, Fujibayashi S, Nakamura T. Effect of water treatment on the apatite-forming ability of NaOH-treated titanium metal. *J Biomed Mater Res* 2002;63(5):552-530
17. Kasemo B, Lausmaa J. Biomaterial and implant surface: a surface science approach. *Int J Oral Maxillofac Implants* 1988;3:247-259
18. Larsson C, Thomsen P, Aronsson B.O, Rodahl M, Lausmaa J, Kasemo B, Ericson L.E. Bone response to surface-modified titanium implants: studies on the early tissue response to machined and electropolished implants with different oxide thicknesses. *Biomaterials* 1996;17:605-616
19. Klokkevold PR, Nishmura RD, Adachi M, Caputo A. Osseointegration enhanced by chemical etching of the titanium surface: A torque removal study in the rabbit. *Clin Oral Impl Res* 1997;8:442-447
20. Bernard JP, Szmukler-Moncler S, Pessotto S, Vazquez L, Belser UC. The anchorage

- of Brånemark and ITI implants of various lengths. I. An experimental study in the canine mandible. *Clin Oral Implant Res* 2003;14;593-600
21. Wong M, Eulenberger J, Schenk R, Hunziker E. Effect of surface topology on the osseointegration of implant materials in trabecular bone. *J Biomed Mater Res* 1995;29;1567-1575
 22. Gotfredsen K, Berglundh T, Lindhe J. Anchorage of titanium implants with different surface characteristics : An experimental study in rabbits. *Clin Implant Dent Relat Res* 2000;2;120-128
 23. Wang XX, Hayakawa S, Tsuru K, Osaka A. Improvement of the bioactivity of H₂O₂/TaCl₅-treated titanium after a subsequent heat treatment. *J Biomed Mater Res* 2000;52:171-176
 24. Lincks J, Boyan BD, Blanchard CR, et al. Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. *Biomaterials* 1998;19(23):2219-32
 25. Wojciak-Stothard B, Curtis A, Monaghan W, Macdonald K, and Wilkinson C, Guidance and activation of murine macrophages by nanometric scale topography. *Cell Res.*, 1996;223:426-435
 26. Curtis A, and Wilkinson C. Topographical control of cells. *Biomaterials*, 1997;18: 1573-1583
 27. Anselme K, Bigerelle M, Noel B, Dufresne E, Judas D, Iost A, Haldouin P. Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughnesses. *J Biomed Mater Res* 2000;49:155-166
 28. Matsuzaka K, Walboomers XF, Yochinari M, Inoue T, Jansen JA. The attachment and growth behavior of osteoblast-like cells on microtextured surfaces. *Biomaterials* 2003;24:2711-2719
 29. Britland S, Morgan H, Wojciak-Stodart B, Riehle M, Curtis A, Wilkinson C, Synergistic and hierarchical adhesive and topographic guidance of BHK cells. *Exp Cell Res* 1996;228:313-325
 30. Nishiguchi S, Kato H, Fujita H, Oka M, Kim HM, Kokubo T, Nakamura T. Titanium metals form direct bonding to bone after alkali and heat treatments. *Biomaterials* 2001;22:2525-2533
 31. Uchida M, KIM H-M, Kokubo T, Nakamura T. Compositional and structural dependence of apatite formation on titania gel in simulated body fluid. *Sixth World Biomaterials* 2000;1308
 32. Li P, DeGroot K. Calcium phosphate formation within sol-gel prepared titania in vitro and in vivo. *J Biomed Mater Res* 1993;27:1495-5000
 33. Rajaraman R, Rounds DE, Yen SPS, Rembaum A. A scanning electron microscope study of cell adhesion and spreading in vitro. *Exp Cell Res* 1974;88:327-339
 34. Lumbicanonda N, Sammons R. Bone cell attachment to dental implants of different surface characteristics. *Int J Oral Maxillofac Implants* 2001;16:627-636
 35. Deligianni DD, Katsala ND, Koutsoukos PG, Missirlis YF. Effect of surface roughness of hydroxyapatite human bone marrow cell adhesion, proliferation, differentiation and detachment strength. *Biomaterials*. 2001;22:87-96
 36. Zinger O, Zhao G, Schwartz Z, Simpson J, Wieland M, Landolt D, Boyan B. Differential regulation of osteoblasts by substrate microstructural features. *Biomaterials* 2004;6:1-11
 37. Gray S, Kian JB, van Wijnen AJ, Frenkel S, Montecino M. Mechanisms regulating osteoblast proliferation and differentiation.

- In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principle of bone biology. San Diego: Academic Press; 1996. pp69-89.
38. Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS, Ryoo HM Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J. Cell. Biochem. 1996; 61:609-18
 39. Matsuzaka K, Yoshinari M, Shimono M, Inoue T. Effects of mutigrooved surfaces on osteoblast-like cells in vitro: Scanning electron microscopic observation and mRNA expression of osteopontin and osteocalcin. J Biomed Mater Res 2004; 68A:227-234
 40. Nishio K, Neo M, Akiyama H, Nishiguchi S, Kim HM, Kokubo T, Nakamura T. The effect of alkali- and heat-treated titanium and apatite formed titanium on osteoblastic differentiation of bone marrow cells. J Biomed Mater Res 2000;52:652-661

알칼리 처리된 타이타늄 표면에 대한 골아 유사세포의 세포 활성화도

박진우¹, 이덕혜¹, 여신일¹, 박광범², 최석규², 서조영^{1,*}

1. 경북대학교 치의학전문대학원 치주과학교실
2. (주) 메가젠

임플란트와 골 사이의 결합력을 증가시키기 위하여 타이타늄 표면에 변화를 주기위한 많은 연구들이 진행되고 있다. 타이타늄의 표면 구조나 미세 지지도의 변화가 임플란트에 대한 세포의 반응에 영향을 미치며, 골아 유사세포는 표면 조도가 높은 타이타늄 표면에 더 잘 부착하며, 세포외 기질의 합성과 광물화 결정이 더 잘 일어난다고 알려져 있다. 그러나 대부분의 연구들은 마이크로 단위의 미세 지지도에 대한 연구들이고 나노 단위의 미세 지지도에 대한 연구들은 미미하다. 이에 본 연구에서는 **ROS 17/2.8 cell line**을 이용하여 기계적 처리만한 군을 대조군으로 하여 **blasting** 처리한 마이크로 단위의 미세 지지도 표면과 알칼리 처리된 나노 단위의 미세 지지도 표면에 대한 골아 유사세포의 세포 부착양상, 증식 그리고 골아 유사세포의 표식인자 발현양상 등을 상호 비교하여 골아 유사세포에 미치는 영향을 관찰하고자 하였다.

SEM을 이용한 미세 지지도 관찰에서 알칼리 처리군에서는 약 **200 nm**의 초미세 다공성의 양상을 나타내었고, **blasting** 처리한 군에서는 **10 μ m** 이하의 움푹 파인 양상을 보였다. 표면조도 측정에 있어서는 **blasting** 처리한 군에서 기계적 처리와 알칼리 처리된 군보다 더 높은 표면 조도를 보였으며 이는 통계학적으로 유의한 차이를 나타내었다 ($p<0.01$). 표면결정성 분석에서는 알칼리처리 군에서 **anatase**와 **rutile**결정형이 보였으나, **blasting** 처리한 군과 기계적 처리 군에서는 관찰되지 않았다.

골아 유사세포 1시간 배양 후의 전자현미경 관찰에서 모든 군의 세포는 부착 및 전개 과정을 보였고, 3시간 배양에서는 모든 군의 세포가 더 많이 전개되었으나, **blasting** 처리한 군과 알칼리처리 군에서 세포가 다소 더 불규칙한 형태를 나타내었다. 24시간 배양에서는 모든 군의 세포에서 완전히 전개가 일어난 양상을 보였다.

1, 4, 7 일간 세포배양 후 세포활성을 평가하기 위한 **MTT assay**에서는 모든 군에서 시간이 증가함에 따라 세포수가 증가하였으며, 1일째에 **blasting** 처리한 군과 알칼리처리 군에서 기계적 처리 군에 비해 세포활성도가 통계학적으로 유의한 증가를 보였다($p<0.01$).

골아 유사세포 표식인자인 **osteopontin**, **alkaline phosphatase**, **$\alpha 1(I)$ collagen**의 유전자 발현양상을 관찰해 본 결과, **osteopontin**, **alkaline phosphatase**, **$\alpha 1(I)$ collagen**의 유전자 발

현양상이 세 군 모두에서 유의한 차이는 관찰할 수 없었으나, **blasting** 처리한 군과 알칼리처리 군에서 기계적 처리 군에 비해 유전자 발현양상이 다소 증가하는 경향을 보였다.

결론적으로 **blasting** 처리한 마이크로 단위의 미세 지지도 표면과 알칼리 처리된 나노 단위의 미세 지지도 표면이 기계적 처리 군에 비해 골아 유사세포의 기능을 촉진시키나, 알칼리 처리된 나노 단위의 미세 지지도 표면은 **blasting** 처리한 마이크로 단위의 미세 지지도 표면이 골아 유사세포의 기능에 미치는 영향을 압도하지는 않는 것으로 사료된다.

