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Gene Expression of MC3T3-E1 Cells on Fibronectin-immobilized Titanium Using Tresyl Chloride Activation Technique

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Fibronectin (FN) can be immobilized directly on titanium surfaces using tresyl chloride activation technique. The key advantage of tresyl chloride activation technique lies in its simplicity. In this study, we examined the cell attachment and gene expression of MC3T3-E1 cells on FN-immobilized titanium using GeneChip. Cells attached on FN-immobilized titanium at a higher rate than untreated titanium. FN altered the gene expression profile, whereby 62 genes were found to be up-regulated, while 56 genes were found to down-regulate to over twice the level on day 14. FN not only enhanced the expression levels of IBSP and OMD, but also decreased SULF1 mRNA level. Taken together, the immobilization of FN on tresylated titanium promoted early matrix mineralization and bone formation.

Keywords: Osteoblasts, Fibronectin-titanium, GeneChip

INTRODUCTION

Currently, a significant amount of research is focused on the biochemical modification of titanium surfaces. The chief intent of surface modification is to enhance the integrity of tissue-implant interfaces by the immobilization of proteins, enzymes, or peptides for the purpose of inducing specific cell and tissue responses[1]. In the implantation of biomaterials, the first biological reaction at the biomaterial-tissue interface is the adsorption of body fluid proteins including extracellular matrix components on the material surface[2,3]. It is widely accepted that these adsorbed proteins control the subsequent biological responses at the implant-tissue interface. Some studies have reported that preadsorption on the titanium surface with cell-adhesive proteins, such as fibronectin, collagen, and laminin, improved cell activity, initial cell attachment, or cell spreading[4-8].

Simple preadsorption of proteins is insufficient to produce a biologically active metallic implant because of the dynamic exchange between preadsorbed proteins and circulating proteins in the body. The adsorption and desorption behaviors of albumin and fibrinogen on 17 different metal surfaces using ^{125}I-labeled proteins showed that substantial *in vivo* desorption of the preadsorbed proteins occurred within 106 hours for most metals[9].

Some researchers have attempted to immobilize proteins or peptides on titanium surfaces to increase the stability of proteins or peptides by forming a covalent bond between cell-adhesive proteins and titanium. Silane coupling agents — such as aminopropyltriethoxysilane — have been widely used to modify titanium surfaces and to couple proteins or peptides. Fibronectin[10-12], fibrillar collagen[13], or some oligopeptides such as Arg-Gly-Asp-Cys[14,15] were immobilized on titanium or titanium alloys using silane coupling agents, and their biological responses evaluated.

Chemical modification of titanium surfaces by immobilization of proteins or peptides has also been reported. Morikawa *et al.*[16] bound collagen on gold-deposited titanium through a condensation reaction between collagen and a stable monolayer of cysteine. They observed good connection of the collagen-immobilized titanium to the gingival tissue. Besides, Morra *et al.*[17] covalently linked collagen to the titanium surface. A thin film of hydrocarbon plasma was deposited on titanium and an acrylic acid was grafted on the plasma-deposited titanium surface. Then, collagen was coupled with the grafted acrylic acid using a condensation reagent. Implants in rabbit femurs showed a significant increase of bone growth and bone-implant contact in the case of collagen-immobilized surfaces. Alternatively, Matsumura *et al.*[18] and Peng *et al.*[19] designed a method of adhering polyethylene-co-vinyl alcohol

(EVA) to titanium by a hot melt technique, and then immobilized collagen on titanium *via* the EVA film. However, these procedures are complicated and require several steps for immobilization of proteins on the titanium surface. Moreover, some techniques such as plasma treatment need special apparatus.

In contrast, Nilsson *et al.*[20,21] reported on a direct and simple technique of immobilizing enzymes to various hydroxyl groups using highly reactive sulfonyl chlorides, such as 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride). They activated the hydroxyl groups of silica or agarose with the reaction of tresyl chloride, and then coupled the enzyme, such as α-chymotrypsin, to tresyl-agarose. The prominent advantage of this method is its simplicity. Similarly, proteins can be bound to a substrate without any spacer.

Fibronectin is a heterodimeric ECM glycoprotein which has been shown to regulate not only cell adhesion, but also migration and differentiation of various mesenchymal cells[22]. Fibronectin is required for osteoblast differentiation. In cultures treated with antibodies against fibronectin, mineralized nodule formation and gene expression — which are characteristic of mature osteoblasts — were suppressed[23].

Titanium has two types of hydroxyl groups: a basic terminal hydroxyl group and a bridge hydroxyl group[24]. As tresyl chloride reacts with the basic terminal hydroxyl groups of the titanium surface, tresyl chloride-activated titanium would presumably facilitate efficient immobilization of proteins. To date, studies have demonstrated successful direct attachment of fibronectin to titanium by means of tresyl chloride activation technique[25-27]. Tresyl chloride is a liquid which can be applied directly to the titanium surface. As such, the reaction between tresyl chloride and titanium proceeded directly without using any solvent. X-ray photoelectron spectroscopy (XPS) revealed that tresyl chloride reacted with the basic hydroxyl groups on titanium surface, forming tresyl chloride-activated titanium (Ti-O-$SO_2CH_2CF_3$). It was found that fibronectin attached easily to the tresyl chloride-activated titanium surface.

The adsorption behavior of fibronectin on tresyl chloride-activated titanium was monitored by a quartz crystal microbalance-dissipation (QCM-D) technique[27]. QCM-D measurement revealed that a greater amount of fibronectin was adsorbed on the tresyl chloride-activated titanium surface than on untreated titanium. Further, the bond between fibronectin and tresyl chloride-activated titanium was stronger than the bond between fibronectin and untreated titanium.

In view of the encouraging results mentioned above, there now arises a need to study the biological responses of protein-immobilized titanium using tresyl chloride technique. The aim of the present study, therefore, was to evaluate the effects of fibronectin-immobilized titanium on cell attachment, differentiation, and gene expression using GeneChip technology.

MATERIALS AND METHODS

Fibronectin immobilization on titanium substrate

Commercially pure wrought titanium disks (JIS, Japan industrial Specification H 4600, 99.9 mass% Ti, Furuuchi Chemical Corp., Tokyo) with diameters of 15 mm and 48 mm were used. The disks were ground with 600-grit sand papers. Surface roughness of the polished titanium disks, as measured with Handysurf E-30A (Tokyo Seimitsu, Tokyo) with a scale length of 4 mm and a cut-off value of 0.8, was 0.76±0.06 μm. After ultrasonic cleaning in distilled water, fibronectin was immobilized on the polished titanium disks.

The surfaces of the polished titanium disks were completely covered with tresyl chloride (Fluka, Buchs, Switzerland) and then stored at 37℃ for two days. After which, tresylated titanium disks were washed with water followed by water-acetone solution (50 : 50), then dried and stored in a desiccator.

Human plasma fibronectin (Harbor Bio-Products, MA, USA) was dissolved in phosphate-buffered saline (PBS) solution (pH 7.4) at a concentration of 50, 100, 200 μg/ml for the Enzyme-Linked ImmunoSorbent Assay (ELISA), and 100 μg/ml for cell attachment assay and GeneChip analysis. Tresylated titanium disks were immersed in the fibronectin-PBS solution for 24 hours at 37℃, and then rinsed with doubled-distilled water. Finally, the titanium disks were dried with a gentle stream of dry air and stored in a desiccator. Untreated titanium disks were used as a control. Before cell assay, all disks were sterilized by ultraviolet radiation.

Quantification of fibronectin on titanium disks

All measurements were done by ELISA as described[28]. Briefly, human plasma fibronectin was immobilized at three different concentrations (50, 100, and 200 μg/ml) on 15-mm disks (n=4). Anti-human fibronectin derived from rabbit (Cosmobio, Japan) was used for two hours at room temperature. The second antibody, peroxidase-conjugated goat anti-rabbit immunoglobulin (MP Biomedicals, Aurora, OH, USA), was used for one hour at room temperature. A colorimetric substrate for HRP, HRP substrate TMB solution system (Moss, Pasadena, MD) was then added. Absorbance at 450 nm was measured by a spectrophotometer.

Cell attachment assay

Fibronectin-immobilized titanium disks (100 μg/ml)

Table 1 Quantitative real-time RT-PCR using mouse primers

Genbank accession no.	Gene	Primers (5'-3')	bp
NM_008318	IBSP	Forward TACCGAGCTTATGAGGACGAA Reverse GCATTTGCGGAAATCACTCTG	246
NM_012050	OMD	Forward CAAGTTTTCAGGAGCTACCA Reverse ATGGAGGCATAAATGTGTACC	161
NM_172294	SULF1	Forward AGAAGGAGGAAACTATGACC Reverse CTAGGGTTAGACCTGTACTC	148

and untreated titanium disks were placed in tissue culture polystyrene dishes. MC3T3-E1 cells were used for cell adhesion assay, plated at a density of 10^5 cells/cm^2 on 15mm-titanium disks (n=5). After 30 minutes, the disks were washed with PBS solution to remove nonadherent cells and transferred to new wells. Attached cells were trypsinized with 0.25% trypsin and 0.5 mM EDTA, and total cell number was counted using a Model Z1 Coulter particle counter (Beckman Coulter, Fullerton, CA).

RNA extraction and GeneChip analysis

MC3T3-E1 cells were plated at a density of 5×10^4 cells/cm^2 on 48-mm fibronectin-immobilized titanium disks (100 μg/ml) and control. The cells were cultured using Minimum Essential Medium (MEM) alpha (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal calf serum, antibiotics (penicillin-streptomycin), 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate. The medium was changed every 2—3 days. After 14 days, total cellular RNA was isolated using RNeasy kit (QIAGEN, CA).

A quantity of 100 ng of total RNA sample was subjected to two-cycle target labeling according to Affymetrix instructions. Antisense complementary RNA (cRNA) derived from double-strand complementary DNA (cDNA) was labeled in the presence of biotinylated deoxyribonucleotide triphosphate (dNTP) derivatives to produce cRNA probes. The probes were then fragmented and hybridized on GeneChip Mouse Genome 430 2.0 Array. Washing and staining were performed for each sample using a GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA).

Chip performance, background levels, and the presence or absence of signals were assessed using Microarray Suite software (Affymetrix, Santa Clara, CA). Each chip in a given set was normalized by adjusting the probe intensities. The presence or absence of signals was re-evaluated and intensity normalization was performed across all the arrays. Data analysis was performed using the GeneChip Expression Analysis software (Affymetrix, Santa Clara, CA) and GeneSpring software (Silicone Genetics, Redwood, CA).

Real-time RT-PCR analysis

Reverse transcription was performed using SuperScript™ First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) from RNA samples. To quantify mRNA, real-time PCR was performed using a DNA Engine OPTICON™ (MJ Research) with a SYBR Green PCR reagent (QIAGEN, CA) using primer sets of genes of interest, with bone formation-related functions (as shown in Table 1). Each assay was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. PCR products were electrophoresed on 1.5% agarose gel, followed by staining with ethidium bromide to examine the size of PCR products.

Fold change was calculated based on the method as described by Ogura *et al.*[29]. The initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of real-time PCR reaction (C_T-value). The number of transcripts was determined based on the threshold cycles of the genes of interest and GAPDH. ΔC_T (C_T gene of interest — C_T GAPDH) indicated the relative abundance of the transcript numbers of gene of interest. $\Delta\Delta C_T$ (ΔC_T fibronectin — ΔC_T control) indicated the relative n-value compared to the control. The value 2^{-n} indicated the differences in expression of gene of interest between fibronectin-immobilized disks and control.

Statistical analysis

All results were expressed as mean±SD. The results were statistically analyzed by one-way analysis of variance (ANOVA) for quantification of fibronectin and Student's *t*-test for cell attachment assay. P value <0.05 represented a significant difference.

RESULTS

Fibronectin immobilization on titanium

The amount of fibronectin on titanium disks depended on the initial immobilizing concentration of fibronectin. As shown in Fig. 1, fibronectin was found to be 77.5, 277.5, and 856.25 ng on 50, 100 and 200 μg/ml for initial immobilizing concentration,

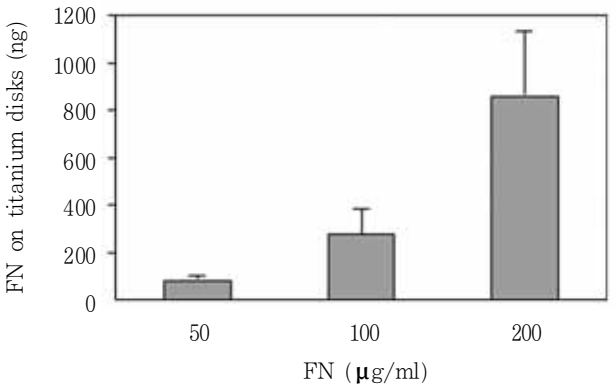

Fig. 1 Immobilization of fibronectin on titanium disks. With the different initial concentrations of fibronectin (50, 100, 200 μg/ml), fibronectin was found in different amounts (77.5, 277.5, 856.25 ng respectively). Results were represented as mean ±SD (n=4). One-way ANOVA showed significant differences among the mean values of fibronectin found on titanium disks (P<0.001).

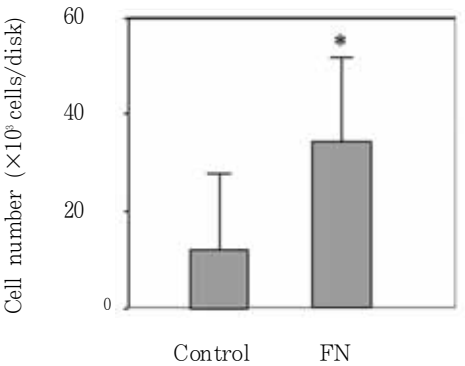

Fig. 2 Cell attachment number on fibronectin-immobilized titanium disks. After 30 minutes, MC3T3-E1 cell attachment number on fibronectin-immobilized titanium disks (100 μg/ml) increased when compared with the control (untreated disks). Results were given as cell attachment number represented as mean±SD (n=5). *: $P<0.05$ when test value was compared to control value.

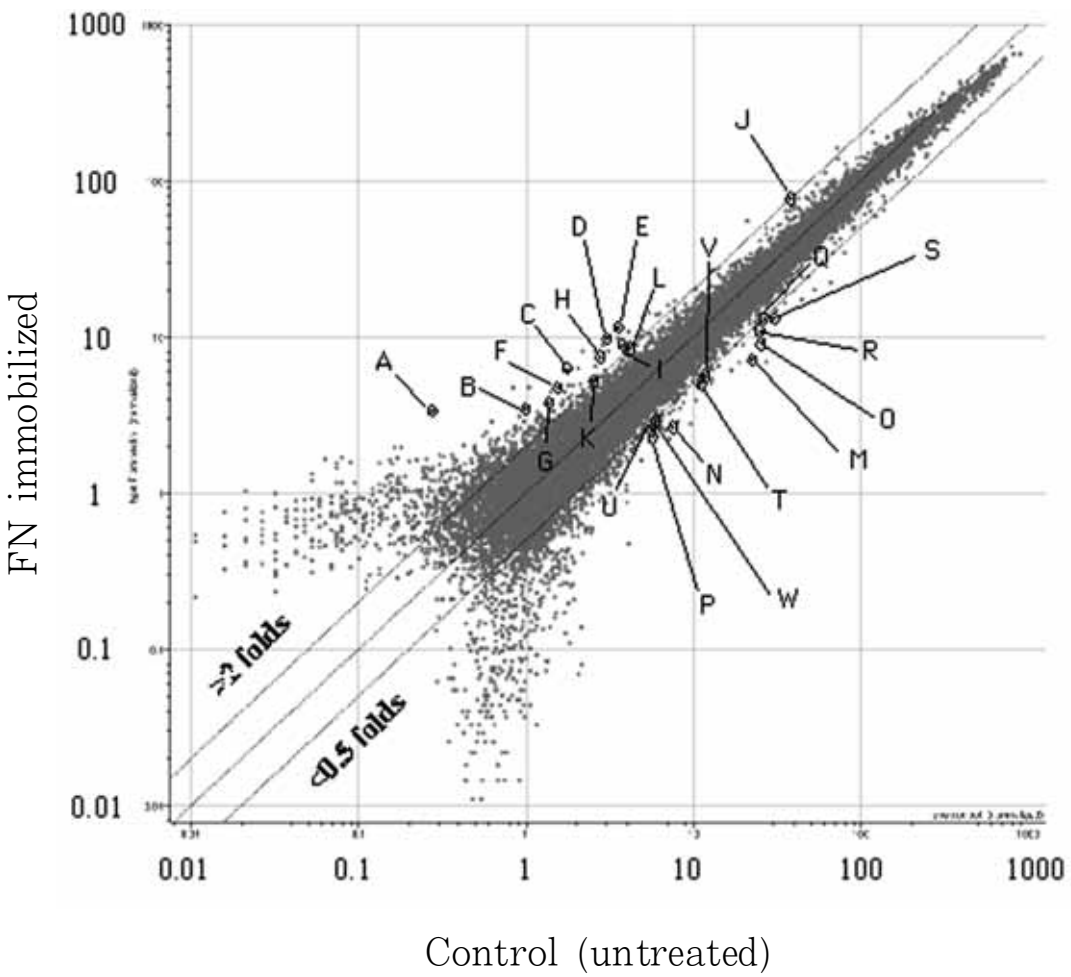

Fig. 3 Scatter plot of gene expression profiling in MC3T3-E1 cells. After 14 days, 62 genes were up-regulated to over twice the level, while 56 genes were found to down-regulate to a level less than twice in fibronectin-immobilized titanium after the cut-off values of low intensity signals.

respectively — thereby demonstrating a dose-dependent trend. One-way ANOVA showed significant differences among the mean numbers of fibronectin found on titanium disks ($P<0.001$). These findings clearly suggested that fibronectin was successfully immobilized on tresylated titanium disks.

Cell attachment on titanium disks

We selected to immobilize titanium disks at 100 μg/ml fibronectin concentration. At this concentration, it could be seen in Fig. 2 that MC3T3-E1 cells attached to fibronectin-immobilized titanium disks at a significantly ($p<0.05$) higher rate than the control (untreated disks).

Fibronectin alters MC3T3-E1 gene expression

In MC3T3-E1 cells, fibronectin caused differences in gene expression. As shown in Fig. 3, the scatter plot of mRNA levels of untreated and fibronectin-immobilized titanium disks after 14 days exhibited differences in gene expression pattern. A number of 62 genes were up-regulated to over twice the level, while 56 genes were found to be down-regulated to a level less than twice in fibronectin-immobilized titanium after the cut-off values of low intensity signals.

Table 2 shows some selected gene IDs, gene names and fold changes in expression level of MC3T3-E1 cells on fibronectin-immobilized titanium

Table 2 Gene expressions of MC3T3-E1 cells on fibronectin-immobilized Ti disks as compared with untreated disks (14 days)

>2 folds

Gene ID	Gene name		Fold change	Spot on plot
NM_007566	Baculoviral IAP repeat-containing 6	Birc6	12.19	A
NM_011499	Serine/threonine kinase receptor associated protein	Strap	3.54	B
NM_008318	Integrin binding sialoprotein	Ibsp	3.54	C
NM_011504	Syntaxin binding protein 3A	Stxbp3a	3.27	D
NM_020559	Aminolevulinic acid synthase 1	Alas1	3.25	E
NM_029404	PHD finger protein 14	Phf14	3.04	F
NM_020009	FK506 binding protein 12-rapamycin associated protein 1	Frap1	2.79	G
NM_025848	Succinate dehydrogenase complex, subunit D	Sdhd	2.68	H
NM_021518	RAB2, member RAS oncogene family	Rab2	2.36	I
NM_012050	Osteomodulin	Omd	2.08	J
NM_021716	Fidgetin	Fign	2.08	K
NM_010331	GPI anchor attachment protein 1	Gpaa1	2.08	L

<0.5 fold

Gene ID	Gene name		Fold change	Spot on plot
NM_028973	Leucine rich repeat containing 15	Lrrc15	0.29	M
NM_024436	RAB22A, member RAS oncogene family	Rab22a	0.36	N
NM_199468	Zinc finger, CCHC domain containing 5	Zcchc5	0.36	O
NM_134156	Actinin, alpha 1	Actn1	0.41	P
NM_172294	Sulfatase 1	Sulf1	0.42	Q
NM_175836	Spectrin beta 2	Spnb2	0.43	R
NM_011607	Tenascin C	Tnc	0.44	S
NM_026144	Dehydrodolichyl diphosphate synthase	Dhdds	0.47	T
NM_016799	Serine/arginine repetitive matrix 1	Srrm1	0.47	U
NM_007707	Suppressor of cytokine signaling 3	Socs3	0.48	V
NM_028188	RUN and SH3 domain containing 1	Rusc1	0.49	W

Table 3 Comparison of gene expression levels using real-time PCR and GeneChip

Gene	Sample	C_T	ΔC_T	$\Delta\Delta C_T$ (n)	Folds (2^{-n})	GeneChip (folds)
GAPDH	Control	27.31				
	Fibronectin	27.06				
IBSP	Control	34.57	7.26			
	Fibronectin	32.91	5.85	−1.41	2.66	3.54
OMD	Control	29.31	2			
	Fibronectin	27.92	0.86	−1.14	2.2	2.08
SULF1	Control	34.05	6.74			
	Fibronectin	35.03	7.97	1.23	0.43	0.42

C_T, cycle number at which fluorescent signal crossed a threshold in the exponential phase of real-time PCR reaction; ΔC_T: C_T gene of interest − C_T GAPDH; $\Delta\Delta C_T$: ΔC_T fibronectin − ΔC_T control; 2^{-n} represented the difference in expression of gene of interest between fibronectin-immobilized relative to control.

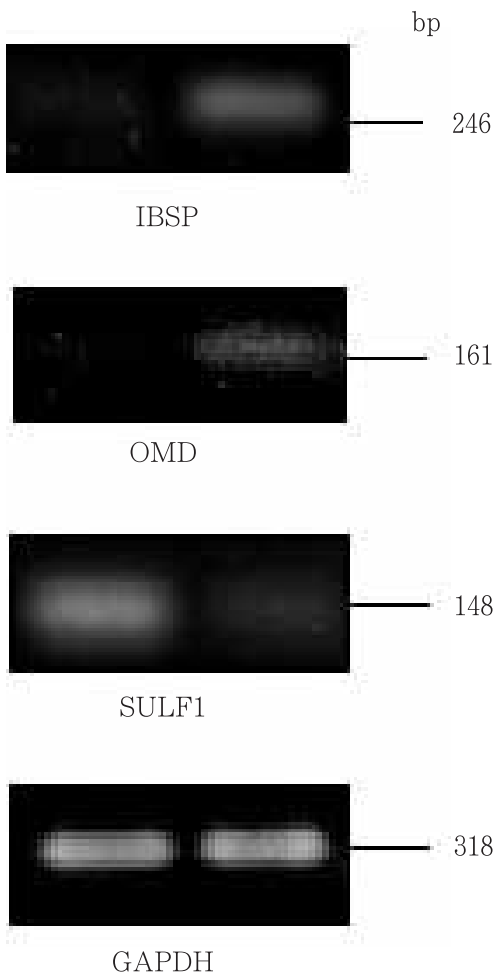

Fig. 4 RT-PCR analysis of fibronectin-modulated genes: Integrin binding sialoprotein (IBSP), Osteomodulin (OMD), and Sulfatase (SULF1). GAPDH was used as an internal control for cDNA quality and PCR efficiency

disks.

Quantitative real-time RT-PCR

To verify the variations shown by GeneChip arrays using real time RT-PCR, we decided to choose several genes that have shown to be involved in osteoblast differentiation and bone formation. The same total RNA samples used for GeneChip analysis were use for real-time RT-PCR. All real-time RT-PCR data are shown in Fig. 4, whereby it was shown that fibronectin markedly increased the mRNA levels of integrin binding sialoprotein (IBSP, BSP) and osteomodulin (OMD, OSAD), but reduced that of Sulfatase1 (SULF1). The sizes of PCR products were 246, 161, and 148 base pairs respectively.

Using SYBR fluorescence system and real-time PCR, we were able to quantitatively analyze IBSP, OMD, and SULF1 with GAPDH. As shown in Table 3, gene expression changes assessed using real-time RT-PCR for IBSP, OMD, and SULF1 revealed 2.66, 2.20, and 0.43 folds respectively. These results correlated with GeneChip array results (3.54, 2.08, and 0.42 folds respectively).

DISCUSSION

There are several methods to bind cell-adhesive proteins to titanium, namely precoating with pyrrole or Au and cysteine, or pretreatment with silane coupling agents[10,11,16,30]. The prominent advantage of tresyl chloride activation technique is its simplicity. The process involves simply casting the tresyl chloride solution on the titanium substrate and then storing at 37℃ for two days. Through this technique, proteins can be immobilized through the interaction between proteins' amino groups and activated hydroxyl group of titanium[26]. It is noteworthy that most of the proteins can be immobilized on titanium using the tresyl chloride activation

technique.

Besides the use of X-ray photoelectron spectroscopy (XPS)[25] to confirm the immobilization of fibronectin on tresylated titanium surfaces, ELISA is a valid alternative means which we chose to use in this study. Results showed clearly that fibronectin was successfully immobilized on tresylated titanium surfaces. The amount of fibronectin on titanium surfaces was dose-dependent. In previous studies, various concentrations of fibronectin were used for surface coating of titanium surfaces. For example, Van den Dolder *et al.*[31] coated titanium fiber mesh with 50 μg/ml fibronectin, while Park *et al.*[32] coated titanium disks with 1 μg/ml fibronectin. In the present study, 100 μg/ml fibronectin was selected to immobilize titanium disks because at this concentration, fibronectin enhanced the attachment of MC3T3-E1 cells to titanium surface as compared with untreated disks — even for a short time, around 30 minutes. Indeed, previous studies have reported that cell adhesion improved when a fibronectin or collagen I coating was used[32].

In several studies, fibronectin-immobilized titanium disks affected some genes which promote cell attachment, osteoblast proliferation and differentiation, and bone formation[33]. In this study, gene expression profiling clearly indicated the effects of immobilizing titanium with fibronectin on bone formation. We found that on day 14, fibronectin markedly increased the mRNA levels of IBSP and OMD, while reduced that of SULF1.

IBSP or BSP is a NCP (non-collagenous protein) member of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) protein family[34], whereby the expression pattern is temporospatially associated with the early stages of the mineralization process[35]. IBSP has been localized in mineralizing tissues such as bone, dentin, cementum, and hypertrophic cartilage. Expression of IBSP gene, which is induced in newly formed osteoblasts, is up-regulated by cytokines that promote bone formation and down-regulated by factors that suppress bone formation. IBSP contains RGD (arginine-glycine-aspartate) sequence which has the ability to bind to cell surface integrins. It also has numerous posttranslational modifications including N- and O-linked glycosylation, tyrosine sulfation, and serine and threonine phosphorylation[36]. These posttranslational modifications are believed to mediate matrix-matrix and cell-matrix relationships. For example, phosphorylation influences crystal growth, whereas glycosylation can affect cell attachment[37]. On this note, IBSP mediates *in vitro* attachment of fibroblasts[38], osteoblasts[39], and osteoclasts[40]. IBSP also has two glutamic acid-rich regions which are believed to be partially responsible for the nucleating activity of IBSP and which could also be involved in the binding capacity of IBSP to hydroxyapatite (HA)[41]. Taken together, IBSP can promote bone formation.

Osteomodulin or Osteoadherin (OMD or OSAD) is a keratan sulfate proteoglycan which belongs to the SLRP (Small Leucine-rich Repeat Proteoglycans) family[42,43]. It is found exclusively in mineralized tissues[44], mouse osteoblasts, odontoblasts, ameloblasts[45] and human odontoblasts[46]. Osteomodulin is also localized extracellularly in alveolar bone, predentine, and enamel matrices of rat and mouse teeth[47]. OMD mRNA level increased in a time-dependent manner during the differentiation process of hMSC to osteoblasts[48] and peak expression occurs late during osteoid formation and mineralization. OMD has high affinity for hydroxyapatite, which is a unique feature of OMD among the SLRP members[42]. It functions as a structural component of the bone matrix. OMD is also considered to be an early marker for terminally differentiated osteoblasts, and SLRP seem crucial for proper bone formation and collagen fibril formation[49].

Proteoglycans are complex macromolecules consisting of a core protein and glycosaminoglycans, such as chondroitin sulfate, keratin sulfate, heparin sulfate, and dermatan sulfate. Proteoglycans play an important role in the attachment and adhesion of osteoblastic cells to extracellular matrix[42,50]. Bone-titanium interface analysis has exhibited a high percentage of sulfur, a component of various glycosaminoglycans[51,52].

The SULF or Sulfatase family is a group of enzymes that catalyze the hydrolysis of sulfate ester bonds from a wide variety of substrates, ranging from complex molecules such as glycosaminoglycans and sulfolipids to steroid sulfates. Most sulfatases are located in lysosomes where they perform — in an acidic environment — the desulfation reactions required in the degradation pathways of glycosaminoglycans, heparan sulfate, chondroitin sulfate, dermatan sulfate, and sulfolipids[53]. The degradation of glycosaminoglycans resulted in decreased interfacial strength between cultured mineralized tissue and titanium[54]. The finding of down-regulation of sulfatase in this study thus suggested a decrease in glycosaminoglycan degradation, further implying an early promotion of matrix mineralization of osteoblastic cells. Expression of genes with unreported functions in osteoblasts was also noted.

As found in a previous study, stem cells have the ability to differentiate to osteoblasts after two weeks. The hMSC could be stained strongly on day 13 or 17 when exposed to Alizarin Red solution[55]. Osteogenesis has been determined by staining with Alizarin Red solution, which can be used to visually detect the presence of mineralization in cell culture

and bone tissue. In light of these findings, we decided to examine gene expression on day 14.

In conclusion, this study enabled the profiling of the expression of genes involved in bone formation directed by fibronectin. Nonetheless, further study on early attachment stage is needed to provide us a better understanding of the interactions between cells and biomaterial-coated titanium surfaces, to the end of developing an optimal biomaterial which can promote osseointegration and osteogenesis.

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