

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

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Abstract (J Korean Assoc Oral Maxillofac Surg 2011;37:214-24)

Objective: This study examined the potential of the *in vitro* osteogenesis of microtopographically modified surfaces, RBM (resorbable blasting media) surfaces, which generate hydroxyapatite grit-blasting.

Methods: RBM surfaces were modified hydroxyapatite grit-blasting to produce microtopographically modified surfaces and the surface morphology, roughness or elements were examined. To investigate the potential of the *in vitro* osteogenesis, the osteoblastic cell adhesion, proliferation, and differentiation were examined using the human osteoblast-like cell line, MG-63 cells. Osteoblastic cell proliferation was examined as a function of time. In addition, osteoblastic cell differentiation was verified using four different methods of an ALP activity assay, a mineralization assay using alizarin red-s staining, and gene expression of osteoblastic differentiation marker using RT-PCR or ELISA.

Results: Osteoblastic cell adhesion, proliferation and ALP activity was elevated on the RBM surfaces compared to the machined group. The cells exhibited a high level of gene expression of the osteoblastic differentiation makers (osteonectin, type I collagen, Runx-2, osterix). imilar data was represented in the ELISA produced similar results in that the RBM surface increased the level of osteocalcin, osteopontin, TGF-beta1 and PGE2 secretion, which was known to stimulate the osteogenesis. Moreover, alizarin red-s staining revealed significantly more mineralized nodules on the RBM surfaces than the machined discs.

Conclusion: RBM surfaces modified with hydroxyapatite grit-blasting stimulate the *in vitro* osteogenesis of MG-63 cells and may accelerate bone formation and increase bone-implant contact.

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

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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 mainly as endosseous implants. The early osseointegration of titanium dental implants is an important factor for their clinical success^{1,2}. The implant loading to bone is influenced by various factors, including surface chemistry, energetics, and surface topography³⁻⁶. These surface parameters are decisive during the bone healing period in the peri-implant region. After implantation, implant surfaces are in 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, and differentiation of osteoblastic cells producing 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 roughness⁶⁻⁸.

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^{7,9}. But among them, surface roughness is one of the most acknowledged parameters improving the bone anchorage of titanium implants¹⁰⁻¹². 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 bone¹³⁻¹⁵. On the other hand, numerous reports indicate that increased surface roughness stimulates osteoblastic cell differentiation and bone formation¹⁶⁻¹⁸.

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There are various methods have been developed to create rough implant surfaces in order to improve the clinical performance of implants and to guarantee a stable mechanical loading. Among them, the popular methods for roughening titanium implants uses a biocompatible and resorbable blasting media, such as hydroxyapatite and β -tricalcium phosphate (β -TCP) ceramic particles or other calcium phosphate ceramic particles^{14,19-21}. These calcium phosphate biomaterials have been contemplated as a potential abrasive materials. Indeed hydroxyapatite and β -TCP are biocompatible and 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^{22,23}. 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 to provide a roughened surface which improve the osteoblastic cell adhesion, proliferation, and differentiation and (ii) to assess the effects of such a 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 resorbable blasting media (RBM) titanium surfaces. After analyzing the properties of the implant surfaces, osteoblastic cell adhesion, proliferation, and differentiation were studied.

II . Materials and Methods

1. Materials

Commercially pure titanium (cpTi) discs with a diameter of 12 mm and 1 mm in thickness (Carpenter, Washington, USA) was used. The samples were cut from a cpTi bar and used as machined discs. Abrasive hydroxyapatite powders (40-80 mesh) were obtained from Himed (NY, USA). Cell culture plastic ware was purchased from Falcon (Becton-Dickinson, Franklin Lakes, NJ, USA). Fetal bovine serum (FBS), DMEM, penicillin/streptomycin, trypsin/EDTA were purchased from HyClone (Salt Lake City, Utah, USA). Phosphate buffered saline (PBS) were purchased from Invitrogen Corporation (Paisley, UK). Triton X-100, Alizarin red-s, p-nitrophenylphosphate (p-NPP), cresyl violet, MgCl₂ were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-

gl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl-2H) tetrasolium inner salt (MTS) and hTGF-beta1 ELISA kit were purchased from Promega (Madison, WI, USA). hOsteocalcin ELISA kit was purchased from Biosource (Nivelles, Belgium). hOsteopontin and hPGE2 ELISA kit were purchased form Assay Designs (MI, USA). QuantiTech Reverse Transcription kit was obtained from Qiagen (CA, USA). PCR premix was purchased from Bioneer (Daejeon, Korea). All other chemicals were from standard laboratory suppliers and were of the highest purity available.

2. 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, China). Decreased grind paper P400, P600, P1200, P2000 were successfully used on a rotative polisher at 250 rotation per minute (rpm) for 1 min. The hydroxyapatite grit-blasted surface (RBM group) was prepared by blasting using hydroxyapatite powders (Himed, 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 with deionized water. Then the samples were ultrasonically cleaned for 30 min 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 20 min 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 Surfpack-SV. This profilometer system consists of a probe which scanned the surface 2.4 mm 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 SEM (JSM-6480LV, Tokyo, Japan) at 20Kv. An 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, VA, USA) is a non-transformed cell line established from human osteosarcoma. This cell were routinely grown in DMEM supplemented with 10% FBS (HyClone, Salt Lake City, Utah) and 0.1 mg/mL penicillin and streptomycin (P/S) (HyClone, Salt Lake City, Utah). Cells were subcultured twice a week using trypsin/EDTA and maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Medium was completely renewed every two days.

5) Cell adhesion

The cell adhesion assay of the MG-63 cells was performed using previous report^{24,25}. 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 Dulbecco's phosphate-buffered saline (DPBS). And the cells were fixed using 4% formaldehyde solution for 1 hr at 4°C and were rinsed three times by DPBS. Then the viable cells were stained using 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, CA, USA) with optical density reading at 590 nm.

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, 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 µL MTS/PMS/Media mixture was added in each well for 1 hr. Finally colorimetric measurement of formazan dye was performed on a DTX 880 with optical density reading at 490 nm. Results were expressed as optical density and relative MTS activity as compared to control.

7) Alkaline phasphatase (ALP) activity

ALP activity was examined as previously described²⁶⁻²⁹, 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 µM dexamethasone, 50 µg/mL L-ascorbic acid, 10 mM b-glycerolphosphate)^{30,31}, 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, USA). For ALP activity measurement with isolated cell supernatants, 100 µg total protein were incubated with 2 mg/mL p-NPP (as a chromogenic substrate, Sigma) using ALP assay buffer.(pH 9.75 in 0.1 M Glycine containing 1 mM MgCl₂) at 37°C for 2 hrs. The degree of colorimetic reaction was measured by DTX 880 for an optical density measurements at 405 nm.

8) Alizarin red-s staining

The alizarin red-s staining with experimental samples was performed as reported elsewhere³²⁻³⁴ 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, Tokyo, Japan) for 20 min at room temperature and rinsed with triple distilled H₂O. Cells were stained with 2% alizarin red-s (Sigma, St. Louis, MO, USA), pH4.2, for 20 min with gentle agitation. After aspiration of the unstained dye, the wells were washed five times with distilled H₂O while shaking for 5 min. For quantification of staining, 500 µL 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 13,000 rpm for 10 min. 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 (200 µL) was measured at 405 nm. 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, 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 and 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 was 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 (OSN), Type I collagen, osteoprotegerin, BSP-2, Runx-2 and Osterix (OSX) 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 72 hrs, total RNA was extracted with Trizol (Invitrogen, CA, USA), according to the manufacturer's instructions. Using the extracted RNA as a template, reverse transcription were carried out with QuantiTech Reverse Transcription kit (Qiagen, CA, USA) and RT reactions were performed in a Mastercycler (Eppendorf, NY, USA). RT reaction mixture was incubated at 42°C for 20 min heated at 94°C for 10 min, and subsequently chilled to 4°C. Then OSN, type I collagen (COL1), runt-related transcription factor 2 (Runx-2) and OSX were amplified by Mastercycler using PCR premix (Bioneer, Daejeon, Korea). (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, 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.

11) Statistical analysis

Each experiment was repeated at least twice with similar results. Results are expressed as mean \pm SD of triplicate deter-

minations. For statistical analysis of experiments (N=6, three groups repeated two times), unpaired t-tests and ANOVA were performed with SPSS (SPSS INC., Chicago, IL, USA). (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$)

III . Results

1. 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.

2. 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. (Figs. 1. A, 1. B) The roughness parameters as measured by optical profilometer are represent in Table 2. 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 (Figs. 1. C, 1. D), machined or RBM surface was basically similar elemental composition. The RBM surfaces possesses the phosphorous and calcium of infinitesimal quantity. (Fig. 1. D, Table 3) This phenomenon is due to hydroxyapatite powder that remain behind after blasting.

Table 1. Primer sequences of osteogenic marker and conditions for polymerase chain reaction

Target gene	Primer sequences (5' \rightarrow 3')		Annealing temperate	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, Osteonectin; COL1, Type I Collagen; Runx-2, Runt-Related Transcription Factor-2; and OSX, Osterix

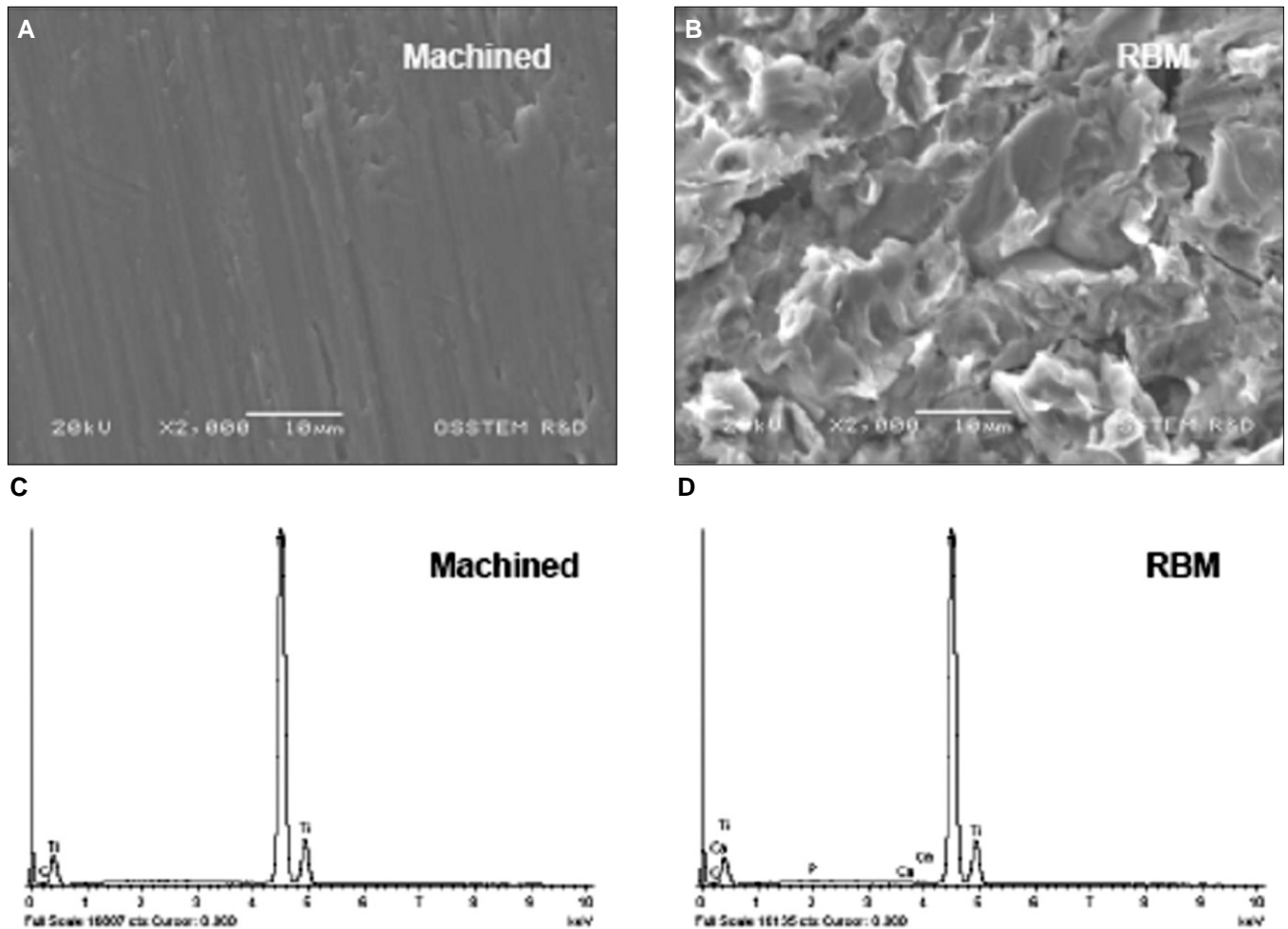


Fig. 1. Surface morphology of titanium discs for osteoblastic cell culture. SEM images of machined titanium (A) and RBM (B). Bar = 10 μ m. EDA Data of machined titanium (C) and RBM (D). Surfaces were examined using the JSM-6480LV SEM at working distance of 10 mm and accelerating voltage of 20 kV.

Table 2. Roughness values of a machined Ti and a RBM surfaces measured by profilometer

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

Ra, average roughness; Ra2max, maximum value of roughness; Rz, average maximum height of the profile; and Rt, maximum height of the height

RBM, resorbable blasting media

3. 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 were significantly higher than the machined surface and the culture plate. (* P <0.05; Fig. 2)

Table 3. 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

EDS, energy-dispersive spectroscopy, RBM, resorbable blasting media

4. 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 day in culture on RBM surface as compared to the machined surface.

5. 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

Table 4. Gradient of MG-63 cell proliferation on a machined Ti and a RBM surfaces during 5 days

	Culture plate	Machine	RBM
Gradient of cell proliferation	0.9493	1.1937	1.2196

RBM, resorbable blasting media

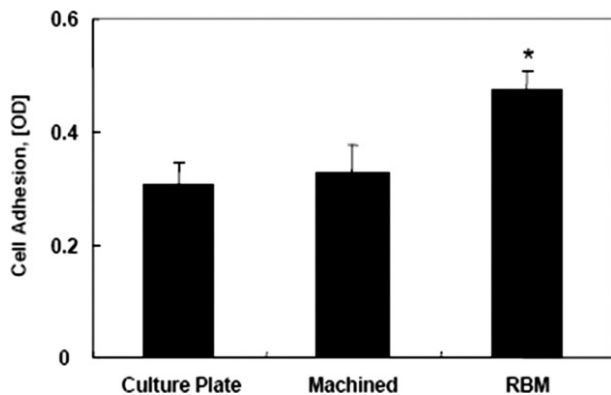


Fig. 2. Effect of surface roughness on the adhesion of MG-63 cells. MG-63 cells were stained with cresyl violet dye and counted using spectrophotometer on surfaces of differing roughness. Data are represented as the average \pm standard deviation. RBM surface, the rough surface, were promoted the cell adhesion compared to smooth machined surface. *: Statistically significant compared with cells cultured on machined surfaces. ($P < 0.05$)

protein content. On RBM surfaces, we observed a significant enhancement of ALP activity ($10.8 \pm 2.6\%$) as compared with machined surface after 7 days. (** $P < 0.001$; Fig. 4. A) 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. 4. B, similar pattern in ALP activity data, 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. 4. C) Moreover, osteopontin (** $P < 0.001$), PGE2 (** $P < 0.001$) and TGF-beta1 (* $P < 0.05$) secretion was greater on RBM surface than the machined surface. (Figs. 4. D, 4. E, and F separately) As a result ELISA data showed that the RBM surface enhances the secretion of various osteoblastic differentiation-related proteins.

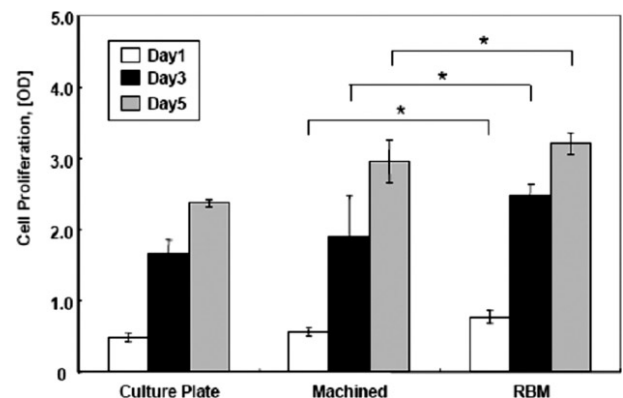


Fig. 3. Results of MG-63 cell proliferation experiments. Data are represented as the average \pm standard deviation. The differences between the number of cells on RBM and machined surfaces during 5 days is statistically significant. *: Statistically significant compared with cells cultured on machined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

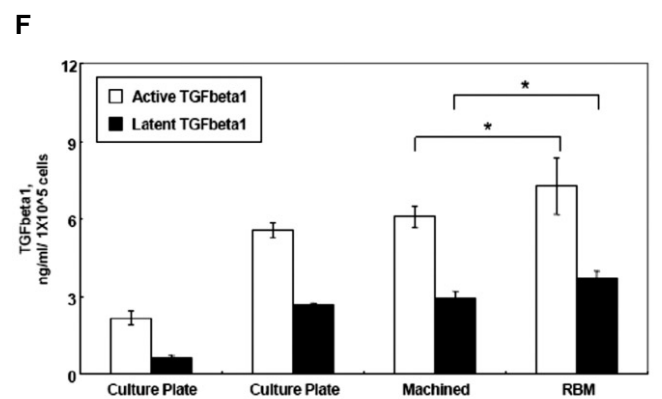
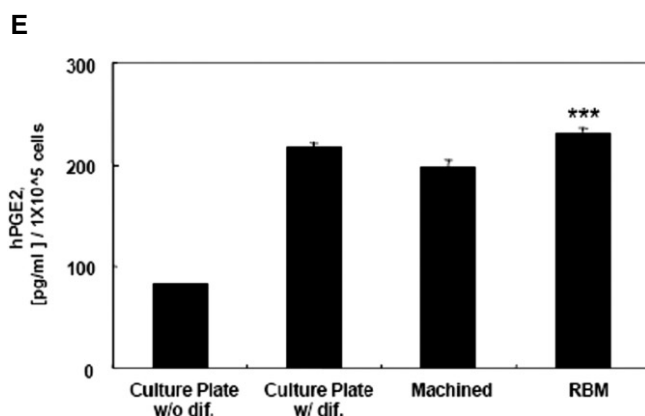
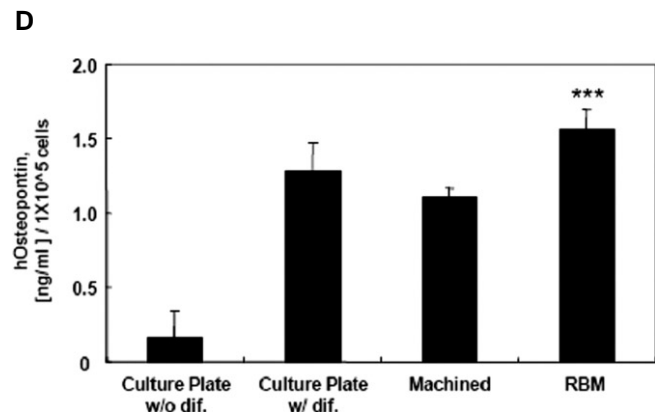
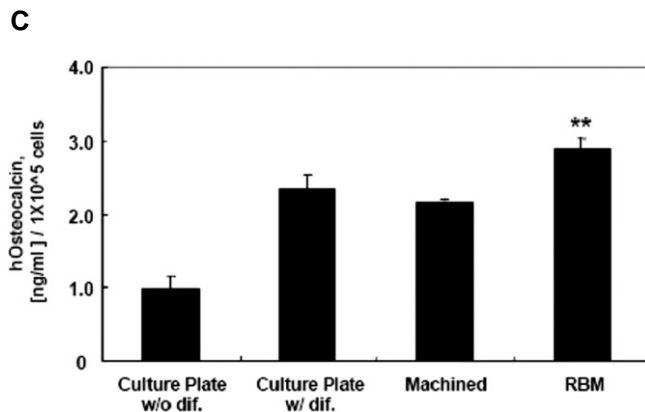
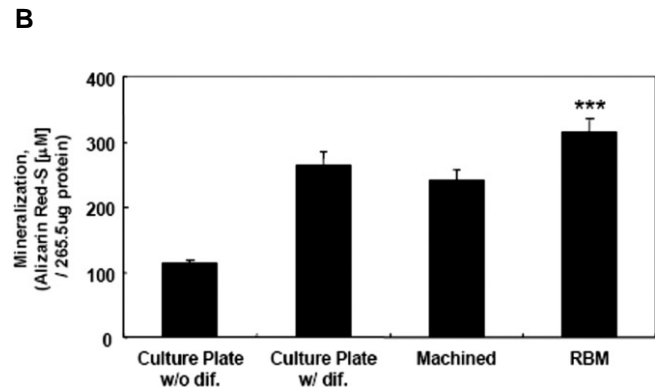
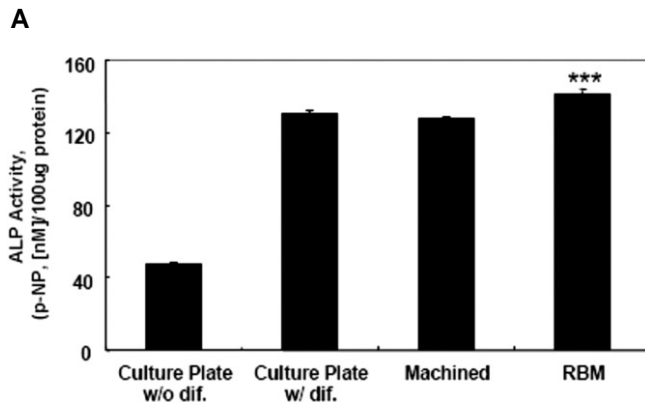
4) Semi-quantitative RT-PCR

Semi-quantitative RT-PCR showed significantly ($*P<0.05$) increased COL1 and Runx-2 (cbfa-1, OSF-2) gene expression in MG-63 grown on RBM, relative to machined surface or culture plate.(Fig. 4. G) There was a slight, but insignificant, increase in OSN and OSX gene expression on the RBM surface relative to culture plate or machined surface.(Fig. 4. G) These results strongly suggested that RBM surface increases

the expression level of osteoblastic differentiation marker gene associated with osteogenesis and osseointegration.

IV . Discussion

Osteoblastic cells play a critical role in the early stages of osseointegration. Several reports have assessed that osteoblastic cell response to smooth surfaces more rapidly than rough



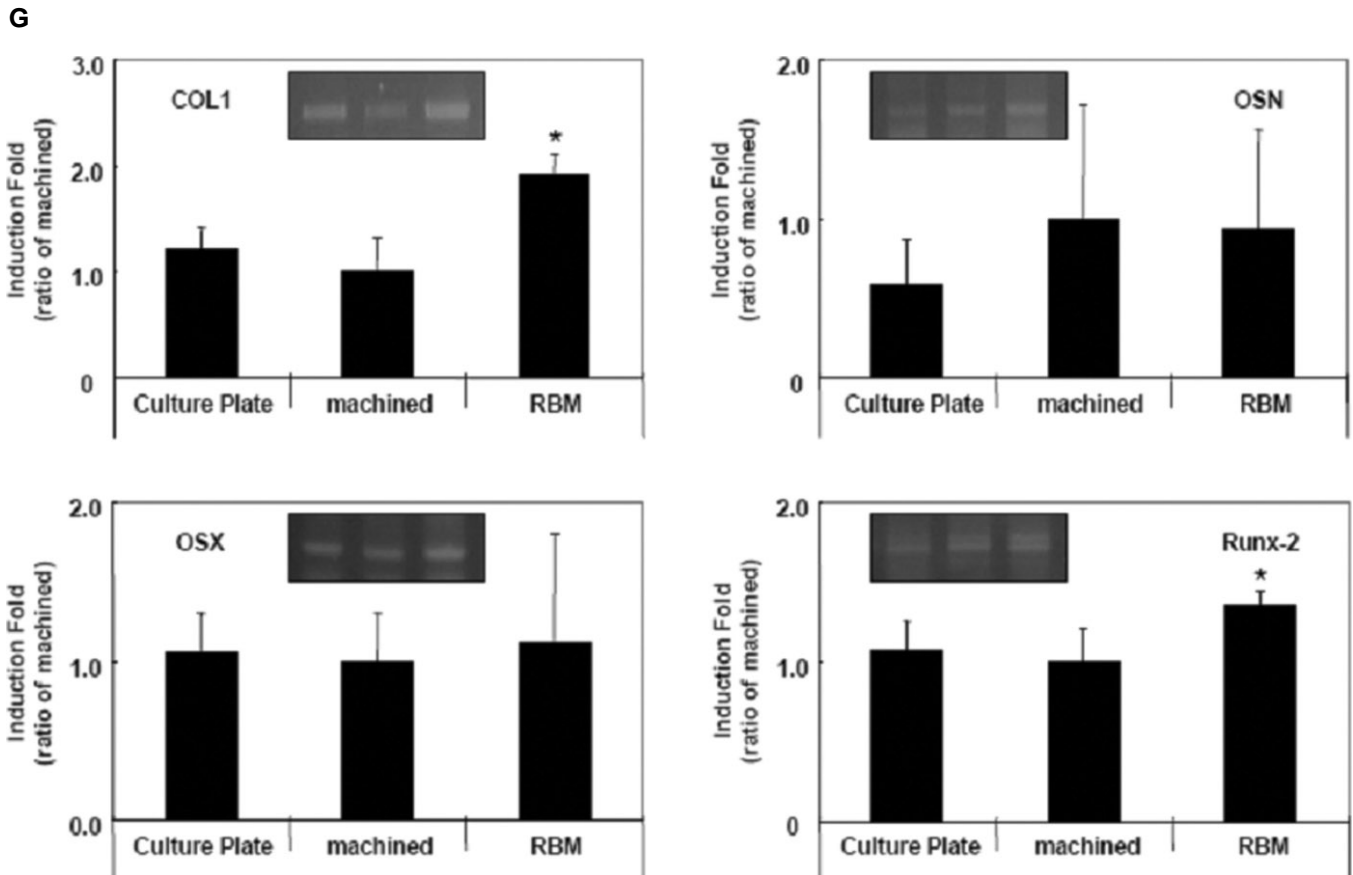


Fig. 4. Effect of surface roughness on the osteoblastic differentiation of MG-63 cells. Data are represented as the average \pm standard deviation. (A) ALP (alkaline phosphatase) activity of MG-63 cells cultured either on machined Ti and RBM surfaces. Culture plate without osteogenic differentiation 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 osteocalcin (C). Osteopontin levels were measured in the conditioned media using an ELISA kit specific for humans osteopontin (D). PGE2 levels were measured in the conditioned media using an ELISA kit specific for humans PGE2 (E). And Active of latent TGF- β 1 (transforming growth factor- β 1) levels were measure in the conditioned media using an ELISA kit specific for human TGF- β 1 (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 72hrs 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. (* $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$)

surfaces while their osteoblastic differentiation was enhanced by rough topography³⁵⁻³⁹. Little is known about the regulatory mechanism of the osteoblastic cell adhesion, proliferation, and differentiation on the titanium discs. This study provided the evidence that the roughness and topography generated by hydroxyapatite-grit blasting (RBM) 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 was 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 remains controversial with respect to osteoblastic phenotype expression^{40,41}, we aimed at testing whether MG-63 preserve their phenotype in direct contact with hydroxyapatite grit-blasted (RBM) surface. Therefore, this study further investigated the phenomenon underlying the surface roughness and topography modulate 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^{37,39}. Like a report of Kim *et al.*³⁶, 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 COL1, OSN, OSX and Runx-2 (cbfa-1/OSF-2). Hydroxyapatite grit-blasted (RBM) surface induced a slight increase in COL1 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 literature⁴²⁻⁴⁵. Bone healing around residual alumina in alumina grit-blasted titanium implants is impaired. On the contrary, Sader *et al.*¹⁹ did not observe any detrimental defect of residual blasting alumina particles *in vivo* and *in vitro* experiment. Furthermore, Wennerberg *et al.*⁴⁶ 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 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 differentiation⁴⁷. 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 was 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 surfaces⁴⁸. 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 reported⁴⁹⁻⁵³. 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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