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Effect of Titanium Coating on Cell Adhesion and Extracellular Matrix Formation in Human Osteoblast-like MG-63 Cells

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Abstract

A variety of titanium (Ti) and its alloys are used in the clinical procedures of bone regeneration for periodontal and dental implant therapies. This study was performed to determine the effect of different surface dental implant materials on biologic responses of a MG-63 human osteoblast-like cell line. MG-63 cells were cultured on Ti coated with hydroxyapatite (HA), calcium metaphosphate (CMP), anodized (A), which compared with non-coated Ti (control). The appearances of surface of dental implant materials and the morphology of these cells were assessed by scanning electron microscopy (SEM). The gene expression profiles of MG-63 cells cultured on Ti were examined by human cDNA microarray (1,152 elements). The expression of several genes was up- and down-regulated by different surfaces of dental implant materials. Interesting, the genes correlated with cellular adhesion and extra cellular matrix (ECM) formation were enhanced, in accordance surface morphology of the dental implant materials used.

Keywords: Titanium, Gene expression, Surface morphology, Dental implant materials

The biologically important Titanium (Ti) and its alloys have been widely used for implants that interact with bone cells *in vitro* and *in vivo*[1]. Medical doctor of orthopedic surgery and dentistry have placed implants, screws and plates, and prostheses to substitute lost teeth, to fix bone fragments, and to replace joints, respectively. Moreover, many surgical instruments, such as drills and saws, are made with Ti alloys. However, it is still unknown that what it is exactly works on osteoblast[2-4].

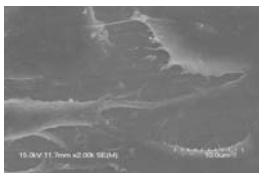
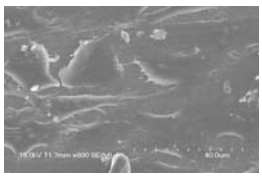
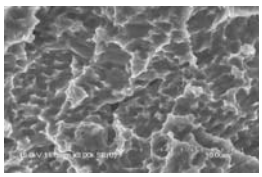
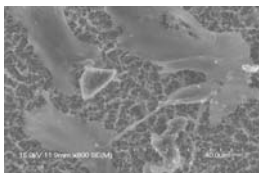
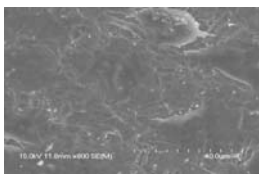
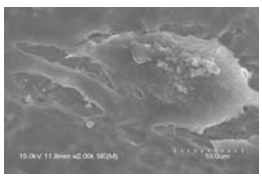
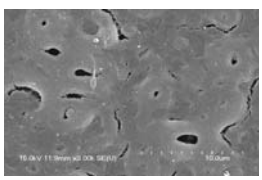
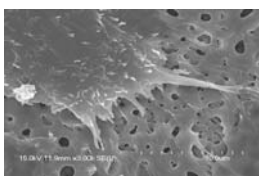
Morphological analysis may affect the formation of a fibrous capsule around implants, inflammatory response at tissue-implant interface, fibroblast attachment, angiogenesis, epithelial down-growth around percutaneous devices, and many cellular processes such as cellular differentiation, transcription, cell metabolism, protein production, and phenotypic expression[1,3,5-7]. Diverse implant surface may contribute to the regulation of osteoblast differentiation by influencing the level of gene expression of key osteogenic factors[7,8]. Morphometric studies had shown differences in bone-implant contact percentages with the varying of surface characteristics, as well as a sensitivity of cells to surface topography[9,10]. Gene expression in response to the placement of implants was correlated with different surface topographies[11-17]. It is speculated that different-coatings on Ti surface conditions would be associated with differential expression and surface morphology, especially in the cellular mechanism of inter cellular adhesion and ECM formation.

In this study, we observed the surface morphology of dental implant materials coated different Ti. In addition, we examined the cell appearance and cell proliferation of MG-63 human osteoblast-like cell culturd on differential Ti using by SEM and MTT assay, respectively. Finally, we investigated the effect of Ti coating on gene expression profiles of MG-63 by cDNA microarray.

The Morphology of MG-63 Cells Cultured on Differential Dental Implant Material by Scanning Electron Microscopy (SEM)

The MG-63 cells were cultured on Ti coated with HA, CMP, A, and smooth for 3 days. The morphology of cell-matrix interaction was observed by SEM and was shown in Table 1. We found cell-matrix in-

Table 1. The appearances of surface of dental implant materials and the cell morphology.

Abbrevition	Coating	Cell culture	
		Before	After
Control	Nothing		
HA	Hydroxyapatite		
CMP	Calcium metaphosphate		
A	Anodized		

Magnification 3,000X, Titanium surface morphology using a scanning electron microscopy (SEM)

teraction and the short pseudopodia in cells cultured on HA, CMP and A (Table 1). However, control which cultured on smooth Ti did not showed cell-matrix interaction and short pseudopodia (Table 1). These results demonstrated that HA, CMP, and A have good attachment potential for osteoblast cells.

The Effect of Ti Coating on Cell Proliferation

MG-63 cells were cultured on Ti coated with HA, CMP, A, and smooth for 1, 3 and 6 days, and the cell proliferation was determined by MTT assay. The proliferation of MG-63 cells cultured on HA and A steadily increased as time-dependent manner (Figure 1). However, cells cultured on CMP grew slower than other Ti such as HA, A and smooth at 6 days (Figure 1). These results demonstrated that differential Ti coating may affect the cell proliferation of osteoblast cells.

Gene Expression Profiles of Human Osteoblast-like Cells Culture on Different Titanium

To identify the gene expression profiles of MG-63 cells cultured on different Ti including HA, CMP, and

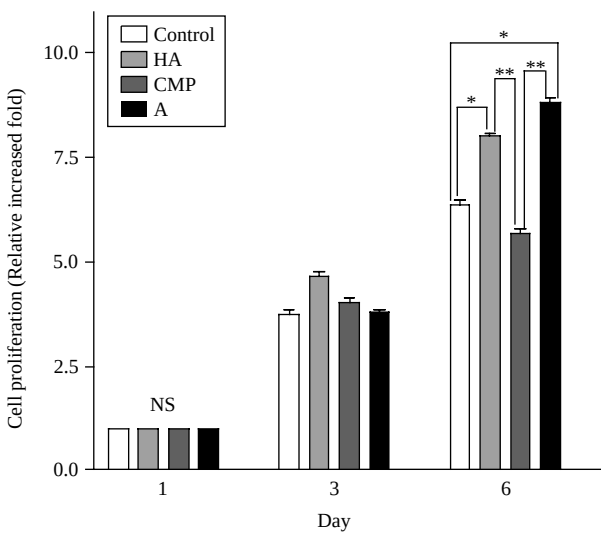


Figure 1. Cell proliferation of MG-63 on various titanium coated. The MG-63 were cultured on different Ti coated with smooth (control), hydroxyapatite (HA), Calcium metaphosphate (CMP), and anodized (A). *, ** indicate statistically significant differences, $*P < 0.05$, $**P < 0.01$ vs control (two-way ANOVA test).

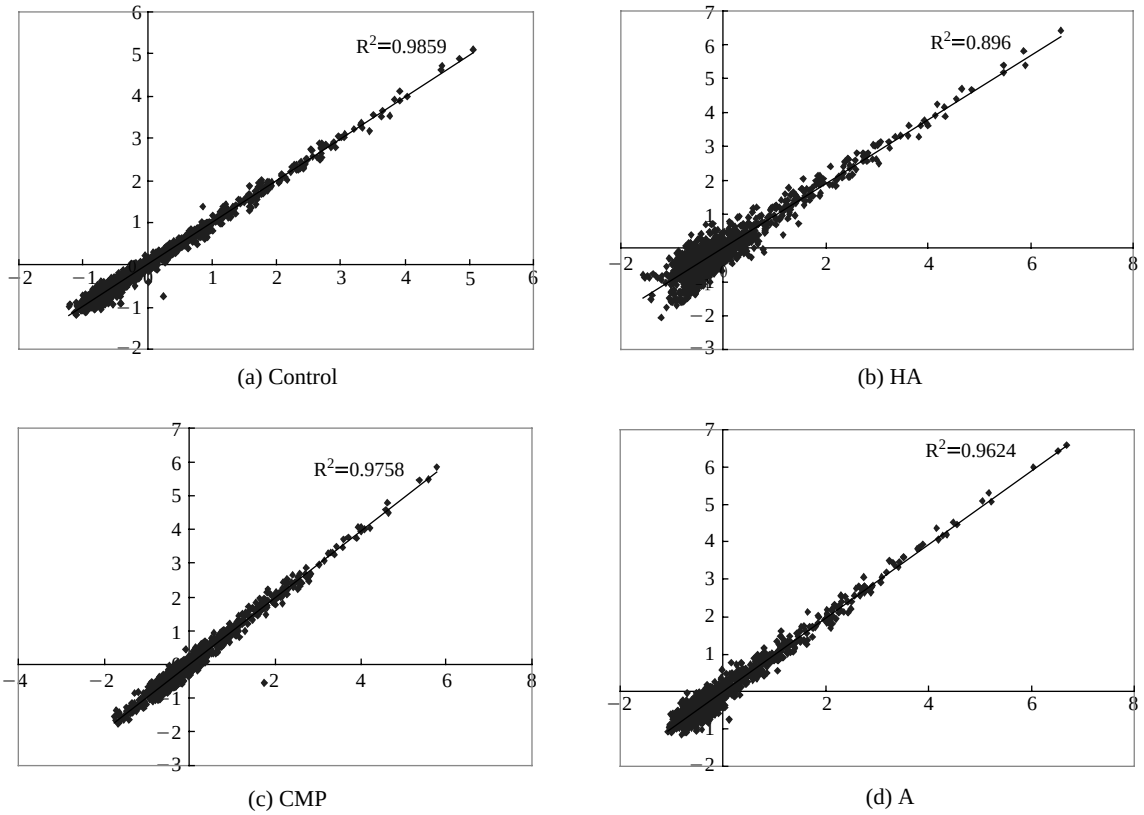


Figure 2. Scatter plot for comparison of expression profile between control and HA, CMP, and A. Expression profiles are shown as bivariated scatter plot of 1,152 gene from the microarray.

A, we performed a cDNA microarray. Figure 2 showed scatter plots of each experimental group for comparing the expression profiles of different-coated on Ti. We used a hierarchical clustering algorithm to group genes on the basis of similar expression patterns and the data is presented in a matrix format (Figure 2).

The global gene expression patterns of MG-63 cells cultured on HA, CMP, and A up-regulated 28 genes, 17 genes and 26 genes (Z-ratio $>$ 2.0), and down-regulated 28 genes, 35 genes, and 35 genes (Z-ratio $>$ $-$2.0), respectively. Cell adhesion related genes including integrin alpha 9 (ITGA9), Villin2 (VIL2), selectin E (SELE), and cadherin were up-regulated on HA, CMP, and A (Table 2). In addition, ECM formation related genes including collagen (COl), elastin (PI), and fibrillin (FBN1) were up-regulated on HA, CMP, and A (Table 2). However, gene expression of cell differentiation, cell cycle, and bone development related genes did not changed by different Ti coating.

Discussion

To avoid unwanted biological effects of different dental implant materials, *in vitro* and *in vivo* biological studies are required before its definite use in humans. Therefore, biological testing of medical and dental devices is needed in order to evaluate the biological behavior of biomaterials. The surface of Ti, one of the dental implant materials, is of paramount importance in influencing the timing of bone healing and the modality of osseointegration.

In this study, we tried to identify the effect of different Ti including HA, CMP, and A on cell proliferation in human osteoblast-like MG-63 cells. The formation of cell attachment to the Ti coated with HA and A seems to be faster than on CMP. Also, cell proliferation on HA and A more increased than on CMP. In relation to these observations, it is known that the formation of cell-implant contacts may not be hampered on rough surfaces 1, 18, and that rough surface may affects proliferation, differentiation, local factor production[19-21].

Osteoblastic cells began to secrete several ECM proteins, which are necessary for adhesion due to their specific binding to cell surface receptors. Moreover, ECM proteins can attach on the dental implant surface. We also attempted to a cDNA microarray to

Table 2. Up- and down-regulated genes of different Ti surface coated with HA, CMP and A.

Genes	Abb.	Z-ratio		
		HA	CMP	A
Metabolism related group				
proteasome (prosome, macropain) subunit, alpha type, 1	PSMA1	0.29	3.31	−1.03
8-oxoguanine DNA glycosylase	OGG1	−0.22	1.28	−3.60
acetyl-coenzyme A acetyltransferase 2	ACAT2	−0.80	1.30	−1.62
ubiquitin-conjugating enzyme E2I (homologous to yeast UBC9)	UBE2I	0.54	0.42	−0.44
damage-specific DNA binding protein 1 (127 kD)	DDB1	0.13	−1.87	−0.97
Cell proliferation and cell cycle related group				
singed (Drosophila)-like (sea urchin fascin homolog like)	SNL	0.20	0.89	−1.04
cyclin B1	CCNB1	−0.14	−3.21	0.72
hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	−0.99	−1.06	−0.77
midkine (neurite growth-promoting factor 2)	MDK	−0.73	−2.59	−1.10
Wee1+(S. pombe) homolog	WEE1	0.61	−2.32	0.08
polo (Drosophia)-like kinase	PLK	0.04	−1.77	0.43
Cell differentiation and bone development related group				
similar to latent transforming growth factor beta binding protein 1	TGFBBP1	0.42	1.19	−2.05
bone morphogenetic protein 2	BMP2	0.35	1.23	−2.52
GDF-1 embryonic growth factor	GDF1	−0.99	0.94	−0.99
catenin (cadherin-associated protein), beta 1 (88 kD)	CTNNB1	0.56	1.48	−1.32
fibroblast growth factor 12	FGF12	−0.05	−4.47	−0.03
fibroblast growth factor receptor 3	FGFR3	−0.26	1.14	0.21
Cell adhesion related group				
villin2	VIL2	2.82	1.65	2.88
integrin, alpha 9	ITGA9	2.68	0.92	3.29
selectin E	SELE	3.23	1.09	2.02
cadherin	–	3.70	0.75	2.41
ECM formation group				
collagen	COl	2.64	0.88	3.37
elastin	PI	2.00	0.47	2.66
fibrillin	FBN1	2.39	1.06	2.10

determine the effects of different Ti on gene expression profiles in MG63 cells. Cell adhesion and ECM formation related genes were up-regulated on HA and A, but did not changed on CMP compared with smooth[22]. For example, the expression of ITGA9, VIL2, and cadherin increased on HA and A. ITGA9 encodes an alpha integrin. Integrin, composed of an alpha chain and a beta chain, mediates cell-cell adhesion and cell-matrix adhesion[23]. In addition, overexpression of integrin promotes human osteosarcoma cell populated collagen lattice contraction and cell movement. VIL2 serves as an intermediate between the plasma membrane and the actin cytoskeleton. It, also, plays a key role in cell surface structure adhesion, migration, and organization. Catenin beta 1 is an adherens junctions (AJs) protein, which mediates cell adhesion and communicates signals to the neighboring cells[26]. It is well known that structural proteins of ECM were composed of collagen, elastin, and fibrillin that up-regulated on HA and A. Fibrillin is secreted into the ECM by fibroblasts and becomes incorporated into the insoluble microfibrils, which appear to provide a scaffold for deposition of elastin[27,28]. Thus, it appears that the genes up-regulated by Ti coated with HA and A were key molecules of cell adhesion and ECM formation.

In conclusion, we observed that cell-matrix interaction and the formation of short pseudopodia induced on Ti coated with HA and A in osteoblast-like cells. Also, we found that the cell proliferation increased as time-dependent manner only in Ti coated with HA and A, those in Ti coated with CMP did not increased. Consequently, our microarray analysis indicated that cell adhesion and ECM formation related genes were up-regulated only in Ti coated with HA and A. Moreover, genes that related to cell differentiation, cell cycle, and bone development did not significantly changed by different Ti coating such as HA, CMP, and A. These observation supports that different Ti coating may affect cell proliferation by regulating gene expression of ECM and cell adhesion molecules. This study may provide a great deal of useful information for the improvement of present dental implant materials.

Materials and Methods

Titanium Preparation

Four Ti substrates including HA, CMP, A, and non-coated Ti were kindly given from Osstem Co. (Seoul, Korea). The materials were Ti discs with a diameter of 12 mm, a thickness of 1 mm, in a coin-shaped circle. The Ti samples used in the experiments had different surfaces (control: smooth on Ti, HA: hydroxyapatite coating on Ti, CMP: Calcium metaphosphate coating surface Ti, A: anodized surface Ti). After surface preparation, these Ti were washed with distilled water, and then rinsed thoroughly in 70% ethanol and absolute ethanol. Prior to cell culturing, the discs were sterilized by γ-rays.

Cell Culture

Human osteoblast-like cell, MG-63, were cultured on different Ti surfaces. The MG-63 cells (KCLB® Korean Cell Line Bank) were cultured in minimum essential medium (MEM) (Biowhittaker, Belgium) with 10% fetal bovine serum, and antibiotics (Penicillin 100 U/mL and Streptomycin 100 μg/mL, Invitrogen, Milano, Italy) were seeded at 1×10^4/mL in a humidified atmosphere of 5% CO_2 at 37˚C.

Scanning Electron Microscopy (SEM)

SEM (S-4700, HITACHI, Tokyo, Japan) was employed in order to determine the morphological characteristics of cells in culture. The advantages associated with SEM include its large depth of focus, high lateral resolution down to the nanometer range, the feasibility to study structures with high aspect ratios, and the direct production of surface images.

Cell Proliferation

After treatment with Ti plate on MG-63cells, cell proliferation was determined by the MTT assay. Briefly, the cells were plated in 24-multiwell plate, treated with 1 or 2.5 μg/cm l of MG-63 for 1-6day, and incubated for 4 hr at 37˚C with 20 μL per well of 5 mg/mL MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution (Sigma Aldrich, USA). To dissolve the insoluble purple formazan crystal that was then formed, the medium was replaced with dimethyl sulfoxide (DMSO). The absorbance in each well was then recorded at 540 nm by using an ELISA reader.

Human cDNA Microarray

A MG-63 cDNA microarray was derived principally from a commercially available master set of approximately 15,000 human verified-sequences (Research Genetics, Inc., Huntsville, AL). The 15,000 human cDNA clone set was sorted for a list of genes (1,152 elements) representing families such as differentiation, development, proliferation, transformation, cell cycle progression, immune response, transcription and translation factors, oncogenes, and molecules involved in cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying is based on the procedures of DeRisi *et al*.

RNA Preparation and cDNA Radiolabeling

The RNA was isolated from cultured cells which adhered to the retrieved implants of different surfaces (control, HA, Ano, and Zr) with Trizol (Invitrogen, Milano, Italy). RNA was quantified via UV spectrophotometry (spectrophotometer-DU650; Beckman, Somerset, NJ, USA). After quantification, 3-10 μg of total RNAs prepared from the MG63-treated dental materials with different surfaces (control, HA, Ano, and Zr) were used for each sample for adjustment of different cell numbers. To synthesize ^{33}P-labeled cDNAs, quantified RNA were labeled in a reverse transcription reaction containing 5X first strand PCR buffer, 1 μg of 24-mer poly dT primer, 4 μL of 20 mM each dNTP excluding dCTP, 4 μL of 0.1 M DTT, 40 U of RNase inhibitor, 6 μL of 3,000 Ci/mmol a-^{33}P dCTP to a final volume of 40 μL. The mixture was heated at 65˚C for 5 min, followed by incubation at 42˚C for 3 min. Two μL (specific activity: 200,000 U/mL) of Superscript II reverse transcriptase (Invitrogen, Milano, Italy) was then added and the samples were incubated for 30 min at 42˚C, followed by the addition of 2 μL of Superscript II reverse transcriptase and another 30 min of incubation. Fiveul of 0.5 M EDTA was added to chelate divalent cations. After the addition of 10 μL of 0.1 M NaOH, the samples were incubated at 65˚C for 30 min to hydrolyze remaining RNA. Following the addition of 25 μL of 1 M Tris (pH 8.0), the samples were purified using Bio-Rad 6 purification columns (Hercules, CA, USA). This resulted in 5×10^6 to 3×10^7 cpm per reaction[23].

Hybridization and Scanning

cDNA microarrays were pre-hybridized in hybridization buffer containing 4.0 mL Microhyb (Invitrogen, Milano, Italy), 10 μL of 10 mg/mL human Cot 1 DNA (Invitrogen, Milano, Italy), and 10 μL of 8 mg/mL poly dA (Pharmacia, Peapack, NJ). Both Cot 1 and poly dA were denatured at 95˚C for 5 min prior to use. After 4 h of pre-hybridization at 42˚C, approximately 10^7 cpm/mL of heat-denatured (95˚C, 5 min) probes were added and incubation continued for 17 h at 42˚C. Hybridized arrays were washed three times

in 2X SSC and 0.1% SDS for 15 min at room temperature. The microarrays were exposed to phosphorimager screens for 1-5 days, and the screens were then scanned in a FLA-8000 (Fuji Photo Film Co., Japan) at 50 μm resolution[23,24].

Data Analysis

Microarray images were trimmed and rotated for further analysis using L-Processor system (Fuji Photo Film Co., Japan). Gene expression of each microarray was captured by the intensity of each spot produced by radioactive isotopes. Pixels per spot were counted by Arraygauge (Fuji Photo Film Co., Japan) and exported to Microsoft Excel (Microsoft, Seattle, WA, USA). The data were normalized with Z transformation to obtain Z scores by subtracting each average of gene intensity and dividing with each standard deviation. Z scores provide each of 2,304 spots (two sets of 1,152 genes) genes with the distance from the average intensity and were expressed in units of standard deviation. Thus, each Z score provides flexibility to compare different sets of microarray experiments, by adjusting differences in hybridization intensities. Gene expression difference as compared with untreated control cells were calculated by comprising Z score differences (Z differences) among the same genes. This facilitates comparing each gene that had been up- or downregulated as compared with the control cells. Z differences were calculated first by subtracting Z scores of the controls from each Z score of the sample. These differences were normalized again to distribute their position by subtracting the average Z difference and dividing with the standard deviation of the Z differences. These distributions represent the Z ratio value and provide the efficiency for comparing each microarray experiment[23]. Scatter plots of intensity values were produced by Spotfire (Spotfire, Inc., Cambridge, MA)[25]. Cluster analysis was performed on the Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http:// rana.lbl.gov). Clustering of changes in gene expression was determined by using a public domain cluster based on pair wise complete-linkage cluster analysis[26].

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