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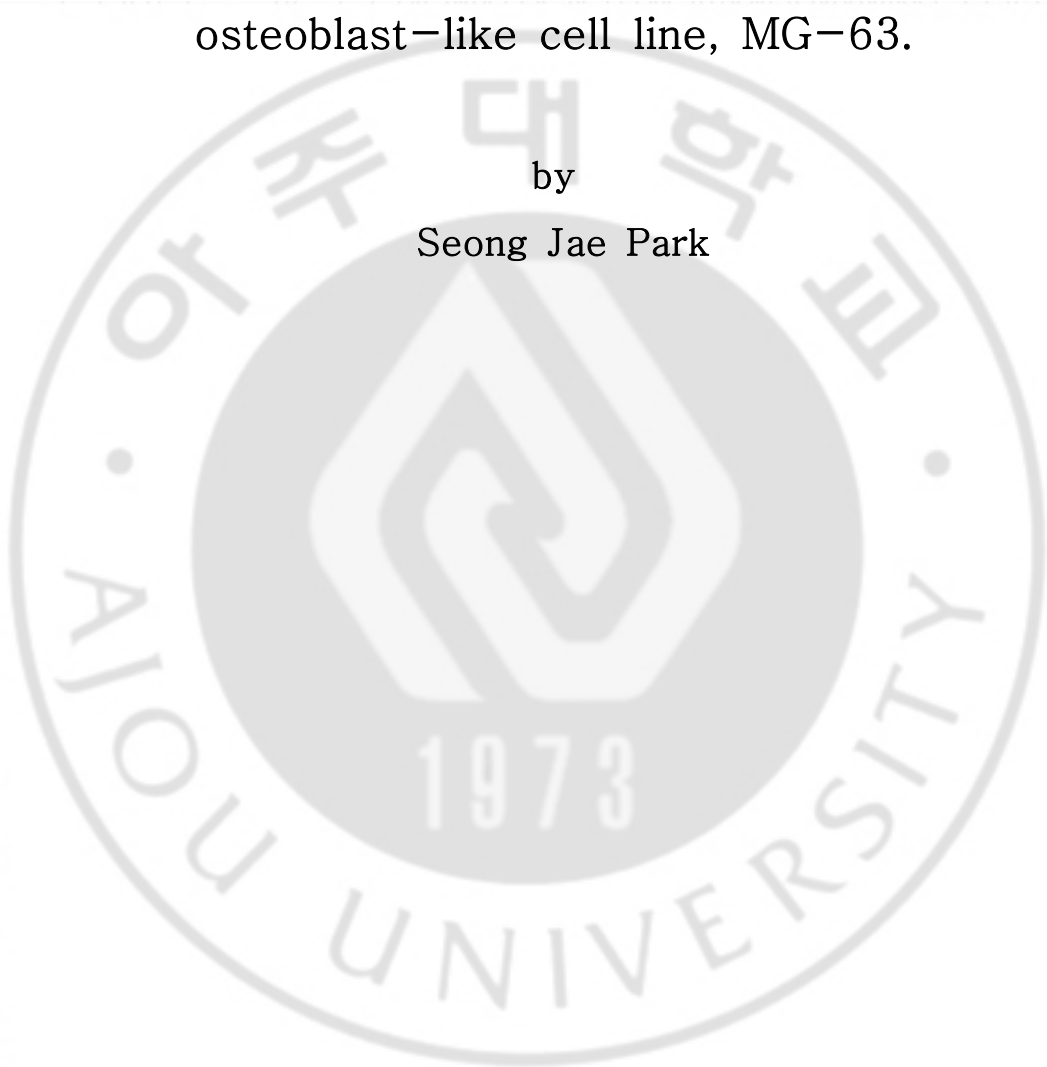
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Effect of implant surface microtopography by
hydroxyapatite grit-blasting
on adhesion, proliferation and differentiation of
osteoblast-like cell line, MG-63.

by

Seong Jae Park



Major in Medicine

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- ABSTRACT -

Effect of Implant Surface Microtopography by Hydroxyapatite Grit-blasting on Adhesion, Proliferation and Differentiation of Osteoblast-Like Cell Line, MG-63.

Objective: The purpose of this study is to evaluate the potential of the *in vitro* osteogenesis of microtopographically modified surfaces, RBM (resorbable blasting media) surfaces that generate a hydroxyapatite grit-blasting.

Methods: First, to produce microtopographically modified surfaces, we made the RBM surfaces using a hydroxyapatite grit-blasting and examined the surface morphology, roughness or elements. And then, to investigate the potential of the *in vitro* osteogenesis, we experimented the osteoblastic cell adhesion, proliferation and differentiation using the human osteoblast-like cell line, MG-63 cells. Osteoblastic cell proliferation was performed to time-course. Also osteoblastic cell differentiation was verified by four different methods of ALP activity assay, mineralization assay using alizarin red-s staining and gene expression of osteoblastic differentiation marker using RT-PCR or ELISA.

Results: Comparing with machined group, osteoblastic cell adhesion, proliferation and ALP activity of RBM surfaces were shown higher. Also, they exhibited high level of gene expression of osteoblastic differentiation makers (osteonectin, type I collagen, Runx-2, osterix). Similar data was represented in the ELISA that RBM surface increased secretion of osteocalcin, osteopontin, TGF-beta1 and PGE₂ which was known to stimulate the osteogenesis. Moreover, alizarin red-s staining revealed that there were significantly more

mineralized nodules on RBM surfaces compared with machined discs.

Conclusions: Our results demonstrated the RBM surfaces modified with hydroxyapatite grit-blasting which stimulate the *in vitro* osteogenesis in MG-63 cells and raises the potential that RBM surfaces that accelerate the bone formation and finally increase bone-implant contact.

Key words: Osseointegration, Osteogenesis, Microtopography, Surface roughness, RBM (resorbable blasting media), Hydroxyapatite, Biocompatibility

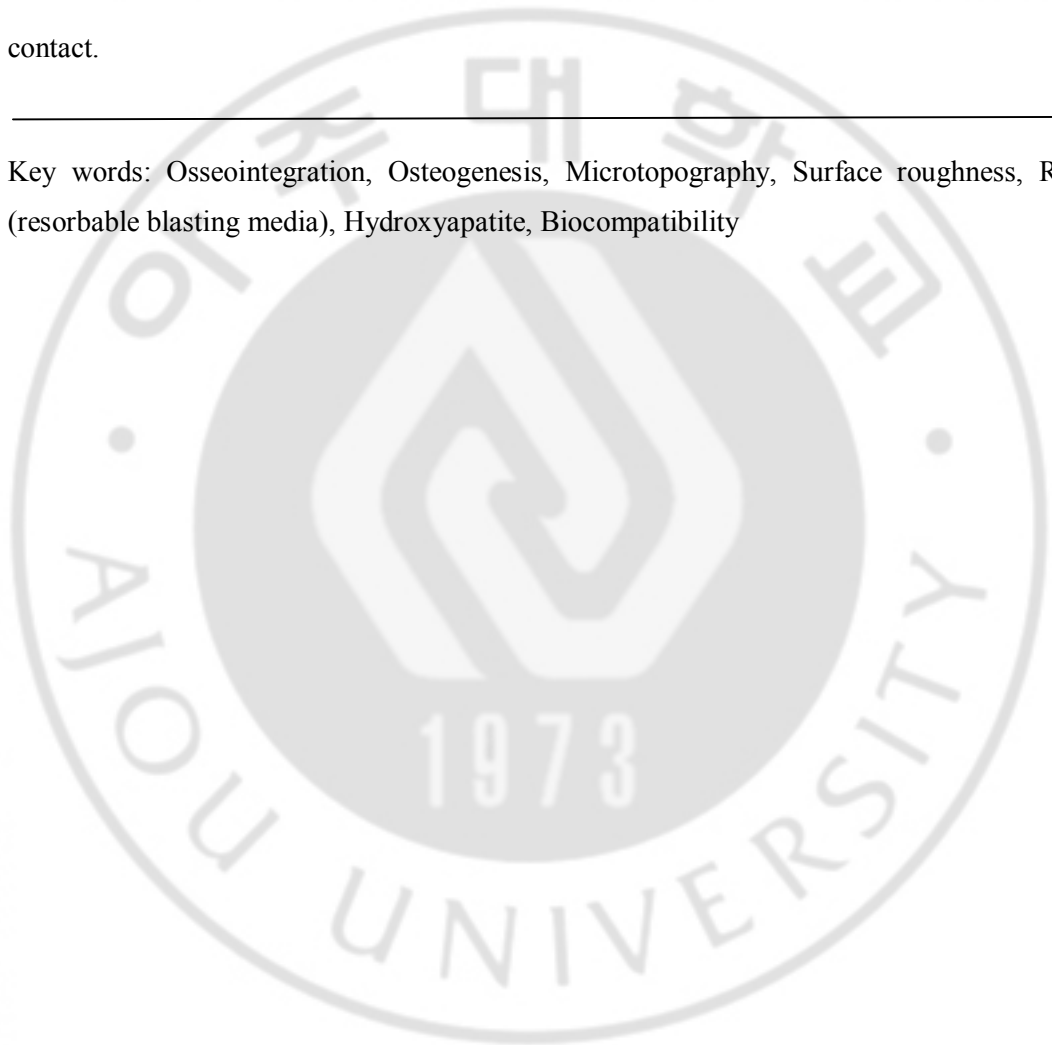
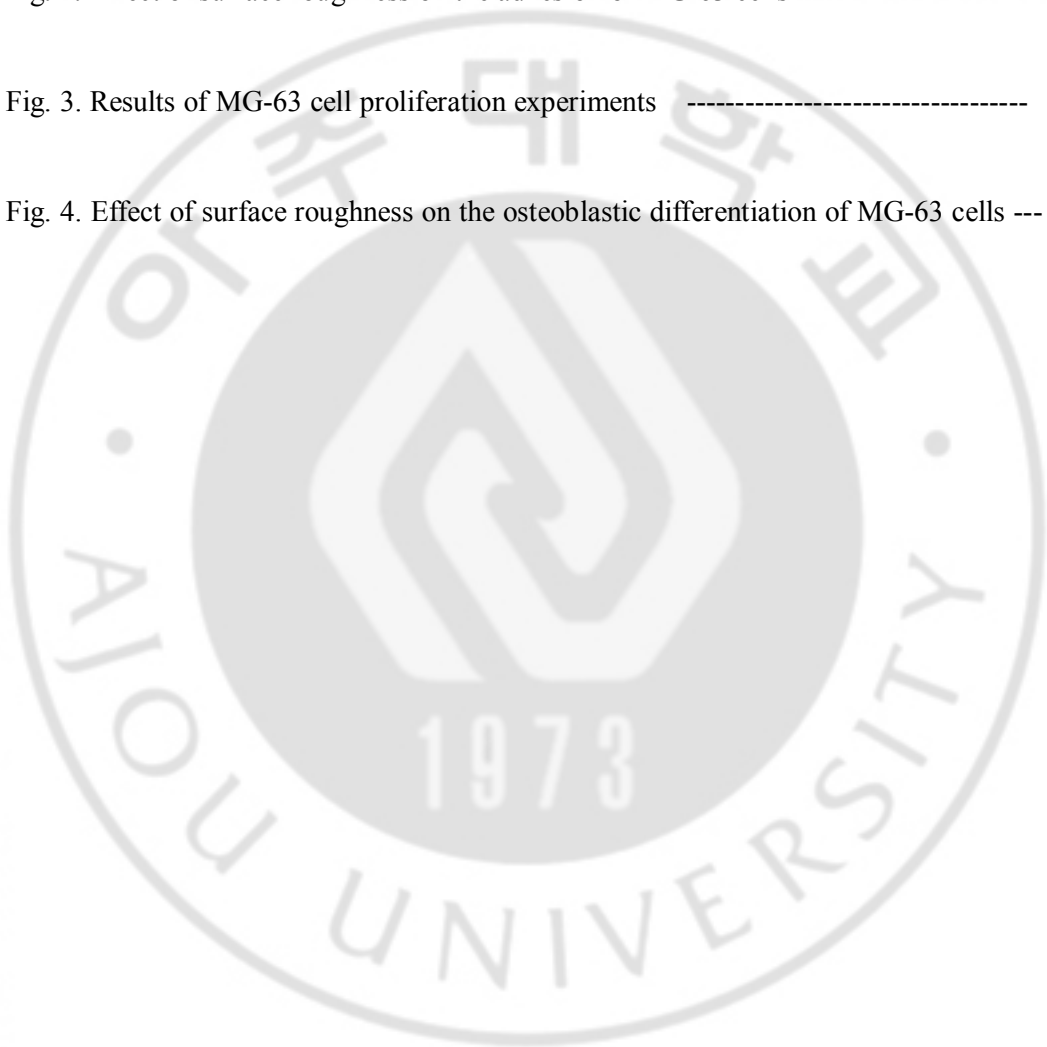


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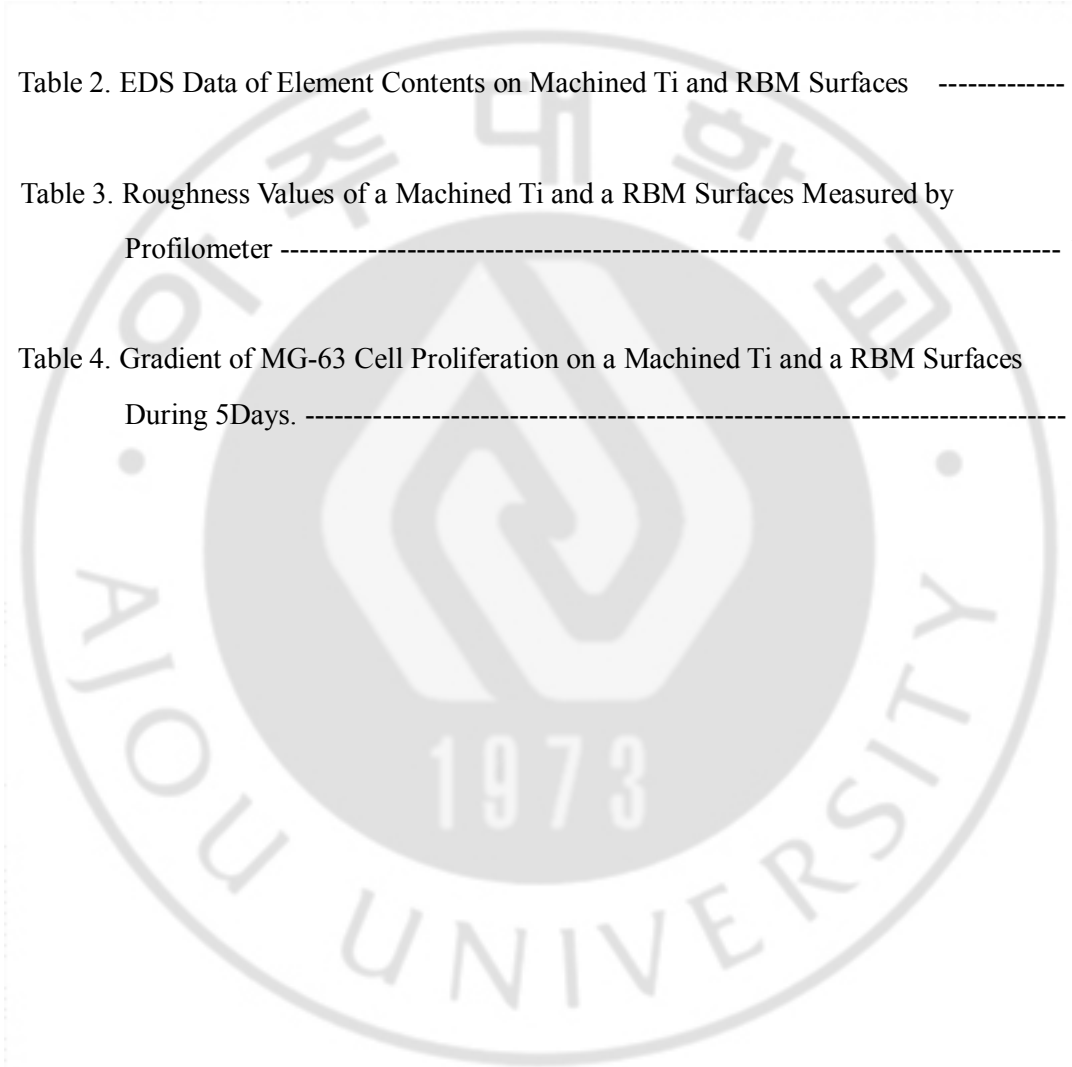
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I . INTRODUCTION

With regards to their biological and physiochemical properties, titanium-based biomaterials have been successfully used in orthopaedic, dental and maxillo-facial surgery, especially in endosseous implants. The early osseointegration of titanium dental implants is an important factor for their clinical success(Davies, 1998; Berglundh et al, 2003). The implant loading to bone is influenced by various factors, including surface chemistry, energetics and surface topography(Albrektsson and Wennerberg , 2004; Esposito et al, 2005; Zhao et al, 2005; Puleo and Thomas, 2006). These surface parameters are decisive during the bone healing period in the peri-implant region. After implantation, implant surfaces contact with body fluids and interact with a number of proteins and different cell types. Cell adhesion is one of the initial stages for subsequent proliferation. Differentiation of osteoblastic cells produces bony tissue and extracellular matrix, which will ensure a high bone-implant contact. It has been shown that osteoblastic cell adhesion, growth and differentiation are related to surface chemistry, energetics and roughness(Cooper et al, 1998; Anselme, 2000; Zhao et al, 2005).

Although many *in vitro* experiments have studied osteoblastic cell behavior on titanium surfaces, the optimal surface parameters for adhesion, proliferation and differentiation of osteoblastic cells have not yet been completely understood(Kieswetter, 1996; Cooper et al, 1998). But among them, surface roughness is one of the most acknowledged parameters improving the bone anchorage of titanium implants(Predecki et al, 1972; Thomas and Cook, 1985; Carlsson et al, 1988). A different evidence indicate that surface roughness of

titanium implants strongly affects the behavior of osteoblastic cells. First of all, rough surfaces encourage the entrapment of fibrin protein, adhesion of osteogenic cells and mechanical stability of implants in host bone(Lauer *et al*, 2001; Mustafa *et al*, 2001; Ellingsen *et al*, 2004). On the other hand, numerous reports indicate that increased surface roughness stimulates osteoblastic cell differentiation and bone formation(Wennerberg *et al*, 1996;Anselme, 2000 *et al*; Anselme, 2000).

To improve the clinical performance of implants and to guarantee a stable mechanical loading, there are various methods have been developed to create rough implant surfaces. Among them, the popular methods for roughening titanium implants use a biocompatible and resorbable blasting media, such as hydroxyapatite and β -tricalcium phosphate (β -TCP) ceramic particles or other calcium phosphate ceramic particles(Mustafa *et al*, 2001; Citeau *et al*, 2005; Le Guehenec *et al*, 2007). These calcium phosphate biomaterials have been contemplated as a potential abrasive materials. Indeed hydroxyapatite and β -TCP are biocompatible. They are well known to form a direct bond with surrounding tissue after bone implantation. As these calcium phosphate materials are soluble in acids, they can be easily removed from the implant surface after blasting(Sanz *et al*, 2001; Novaes *et al*, 2002). As these treatments generate surfaces with random topography and various chemical compositions, the optimal surface properties that result in rapid osteoblastic cell adhesion, proliferation and differentiation.

In view of the above-mentioned data, the main objectives of this work were (i) to develop a new technique of blasting using hydroxyapatite particles that provide a roughened surface which improves the osteoblastic cell adhesion, proliferation and differentiation and

(ii) to assess effects of the roughened surface with respect to osteoblastic cells. In this attempt, we examined the roughness of pure titanium discs prepared by hydroxyapatite grit-blasting technique. Two groups were investigated: polished (machined) and hydroxyapatite grit-blasted (RBM) titanium surfaces. After analyzing the properties of the implant surfaces, osteoblastic cell adhesion, proliferation and differentiation were studied.



II. MATERIALS AND METHODS

A. Materials

Commercially pure titanium (cpTi) discs are 12mm in diameter and 1mm in thickness (Carpenter, Washington, Alabama, USA). The samples were cut from a cpTi bar and used as machined discs. Abrasive hydroxyapatite powders (40-80 mesh) (Himed, New York, NY, USA), Cell culture plastic (Falcon, Franklin Lakes, NJ, USA), Fetal bovine serum (FBS), DMEM, penicillin/streptomycin, trypsin/EDTA (HyClone, Salt Lake City, Utah, USA), Phosphate buffered saline (PBS) (Invitrogen Corporation, Inchinnan, Paisley, UK), Triton X-100, Alizarin red-s, p-nitrophenylphosphate (p-NPP), cresyl violet, MgCl₂ (Sigma, St. Louis, MO, USA), 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl-2H) tetrasolium inner salt (MTS) and hTGF-beta1 ELISA kit (Promega, Madison, WI, USA), hOsteocalcin ELISA kit (Biosource, Nivelles, Belgium), hOsteopontin and hPGE2 ELISA kit (Assay Designs, Ann Arbor, MI, USA), QuantiTech® Reverse Transcription kit (Qiagen, Valencia, CA, USA), PCR premix (Bioneer, Daejeon, Korea) were prepared. All other chemicals were from standard laboratory suppliers and were of the highest purity available.

B. Methods

1. Preparation of titanium discs

CpTi discs were cleaned with acetone and ethanol. Discs were thereafter randomly divided into two groups. The polish group was obtained by polishing with silicon carbide abrasive paper (Jinan Abrasive Cloth Factory, Jinan, Shandong, China). Decreased grind

paper P400, P600, P1200, P2000 were successfully used to a rotative polisher at 250 rotation per minute (rpm) for 1min. The hydroxyapatite grit-blasted surface (RBM group) was prepared by blasting using hydroxyapatite powders (Himed, New York, NY, USA) of 40-80 mesh with a microhardness. The hydroxyapatite powders were blasted at air pressure of 5 bars for 10 sec. Hydroxyapatite grit-blasted (RBM) discs were then passivated by immersion for 10 min in 15% nitric acid at room temperature and rinsed deionized water. Then the samples were ultrasonically cleaned for 30min in Et-OH, washed 3 times in DW. Finally they were dried at 80°C dry oven. Before use in the cell-based assays, all discs were packaged and sterilized using autoclave machined for 20min at 120°C.

2. Surface roughness analysis

Three cpTi discs of each group were randomly selected and roughness measurements were carried out with a SV-3000 S4 digital profilometer (Mitutoyo, Tokyo, Japan) linked to an acquisition software Surfpak-SV. This profilometer system consists of a probe which scanned the surface 2.4mm in length. Results were expressed 2D parameter, as a Ra (average roughness), Rz (average maximum height of the profile) and Rt (maximum height of the profile). These parameters expressed in μm , gave a general description of surface roughness.

3. Scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS)

The different surface morphologies of cpTi and RBM surface were examined using a scanning electron microscopy (SEM, JEOL, Tokyo, Japan) at 20Kv. An energy-dispersive spectrophotometer (EDS) attached with the JSM-6480LV SEM has also been used to

characterize the elemental atomic composition of samples. Using the INCA-sight system of Oxford Instrument, elemental analysis of a defined area on the cpTi and RBM surface can easily be determined to a high degree of precision (~5 wt %).

4. Cell culture

The MG-63 cells (ATCC, Manassas, VA, USA) is a non-transformed cell line established from human osteosarcoma. This cell was routinely grown in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Salt Lake City, Utah, USA) and 0.1 mg/ml penicillin and streptomycin (P/S) (HyClone, Salt Lake City, Utah, USA). Cells were subcultured twice a week using trypsin/EDTA and maintained at 37°C in a humidified atmosphere of air including 5% CO₂. Medium was completely renewed every two days.

5. Cell adhesion

The cell adhesion assay of the MG-63 cells performed using previous report (Ginsberg and Che, 2004; Hutton et al, 2008). Briefly, the cells were grown to approximately 80% confluence prior to cell adhesion assay. Mg-63 cells were cultured either onto the various titanium disc or in the culture plastic in 24 multi-well plate at a density of 1×10^5 cells. After 1hr, culture media were removed and rinsed three times by DPBS. The cells were fixed using 4% formaldehyde solution for 1hr at 4°C and were rinsed three times by DPBS. Then the viable cells stained with cresyl violet which stained ribonucleic acid and were eluted using citric acid. Finally colorimetric measurement of cresyl violet was performed on a DTX 880 (Beckman Coulter, Fullerton, CA, USA) with optical density reading at 590nm.

6. Cell Proliferation

The cell proliferation assay of the MG-63 cells was performed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. MG-63 cells were cultured either onto the various titanium disc or in the culture plastic in 24 multi-well plate at a density of 1×10^5 cells. After indicated times, culture media was removed and 500 μ l MTS/PMS/Media mixture was added in each well for 1hr. Finally colorimetric measurement of formazan dye performed on a DTX 880 with optical density reading at 490nm. Compared to control, results were expressed as optical density and relative MTS activity.

7. Alkaline phosphatase (ALP) activity

ALP activity was examined as previously described (Engvall, 1980; Jung and Pergande, 1980; Buxton and Murdoch, 1982; Jones et al, 1989), in MG-63 cells cultured either onto the various titanium disc or culture plastic in 24 multi-well plates (1×10^5 cells/well). Osteogenic medium (0.5 μ M dexamethasone, 50 μ g/ml L-ascorbic acid, 10mM β -glycerolphosphate) (Chen et al, 2008; Rausch-Fan X et al, 2008), was completely renewed every two days and after indicated times. Cells were washed twice with DPBS and collected in 0.1% ALP lysis buffer. In order to extract the dissolved proteins from the crude cell lysates, the supernatants were centrifuged at 13,000 rpm for 10 min. The protein concentrations were determined using a BCA reagent (Pierce, Rockford, IL, USA). For ALP activity measurement with isolated cell supernatants, 100 μ g total protein were incubated with 2mg/ml p-NPP (as a chromogenic substrate, Sigma) using ALP assay buffer. (pH 9.75

in 0.1M Glycine containing 1mM MgCl₂) at 37°C for 2hr. The degree of colorimetric reaction was measured by DTX 880 for an optical density measurements at 405nm.

8. Alizarin red-s staining

The alizarin red-s staining with experimental samples was performed as reported elsewhere (Sudo H et al, 1983; Gregory CA et al, 2004; Malladi P et al, 2006) with a minor modifications. Briefly, MG-63 cells were cultured either onto the various titanium disc or culture plastic in 24 multi-well plates (1×10^5 cells/well). Osteogenic medium was completely renewed every two days and after indicated times. Cells were washed twice with DPBS and fixed in 10% (v/v) formaldehyde (Junsei, Chuo-ku, Tokyo, Japan) for 20min at room temperature and rinsed with triple distilled H₂O. Cells were stained with 2% alizarin red-s (Sigma-Aldrich, Oakville, ON, Canada), pH4.2, for 20min with gentle agitation. After aspiration of the unstained dye, the wells were washed five times with distilled H₂O while shaking for 5min. For quantification of staining, 500µl 10% (v/v) acetic acid was added to each well, and the plate was heated to 70~80°C for 1hr. The cells were then scraped from the plate and centrifuged at 13000rpm for 10min. The supernatant was transferred to a new e-tube and the 0.4 volume of 10% (v/v) ammonium hydroxide added to neutralize the acid. Absorbance of extracted alizarin red-s in acetic acid solution (200µl) was measured at 405nm. Amount of alizarin red-s was determined according to an alizarin red-s standard curve and normalized to the total protein of the cell lysates using BCA method (Pierce, Rockford, IL, USA).

9. ELISA

MG-63 cells were cultured either onto the various titanium disc or culture plastic in 24 multi-well plates (1×10^5 cells/well). Osteogenic medium was completely renewed every two days. After indicated times, supernatants were collected using centrifuge (at 13,000 rpm for 2 min) to eliminate a cell debris. The ELISA of the MG-63 cells performed using each ELISA kits (hTGF-beta1 ELISA kit; Promega, hOsteocalcin ELISA kit; Biosource, hPGE₂ ELISA kit; Assay Design, hOsteopontin ELISA kit; Assay Design) according to the manufacturer's protocol.

10. Semi-quantitative RT-PCR

Changes in osteoblast gene expression for osteonectin, Type I collagen, osteoprotegrin, BSP-2, Runx-2 and Osterix were analyzed using semi-quantitative RT-PCR. MG-63 cells cultured either onto the various titanium disc or culture plastic in 24 multi-well plates (1×10^5 cells/well). Osteogenic medium was completely renewed every two days. After 72hr, total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Using the extracted RNA as a template, reverse transcription was carried out with QuantiTech[®] Reverse Transcription kit (Qiagen, Valencia, CA, USA) and RT reactions were performed in a Mastercycler (Eppendorf, Westbury, NY, USA). RT reaction mixture was incubated at 42°C for 20min heated at 94°C for 10min, and subsequently chilled to 4°C. Then osteonectin (OSN), type I collagen (COL1), runt-related transcription factor 2 (Runx-2) and Osterix (OSX) were amplified by Mastercycler using PCR premix (Bioneer, Daejeon, Korea) (see Table 1). The captured image of amplification of

osteogenic marker gene expression products from 2% agarose gel stained with ethidium bromide was analyzed using a Doc-It system (UVP, Upland, CA, USA). Semi-quantitative analysis was achieved by calibration. The level of specific osteogenic marker bands was normalized for the housekeeping β -actin cDNA content.

Table 1. Primer Sequences of Osteogenic Marker and Conditions for Polymerase Chain Reaction

Target Gene	Primer Sequences(5'→3')	Annealing Temperature	Number of Cycle
OSN	Forward ACA TGG GTG GAC ACG G	50°C	35
	Reverse CCA ACA GCC TAA TGT GAA		
COL1	Forward ATC CGC AGT GGC CTC CTA AT	52°C	35
	Reverse TCC CCT CAC CCC AGT AT		
Runx-2	Forward AGG TGA CTG GCG GGG TGT AA	53°C	35
	Reverse CTT CTG CCT CTG GCC TTC CA		
OSX	Forward CAG CTG CCC ACC TAC CCA TC	53°C	35
	Reverse CCA CTA CCC CCA GTG CTT GC		
β-Actin	Forward GTG GGG CGC CCC AGG CAC CAG GGC	58°C	30
	Reverse CTC GTT AAT GTC ACG CAC GAT TTC		

OSN, Osteonection; **COL1**, Type I Collagen, **Runx-2**, Runt-Related Transcription Factor-2; and **OSX**, Osterix

11. Statistical analysis

Each experiment was repeated at least twice with similar results. Results are expressed as mean \pm STDEV of triplicate determinations. For statistical analysis of experiments (N=6, three groups repeated two times), unpaired t-tests and ANOVA were performed using SPSS.

(*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$)

III. RESULTS

A. Surface Parameter Tuning for Optimization of RBM

The purpose of this investigation was to find an optimal RBM surface that show the appropriated cell response. To decide the optimal RBM surface, we established surface roughness as a key parameter that influence to RBM surface characteristics. Briefly, cell adhesion, proliferation and osteoblastic differentiation displays tendency that increase as RBM surface roughness grows. (data not shown) Through this approach, we select the optimal RBM surface that has a 1.5 μ m range surface roughness and a following experiments used selected RBM condition.

B. Surface Characterization of Optimized RBM

SEM indicates a quite discrepancy between each of the two surfaces. RBM surfaces exhibited a highly rugged and irregular surface (Fig. 1A, B). The roughness parameters as measured by optical profilometer are represent in Table 3. The values refer to the mean of ten measurements for each of the two types of surfaces. The surfaces scored from rougher to more smoother as follows: culture plate < machined < RBM. According to the EDS data (Fig. 1C, D), machined or RBM surface was basically similar elemental composition. The RBM surfaces possesses the phosphorous and calcium of infinitesimal quantity (Fig. 1D, Table 2.). This phenomenon is due to hydroxyapatite powder that remains behind after blasting.

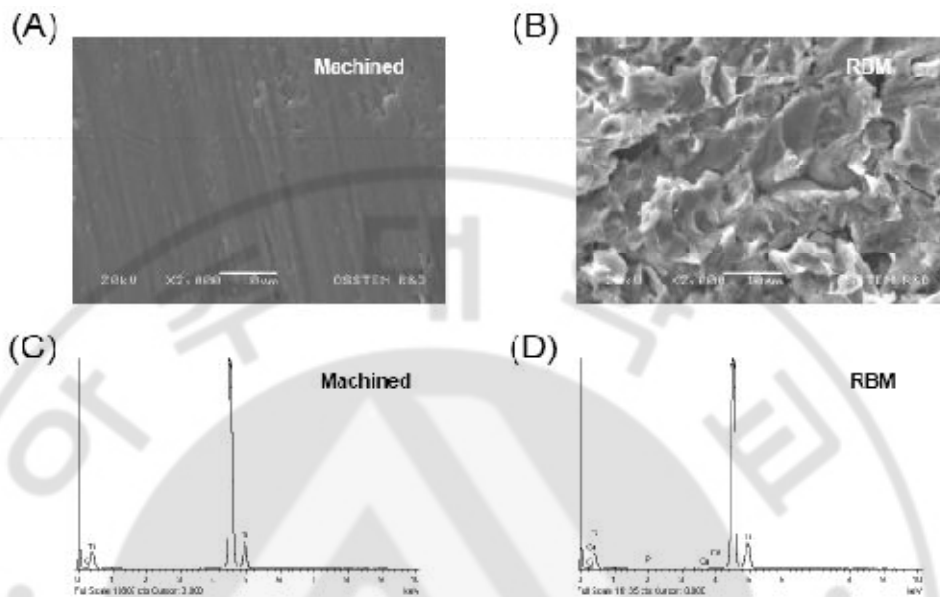


Fig. 1. Surface morphology of titanium discs for osteoblastic cell culture. SEM images of machined titanium (A) and RBM (B). Bar = 10 μ m. EDA Data of machined titanium (C) and RBM (D). Surfaces were examined using the JSM-6480LV SEM at working distance of 10 μ m and accelerating voltage of 20 kV.

Table 2. EDS Data of Element Contents on Machined Ti and RBM Surfaces

	Element,(Weight %)				Element,(Atomic %)			
	C	p	Ca	Ti	C	P	Ca	Ti
Machined	1.44	0	0	98.56	5.52	0	0	94.48
RBM	1.48	0.31	0.15	98.06	5.65	0.46	0.18	93.72

C, Carbon; P, Phosphorous; Ca, Calcium and Ti, Titanium

Table 3. Roughness Values of a Machined Ti and a RBM Surfaces Measured by profilometer

	Machined	RBM
Ra, [μm]	0.222(\pm 0.039)	1.523(\pm 0.156)
Ra2max, [μm]	0.244(\pm 0.032)	1.663(\pm 0.163)
Rz, [μm]	1.566(\pm 0.199)	10.514(\pm 1.177)
Rt, [μm]	1.727(\pm 0.278)	11.648(\pm 1.378)

Ra, average roughness; R2max, maximum value of roughness; Rz, average maximum height of the profile; and Rt, maximum height of the height

C. Cell Adhesion

The cell adhesion data of the MG-63 cells on the different surfaces are presented in Fig. 2. Statistical testing of the data, using a two-way analysis of variance (ANOVA) and a multiple comparison test (Turkey), revealed that after 1hr of incubation in the RBM surfaces, the adherent cells became significantly higher than the machined surface and the culture plate (* $P < 0.05$; Fig. 2).

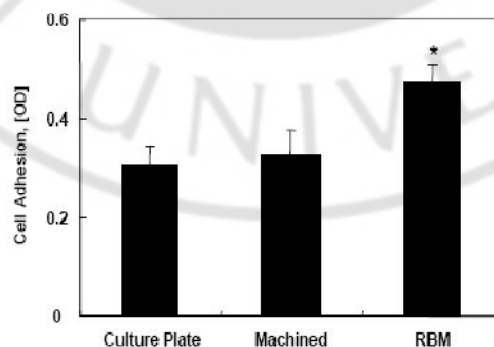


Fig. 2. Effect of surface roughness on the adhesion of MG-63 cells. MG-63 cells were

stained with cresyl violet dye and counted using spectrophotometer on surfaces of differing roughness. Data are represented as the average \pm standard deviation. RBM surface, the rough surface, were promoted the cell adhesion compared to smooth machined surface. *: Statistically significant compared with cells cultured on machined surfaces ($p < 0.05$)

D. Cell Proliferation

To examine the proliferation of osteoblastic cells in RBM surface, we carried out a measurement of MTS activity on MG-63 cells after 1, 3, 5 days in culture. Results indicated that cell proliferation on RBM surface was increased after 1 days of culture than the machined surfaces (* $P < 0.05$; Fig. 3). This phenomenon was observed similar at 3 days and 5 days. Also gradient of cell proliferation curve was more steep in RBM surfaces (Table 4). Therefore these results showed that proliferation of MG-63 cells was increased as early as 1 days in culture on RBM surface, compared to the machined surface.

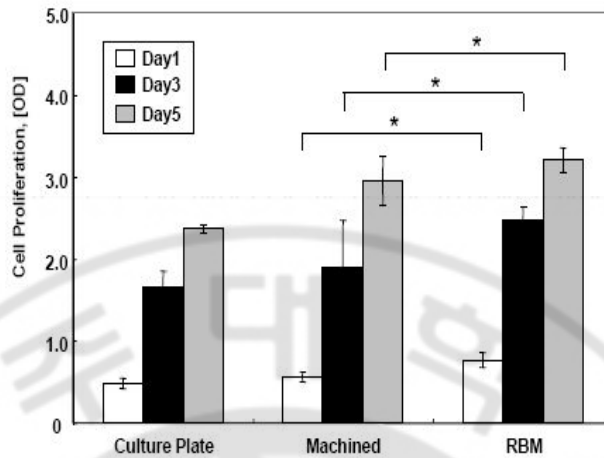


Fig. 3. Results of MG-63 cell proliferation experiments. Data are represented as the average \pm standard deviation. The differences between the number of cells on RBM and machined surfaces during 5 days is statistically significant. *: Statistically significant compared with cells cultured on machined surfaces ($p < 0.05$).

Table 4. Gradient of MG-63 Cell Proliferation on a Machined Ti and a RBM Surfaces During 5 Days.

	Culture Plate	Machined	RBM
Gradient of Cell Proliferation	0.9493	1.1937	1.2196

E. Cell Differentiation

1. ALP activity

Generally, ALP activity was affected by the surface microtopography. In addition

osteoblastic cell differentiation was assessed by measuring the ALP activity normalized to total protein content. On RBM surfaces, we observed a significant enhancement of ALP activity ($10.8 \pm 2.6\%$), compared to machined surface after 7 days (** $P < 0.001$; Fig. 4A). These results indicate that osteoblastic cells cultured in direct contact with RBM surface increased their capability to express ALP

2. Mineralization (Quantification of alizarin red-s staining)

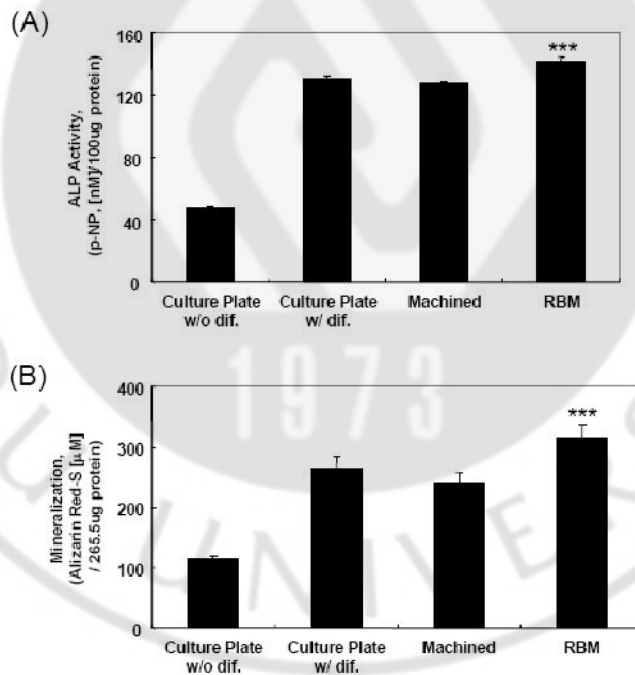
We used the alizarin red-s staining for quantification of mineralization induced by osteoblastic cells. As shown Fig. 4B, similar pattern in ALP activity data and MG-63 cells cultured on RBM surfaces had increased mineralization dramatically (** $P < 0.001$).

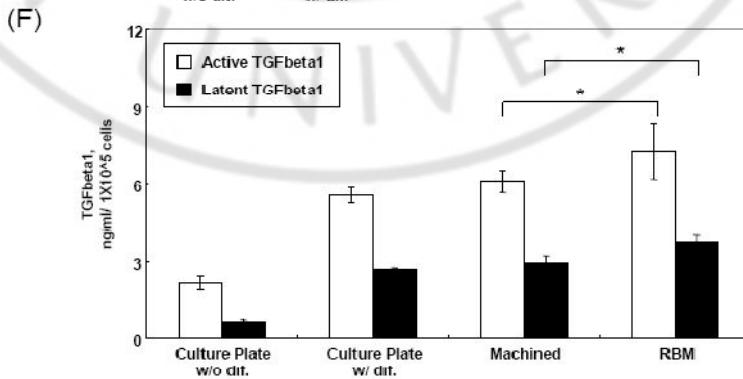
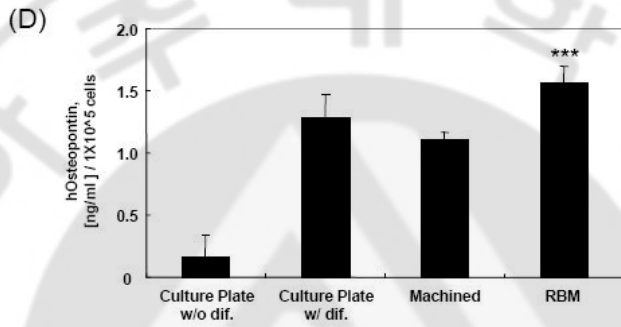
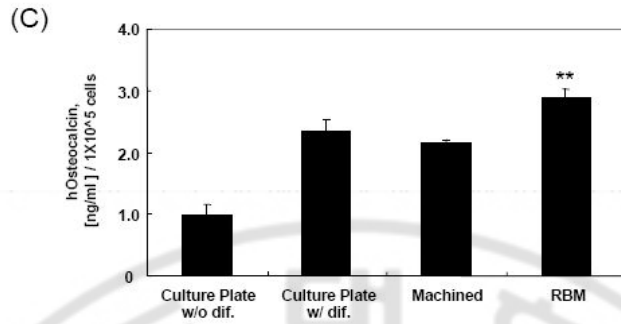
3. ELISA

According to the acceleration of the differentiation, various osteoblastic differentiation-related proteins, such as extracellular matrix proteins or other signaling factors, were secreted. So we checked the expression levels of osteoblastic differentiation-related proteins using ELISA methods. Osteocalcin secretion by MG-63 cells was slightly greater (** $P < 0.01$) on RBM surface than machined surface (Fig. 4C). Moreover, osteopontin (** $P < 0.001$), PGE₂ (** $P < 0.001$) and TGF-beta1 (* $P < 0.05$) secretion were greater on RBM surface than the machined surface (Fig. 4D, 4E, and 4F separately). As a result ELISA data showed that the RBM surface enhances the secretion of various osteoblastic differentiation-related proteins.

4. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR showed significantly (* $P < 0.05$) increased type I collagen and Runx-2 (runt-related transcription factor 2, cbfa-1, OSF-2) gene expression in MG-63 grown on RBM, relative to machined surface or culture plate (Fig. 4G). There was a slight, but insignificant, increase in OSN (osteonectin) and OSX (osterix) gene expression on the RBM surface relative to culture plate or machined surface (Fig. 4G). These results strongly suggested that RBM surface increases the expression level of osteoblastic differentiation marker gene associated with osteogenesis and osseointegration.





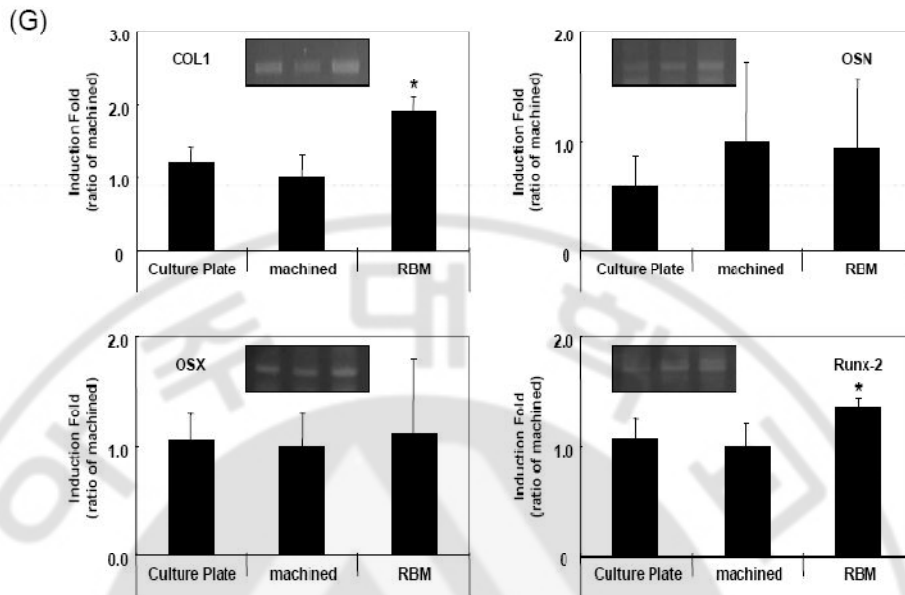


Fig. 4. Effect of surface roughness on the osteoblastic differentiation of MG-63 cells.

Data are represented as the average \pm standard deviation. (A) ALP (alkaline phosphatase) activity of MG-63 cells cultured either on machined Ti and RBM surfaces. Culture plate without osteogenic differentiation was served as a internal negative control. And a culture plate with osteogenic differentiation was a internal positive control. On RBM surface, the ALP activity enhanced about 10% than machined surface (B). Mineralization properties of the machined Ti and RBM surface. MG-63 cells were cultured as before for 14 days with osteogenic differentiation medium. Total calcium deposition measured by alizarin red-s stain via extraction with acetic acid. (C-F) Effect of surface roughness on osteogenesis-related local factor levels determined by using an ELISA kit. Osteocalcin levels were measure in the conditioned media using an ELISA kit specific for human osteoclastin (C). Osteopontin

levels were measured in the conditioned media using an ELISA kit specific for human osteopontin (D). PGE₂ levels were measured in the conditioned media using an ELISA kit specific for humans PGE₂ (E). And Active of latent TGF-β1 (transforming growth factor-β1) levels were measure in the conditioned media using an ELISA kit specific for human TGF-β1 (F). (G) RT-PCR analysis of roughness-influenced osteogenic marker gene. Type I collagen, osteonectin, osterix and Runx-2 RNA levels in MG-63 cells exposure for 72hr on a machined Ti or RBM surface. RT-PCR products were subjected to electrophoresis on 2% agarose gel and visualized UV exposure. A representative analysis is shown inside of the each figure. And the level of specific bands was normalized for the β-actin cDNA content. Statistically significant compared with cells cultured on machined surfaces by unpaired t-tests (* P<0.01, ** P<0.05, *** P<0.001).

IV. DISCUSSION

Osteoblastic cells play a critical role in the early stages of osseointegration. Several reports have assessed that osteoblastic cell response to on smooth surfaces more rapidly than rough surfaces while their osteoblastic differentiation was enhanced by rough topography (Schwartz et al, 1999; Bächle and Kohal, 2004; Anselme and Bigerelle, 2005; Kim, 2006; Kim et al, 2006). Little is known about the regulatory mechanism of the osteoblastic cell adhesion, proliferation and differentiation on the titanium discs. This study provides the evidence that the roughness and topography generated by hydroxyapatite-grit blasting (RBM) which is an important regulating factor of the osteoblastic cell adhesion, proliferation and differentiation. This was examined in six ways, using the cresyl violet staining, MTS assay, ALP activity assay, alizarin red-s staining, ELISA, semi-quantitative RT-PCR. In all six cases, the osteoblastic cell adhesion, proliferation and differentiation were dramatically enhanced in RBM surfaces. These results provided unequivocal evidence that surface roughness and topography generated by RBM surface is an important factor that enhances the osteoblastic cell adhesion, proliferation and differentiation.

While the effects of surface roughness and topography remain controversial with respect to osteoblastic phenotype expression (Wennerberg et al, 1996; Deligianni et al, 2001), we aimed at testing whether MG-63 preserve their phenotype in direct contact with hydroxyapatite grit-blasted (RBM) surface or not. Therefore, this study further investigated the phenomenon underlying the surface roughness and topography modulating the

osteoblastic cell behavior. Briefly, MG-63 cells cultured on very irregular surfaces, such as RBM, exhibited the increased cell adhesion, proliferation and differentiation. MTS activity was increased more steeply on RBM surfaces. ALP is a marker of osteogenic differentiation, bone formation and mineralization. Previous reports have demonstrated the influence of surface roughness on ALP activity (Schwartz et al, 1999; Kim et al, 2006). Like a report of Kim *et al* (Kim et al, 2006), ALP activity was enhanced in parallel with the roughness parameters in our study. Moreover this osteoblastic phenotypic characterization can be based on the analysis of various osteoblastic markers including type I collagen, osteonectin, osteonectin and Runx-2 (cbfa-1/OSF-2). Hydroxyapatite grit-blasted (RBM) surface induced a slight increase in Type I collagen and Runx-2 expression. Also secretion of osteoblastic differentiation-related protein and deposition of mineral was enhanced in RBM surface.

Nevertheless, the biological effects of residual alumina particles on titanium surfaces are controversial in the literature (Piattelli et al, 2003; Diniz et al, 2005; Rodrigo et al, 2006; Canabarro et al, 2008). Bone healing around residual alumina in alumina grit-blasted titanium implants is impaired. On the contrary, Sader *et al.* (Sader et al, 2005) did not observe any detrimental defect of residual blasting alumina particles *in vivo* and *in vitro* experiment. Furthermore, Wennerberg *et al.* (Wennerberg et al, 1996) did not find any significant differences in bone-implant contact for alumina-blasted and machined titanium implants.

Calcium phosphate ceramics were used as the blasting materials in order to avoid the possible negative effects of residual alumina on the osseointegration between titanium and implant. Our results demonstrate the biocompatible and resorbable hydroxyapatite abrasive

particles that can be used to create titanium surface roughness. This grit-blasting process increased surface roughness of titanium implants and offered an osteoblastic cell favorable surfaces. As shown as EDS data (Table 2.), even if infinitesimal calcium and phosphorous were found on the RBM surfaces after blasting and cleaning, they should not be detrimental to the osseointegration periods.

Although both alumina and hydroxyapatite grit-blasting media had comparable roughness, their surface energetics were different. The alumina-blasted surface was more hydrophobic than the hydroxyapatite grit-blasted surface. Hydrophilic surface should be more favorable to the osteoblastic cell adhesion, proliferation and differentiation (Li et al, 2004). These difference may be related to the presence of residual alumina particles on the surfaces. Therefore, grit-blasting titanium surfaces with a biocompatible, resorbable and osteoconductive materials like hydroxyapatite and β -TCP ceramic particles are alternative method for avoiding the presence of residual alumina particles.

In this study, osteoblastic cell adhesion, proliferation and differentiation were greater for the RBM surfaces than for the machined surfaces. This suggests that the RBM surfaces allowed more great osteoblastic cell phenotype than the machined surfaces (Giordano et al, 2006). Moreover we showed that rough implant surface can alter the expression of bone associated regulatory transcription factors and key osseointegration-related proteins. Maybe this occurred as a result of differences in cell adhesion, as a result of integrin-mediated adhesion and regulation of downstream signaling pathways as previously reported (Schneider et al, 1999; Schneider et al, 2001; Schneider et al, 2003; Schneider et al, 2004). This early osteoblastic cell differentiation in contact with roughened

RBM surfaces may be favorable during the early phases of bone healing. Further investigations on the relationship between surface roughness, topography and chemistry should improve the understanding of the osseointegration-related osteoblastic cell behavior.



V. CONCLUSION

In summary, this study examined the effect of microtopographically modified rough surfaces by hydroxyapatite grit-blasting on osteoblastic cell adhesion, proliferation and differentiation. Human osteoblast-like cell line, MG-63 cells, cultured on the RBM surfaces exhibited more osteoblastic cell adhesion, proliferation, differentiated osteoblastic phenotype and produced more local factors that stimulate the osteoblastic differentiation. Our results demonstrated the RBM surfaces stimulate the *in vitro* osteogenesis in MG-63 cells and raise the potential that RBM surfaces accelerate the bone formation and finally increase bone-implant contact.

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- 국문요약 -

골모유사세포주인 MG-63에서 수산화인회석을 이용한 임플란트 표면처리가 세포
의 흡착, 성장 및 분화에 미치는 효과

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박 성 재

(지도교수: 송 승 일)

목적: 본 연구의 목적은 흡수성 분사물질인 수산화인회석을 분사하여 표면을 미세형태학적으로 변형시킨 RBM표면의 골형성 정도를 세포수준에서 평가 하고자 하였다.

연구방법: 우선 미세형태학적으로 변형된 표면들을 만들기 위해 수산화인회석 분사를 이용해 RBM표면을 제작하고 표면형태, 거칠기, 구성요소들을 관찰하였다. 그런 다음 세포수준에서 골형성의 잠재성을 조사하기 위해 골모유사 세포주인 MG-63를 이용하여 골모세포의 흡착, 성장, 분화를 실험하였다. 골모세포 증식은 시간에 따른 변화를 관찰하였고 골모세포 분화는 ALP activity assay, Alizarin red-s staining을 이용한 Mineralization assay, RT-PCR 과 ELISA를 이용한 골모세포 분화 유전자의 발현을 통하여 평가하였다.

연구결과: 골모세포 흡착, 성장, ALP activity는 RBM 표면에서 Machined표면과 비교했을 때 유의성 있게 증가하였다. 그리고 RBM표면에서 높은 수준의 골모세포 분화 Marker의 유전자 발현을 보였다. (osteonectin, type I collagen,

Runx-2, osterix). 비슷한 결과가 ELISA에서 관찰 되었는데, RBM 표면이 골 형성을 자극시키는 것으로 알려진 osteocalcin, osteopontin, TGF-beta1, PGE₂의 분비를 증가시키는 것을 알 수 있었다. 또한 Alizarin red-s staining은 Machined discs와 비교된 RBM표면에서 상당히 유의성 있게 미네랄화 되었음을 보여주었다..

결론: 본 연구의 결과를 통하여 수산화인회석 분사를 이용한 RBM 표면은 MG-63 세포주에서 골 형성을 자극하는 것을 알 수 있었고 이를 통하여 골 형성의 증가와 골과 임플란트의 접착을 증가시킬 것으로 사료 된다.

핵심어: 골유착, 골형성, 미세형태학적, 표면 거칠기, RBM 표면처리, 수산화인회석, 생체적합성

감사의글

이 논문이 완성되는데 있어 부족한 저에게 끊임없는 지도와 격려를 보내주신 송승일 교수님께 진심으로 감사 드리며, 귀중한 조언과 부족한 논문의 심사에 도움을 주신 정규림 대학원장님, 백광우 과장님, 이정근 교수님, 황경균 교수님께 감사의 마음을 전하고 싶습니다.

같이 논문을 준비하는 동안 힘들 때 서로 격려해 주고 많은 힘이 되어 준 배성범 선생님, 김인수 선생님, 이두한 선생님, 양근용 선생님께도 감사를 드립니다.

끝으로 제가 이 자리에 있게 해주신 사랑하는 아버지, 어머니, 항상 격려해 주시고 성원해 주시는 장인어른과 장모님, 곁에서 제가 살아가는데 힘이 되어주는 사랑하는 아내 은희, 큰딸 시연, 둘째 주연, 아들 상언에게 이 논문을 드립니다.

논문 쓰는 동안 우리 병원을 지켜주신 유향희 선생님, 고희진 선생님, 천점임 선생님, 김혜선 선생님께 다시 한번 감사 드립니다.

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