The Effect of Titanium Surface Roughening on Protein Absorption, Cell Attachment, and Cell Spreading

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Purpose: *The purpose of this study was to compare properties of roughened and polished titanium with respect to their ability to attach to cells and bind to protein as well as their cell spreading behavior.* ***Materials and Methods:*** *Three different titanium surface treatments were compared for their ability to support cell attachment and spreading: sandblasted and acid-etched, resorbable blast media, and machine-polished titanium. The surface of the materials was characterized for surface roughness, surface energy, and surface chemistry. Osteoblast-like MG-63 cells were tested for in vitro attachment and spreading in the presence of serum proteins. Cell attachment was assessed by direct counting, dye binding, and microculture titanium assays. Cell spreading was determined by measuring area/cell in phalloidin-AlexaFluor 488 stained cells. Absorption of bovine serum albumin was determined by assay.* ***Results:*** *Scanning electron micrography and x-ray diffractometry confirmed increased surface roughness of the roughened materials. All 3 materials had similar albumin binding kinetics. Three different methods confirmed that roughened surfaces enhance early cell attachment to titanium in the presence of serum. Cells spread better on smoother machined surfaces than on the roughened surfaces.* ***Conclusion:*** *Roughened titanium surfaces exhibited better early cell attachment than smooth surfaces in the presence of serum. The cells attached to roughened titanium were less spread than those attached to machined titanium. Although albumin binding was not different for roughened surfaces, it is possible that roughened surfaces preferentially bound to serum adhesive proteins to promote early cell attachment.* Int J Oral Maxillofac Implants 2008;23:675–680

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Roughened titanium surfaces such as sandblasted and acid-etched (SAE) and resorbable blast media (RBM) are widely used in titanium dental implants.[1–11] Roughened titanium dental implants exhibit stronger bone-implant contact area and increased pull-out strength.[1] The enhanced performance of roughened titanium is thought to be due to the increased surface area available for cell interactions. However, it is not known what properties of roughened surfaces are the most important for enhanced performance. Moreover, data in the literature on the biological effects of surface properties on cell attachment are contradictory.[12–14] Some studies show that smooth surfaces favor cell proliferation and alkaline phosphatase activity, whereas others show greater cell proliferation and alkaline phosphatase activity on rough surfaces.[15–18] After in vivo implantation, increased moderate surface roughness in screws provided higher bone-to-metal contact compared to high surface roughness, whereas increased microroughness enhanced bone-titanium fixation as measured by screw removal torque.[19,20]

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Thus, for the present study, it was decided to compare properties of roughened and polished titanium with respect to their ability to attach to cells and bind to protein, as well as their cell spreading behavior. The investigators determined the protein binding, cell attachment, and cell spreading activity of roughened surface treated titanium with machine-polished titanium.

MATERIALS AND METHODS

Grade 2 pure titanium disks were divided into 3 groups: SAE, RBM, and M (machine finished). SAE and RBM surfaces were obtained from MEGAGEN (Daegu, Korea). Sanding conditions for RBM surfaces were as follows: apatitic abrasive (Himed Co, Old Bethpage, NY) was used to roughen surfaces. The apatitic particles were smaller than 150 mesh and accelerated with a pressure of 5 atmospheres. SAE samples were acid-etched after sandblasting in etching solution ($HCl:H_2SO_4$ = 4:1) at 80°C for 5 minutes. The materials were autoclave sterilized.

Surface roughness of triplicate samples was measured by profilometry (Surtronic 3; Taylor-Hobson, Leicester, United Kingdom). Contact angles of 12 samples in each group were measured using double-distilled deionized water, glycerol, diiodomethane, and bromonaphthalene. The surface energy of each surface was calculated using the Zisman method, in which the cosines of these angles were plotted against the known surface tension values of the liquids as a Zisman plot. The critical surface tension or surface energy was then extrapolated to cosine = 1, which is complete wetting. The surface energies of the surfaces were statistically compared using analysis of variance (ANOVA), and difference was considered significant if $P < .05$. Surface crystal structure of each surface was analyzed using an Advance D8 x-ray diffractometer with Cu Kα radiation from 10 degrees (2θ) to 70 degrees (2θ). The samples were scanned at incident grazing angle (1 degree), and the scan rate was at 0.01 degree per minute at 40 kV and 40 mA.[21,22]

One hundred microns of bovine serum albumin (fraction V; 1mg/mL protein/saline solution) was pipetted onto each surface and incubated in a sterile humidified incubator at 37°C for 180 minutes. At various time points between 15 and 180 minutes, nonadherent proteins were removed and saved, and the volume was recorded. Protein concentrations were analyzed using the micro bicinchoninic acid (BCA) protein assay and measured using a microplate reader at 595 nm. Protein concentration was calculated by correlating the reading to a standard curve. The degree of adsorption was determined by subtracting the residual protein from the initial added protein.

Osteoblast-like MG-63 cells were used to determine cell attachment and spreading. Cells were propagated in Liebovitz L-15 medium containing 10% fetal bovine serum. Freshly fed subconfluent MG-63 cells were harvested by release with 5 mmol/L EDTA-PBS (phosphate-buffered saline; pH 7.4) to eliminate the presence of proteases, which may affect cell surface or substrate bound proteins. Cells were attached at 3×10^4 cells/mL in Liebovitz L-15 medium containing 10% fetal bovine serum onto 1-cm diameter titanium disks that were immobilized in 1% agarose dissolved in serum-free L-15 by heating to boiling in a water bath. The titanium disks were partially embedded; the upper surface remained exposed. Three disks were placed in one 60-mm plate, one each of RBM, SAE, or M. After 3 to 6 hours of incubation, the nonadherent cells were washed away by 3 gentle changes of serum-free L-15 medium. The cells were fixed by addition of neutral buffered formalin for 10 minutes, permeabilized with 0.1% triton X-100 in PBS, washed with PBS, then stained with phalloidin-AlexaFluor 488 in PBS. Fifteen randomly selected microscopic fields per disk were photographed, then cells were counted or the cell volume determined using NIH Image software (NIH, Bethesda, MD). A repeat experiment showed identical results.

Independent confirmation of the direct cell count experiments were provided by 2 experiments in which the number of cells attached to RBM, SAE, and M titanium disks were determined by colorimetric assays in triplicate. The first method estimated cells bound to disks by crystal violet dye binding after cell attachment to surfaces as described. The disks were removed after 3 gentle washes to remove unattached cells, fixed in 95% ethanol for 10 minutes, and stained with crystal violet (0.1% in water) for 30 minutes. The excess crystal violet was removed with 3 washes of distilled water. The attached cells were estimated by absorbance at 595 nm determined in 3 implants of each type after solubilizing the crystal violet with 0.2% triton X-100 in water. The second method estimated cells bound to disks by microculture tetrazolium assay (ATCC) after cell attachment to surfaces as described. The disks were removed after 3 gentle washings with serum-free L-15. MTT reagent in serum-free L-15 was then added to 60-mm plates containing RBM, SAE, or M and incubated for 2 hours at 37°C. After washing with serum-free L-15, the MTT product was solubilized with 0.2% triton X-100, and the absorbance at 570 nm was read to determine the total amount of MTT color reagent metabolized by cells adherent to triplicate disks of each type. Differences between groups were determined by Student *t* test.

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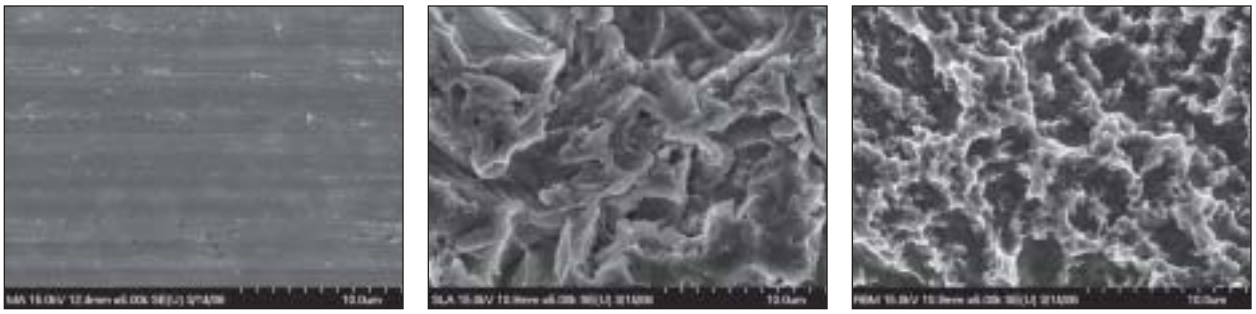

Fig 1 SEM image of the *(left to right)* M, RBM, and SAE surfaces.

Table 1 Characteristics of Machined, RBM, and SAE Surfaces

	Machined		RBM		SAE	
	Mean	SD	Mean	SD	Mean	SD
Surface roughness (μm, n = 3)	0.22	0.03	1.53	0.15	1.74	0.20
Surface energy (dyn/cm, n = 12)	34.3	4.6	35.8	7.6	25.3	5.3

Surface roughness was significantly different between the machined and roughened surfaces ($P < .05$). Surface energy was different between SAE and machined surfaces ($P < .05$) and between SAE and RBM ($P < .05$).

The materials used for in vitro experiments were characterized by scanning electron microscopy (SEM), by profilometry for surface roughness and surface energy, and by x-ray diffractometry (XRD) to determine surface crystal structure. Figure 1 shows SEM images of the M, SAE, and RBM surfaces examined in these studies. The roughened surface of RBM and SAE materials is readily apparent in these images. The measurements in Table 1 quantitatively confirm increased surface roughness of RBM and SAE material compared to machine polished titanium. Figure 2 shows typical data for XRD analysis. The XRD patterns showed that surfaces of the machined, RBM, and the SAE were alpha Ti based on crystal structure evidenced by the main peaks of 100, 101, and 102. No other crystal structure peaks were observed.

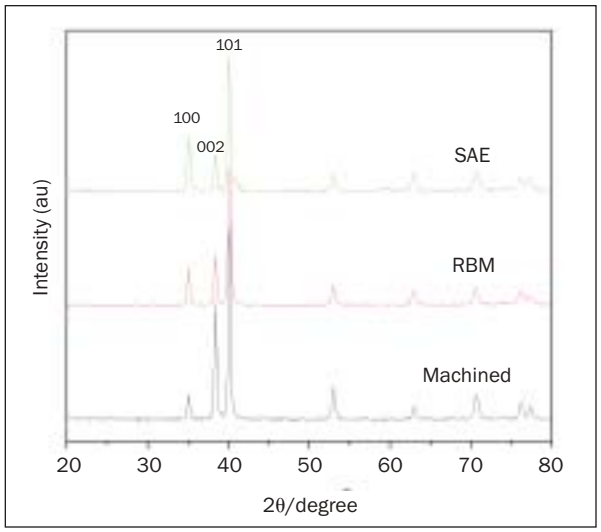

Fig 2 XRD patterns. All peaks are assigned to alpha-titanium.

RESULTS

There were no significant differences in the total protein binding ability of the surfaces as assessed by ability to bind to albumin. Figure 3 shows that binding of bovine serum albumin, the most abundant serum protein, was identical for all 3 materials from 15 to 180 minutes of incubation with bovine serum albumin. Thus, the increased surface area of the RBM and SAE surfaces compared to machined surfaces did not affect protein absorption.

The roughened surfaces were characterized for MG-63 cell attachment in L-15 medium containing 10% fetal bovine serum (Fig 4). MG-63 cells attached best to RBM and SAE roughened surfaces compared to machine-polished titanium. RBM had the highest activity for cell attachment, with SAE next. Cell attachment was enhanced on RBM when characterized by 3 different methods, cell metabolic activity (Fig 4a), crystal violet dye binding (Fig 4b), and direct counting (Fig 4c).

As seen in Fig 5, the area of cells attached to the machine-polished titanium appeared much greater than the area of those on on SAE or RBM. The cell area was quantified using NIH Image software to see if the difference was significant. Cell spreading on

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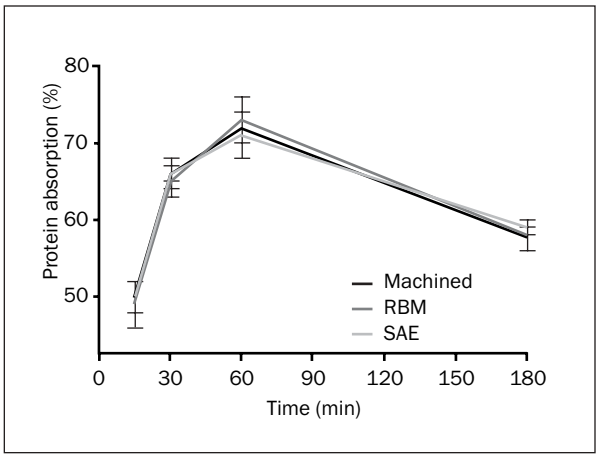

Fig 3 Absorption of bovine serum albumin to titanium surfaces. Error bars are standard error of the mean.

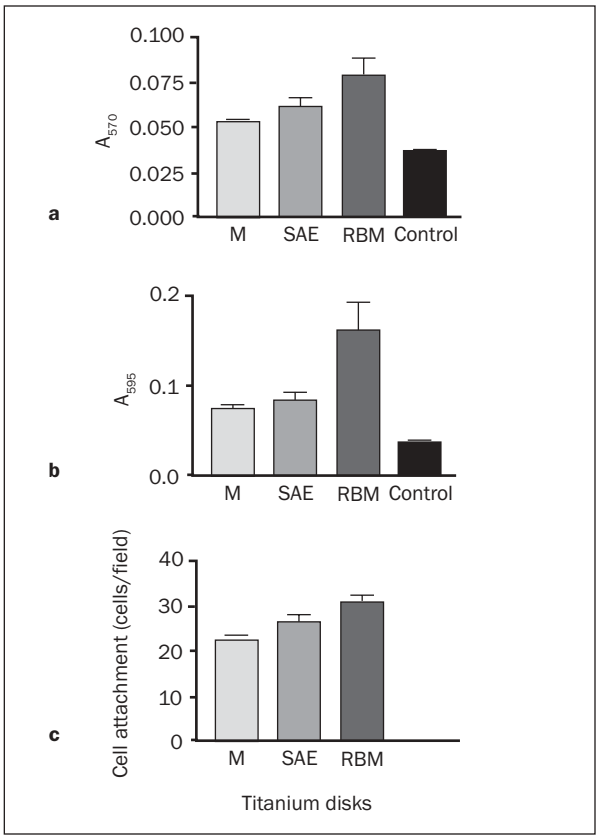

Fig 4 *(right)* Cells attach better to RBM and SAE roughened titanium than to machine-polished titanium. Osteoblast-like MG-63 cells were allowed to attach in the presence of 10% fetal bovine serum for 4 hours, and then cell number was assessed by 1 of 3 methods. *(a)* Metabolic conversion of MTT to a colored product and absorbance at 570 nm. The control is the amount of absorbance in an empty multiwall plate without disks. *(b)* Crystal violet staining and absorbance at 595 nm. The control is the amount of absorbance in an empty multiwall plate without disks. *(c)* Cells are counted after labeling with Phalloidin-Alexafluor 488. The average number of attached cells in 15 microscopic fields is shown. Error bars are standard error of the mean.

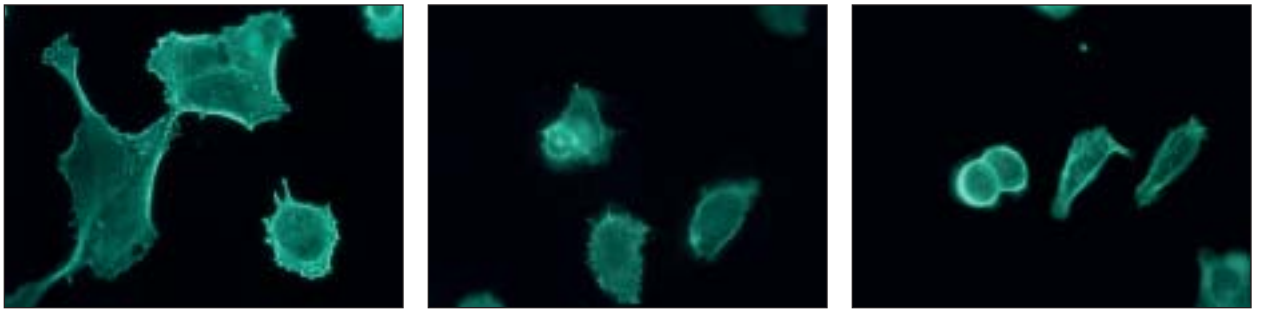

Fig 5 Fluorescent microscopic images of phalloidin-AlexaFluor 488 stained cells attached to *(left to right)* M, SAE, and RBM titanium surfaces. A microscopic field of view of the attachment quantified in Fig 4c is shown.

machined titanium was significantly greater than on the SAE or RBM surfaces ($P < .05$). The SAE and RBM surfaces had similar cell spreading at 4 hours (Fig 6). Additional incubation time did not change the enhanced spreading on machine-polished surfaces, as cell area was significantly greater after an additional 2 hours of incubation (not shown).

DISCUSSION

To determine the relationship between cell attachment, protein absorption, and surface characteristics of roughened titanium implant materials, 3 different titanium surface treatments were compared for the ability to mediate osteoblast-like cell attachment,

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cell spreading, and protein absorption. The RBM and SAE titanium surfaced materials consistently mediated faster cell attachment than the machine-polished titanium. RBM materials tended to support the fastest cell attachment compared to SAE under the conditions of the experiments presented. There was an inverse relationship between cell attachment and cell spreading. Cells exhibited the greatest surface area on machine polished compared to the roughened surfaces.

This report suggests that roughened surfaces provide the best attachment for osteoblasts. This may be a function of the increased surface area of the roughened surfaces. It is also possible that cell attachment may have been augmented by proteins in the serum. The titanium surface would have immediately been coated with serum proteins as well as the ions, minerals, and salts in cell culture medium. The experiment was performed in the presence of the typical concentration of fetal calf serum used to support maximal growth of the MG-63 cell line. Serum contains multiple cell attachment proteins in addition to fibronectin and may more accurately reflect the state of the titanium surface when implanted in vivo compared to serum-free medium. The coating of a surface with attachment proteins would affect cell attachment. It was previously observed that a short 15-minute preabsorption of fibronectin, a serum attachment protein, enhanced cell attachment to machine polished titanium.[21] It also was previously observed that fibronectin-treated machine polished titanium did not have a discernible difference from untreated titanium on the cell morphology.[23] This suggests the differences observed in cell spreading may have been due to titanium surface roughening. It is clear from this study that roughened surfaces enhance early cell attachment in the presence of serum, which conflicts with a previous report.[24]

The results indicate that roughened surfaces bind preferentially to serum cell attachment proteins such as vitronectin or fibronectin, even though they did not bind more avidly to bovine serum albumin. Kieswetter et al suggested that "surface energy plays a key role in the proteinaceous layer that is absorbed."[15] Perhaps the difference between RBM and SAE were not reflected in the amount of BSA absorbed, but by the amount and/or activity of the serum adhesion proteins.

To validate the accuracy of the cell attachment assessments, 3 different methods were used. The different methods to analyze cell attachment were complementary. Cell metabolism of attached cells is measured by the MTT assay, while cell staining by crystal violet estimated dye-binding components of the cell, and direct cell counting after phalloidin staining determined the number of intact, f-actin–containing cells. The 3 assays provided identical conclusions; all showed more cells bound to RBM and SAE surfaces than to smooth machine-polished titanium oxide. This result conflicts with a report by Rosa and Beloti that roughened surfaces blasted with 25, 75, and 250 μm Al_2O_3 were not different from smooth surfaces for cell attachment after 4 hours.[14] Park et al found no differences for attachment on roughened versus smooth surfaces.[24] The different conclusions may be due to differences in cell type. Cells used by Rosa and Beloti were human bone marrow cells, and Park et al used human palatal embryonic cells, while this study used a human osteoblast-like cell line, MG-63. Second, this study directly assessed cells attached to the titanium oxide surface, whereas Rosa and Beloti used the number of cells released by trypsin digestion to estimate the adherent cells,[14] and Park et al used the number of nonadherent cells to estimate cell adhesion.[24] The present study used a transformed osteoblast-like osteosarcoma cell line, which may not share all of the features of preosteoblasts that are present in marrow-derived[14] or palatal mesenchyme cells.[24] It is possible that MG-63 cells express different surface adhesion proteins than nontransformed cells. The current study determined adherent cells in the presence of serum, whereas other studies were performed in the absence of serum. In other studies, the cells attached were estimated by determining cells trypsin-released

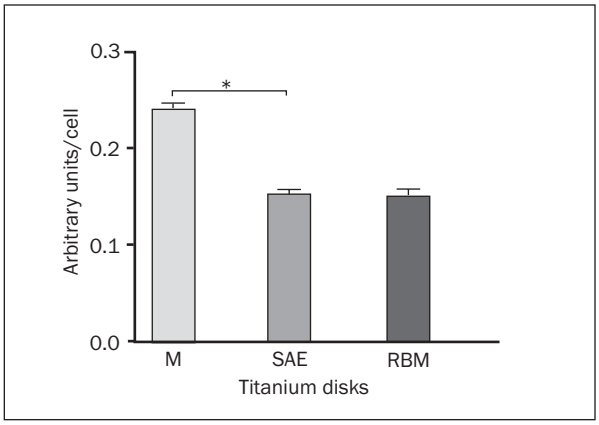

Fig 6 Cell spreading is enhanced on polished titanium surfaces compared to SAE or RBM roughened surfaces. Phalloidin-Alexa-Fluor 488 was used to label MG-63 osteoblast-like cells sarcomas after 4 hours. The cells spread significantly more on polished metal (M) than on alumina-roughened (SAE) or hydroxyapatite-roughened (RBM) surfaces in the presence of 10% serum (* indicates different $P < .05$).[15] Microscope fields were evaluated for spreading by NIH image software, which assigned area units for each cell and expressed spreading as the average arbitrary units/cell. Error bars are standard error of the mean.

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from surfaces or cells that were nonadherent, while the present study directly determined adherent cells by 3 different methods.

The present studies in serum are representative of the in vivo situation, because among the earliest events that occurs will be the coating of titanium surfaces with serum proteins. This coating occurs as the implant contacts blood at the implant site and is coated with the abundant cell attachment proteins fibrinogen, fibronectin, and vitronectin. The findings correlate with improved performance of titanium implants with roughened surfaces compared to smooth surfaces. The results also confirm other studies that have shown improved cell attachment on roughened surfaces.[12,13]

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REFERENCES

1. Yang Y, Oh N, Liu Y, et al. Enhancement of osseointegration using surface modified titanium implants. J Miner Metals Mater Soc 2006;58:71–76.
2. Cochran D. A comparison of endosseous dental implant surfaces. J Periodontol 1999;70:1523–1539.
3. Mante M, Daniels B, Golden E, Diefenderfer D, Reilly G, Leboy PS. Attachment of human marrow stromal cells to titanium surfaces. J Oral Implantol 2003;29:66–72.
4. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez BS, Arvidson K. Determining optimal surface roughness of TiO(2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. Clin Oral Implants Res 2001;12:515–525.
5. Deligianni D, Katsala N, Ladas S, Sotiropoulou D, Amedee J, Missirlis YF. Effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein adsorption. Biomaterials 2001;22:1241–1251.
6. Degasne I, Baslé MF, Demais V, et al. Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. Calcif Tissue Int 1999;64:499–507.
7. Rosa A, Beloti MM. Rat bone marrow cell response to titanium and titanium alloy with different surface roughness. Clin. Oral Implants Res. 2003;14:43-48.
8. Kakuta S, Miyaoka K, Fujimori S, Lee WS, Miyazaki T, Nagumo M. Proliferation and differentiation of bone marrow cells on titanium plates treated with a wire-type electrical discharge machine. J Oral Implantol 2000;26:156–162.
9. Korovessis P, Deligianni DD. Role of surface roughness of titanium versus hydroxyapatite on human bone marrow cells response. J Spinal Disord Tech 2002;15:175–183.
10. Leven R, Virdi AS, Sumner DR. Patterns of gene expression in rat bone marrow stromal cells cultured on titanium alloy disks of different roughness. J Biomed Mater Res A 2004;70:391–401.
11. Soskolne W, Cohen S, Sennerby L, Wennerberg A, Shapira L. The effect of titanium surface roughness on the adhesion of monocytes and their secretion of TNF-alpha and PGE2. Clin Oral Implants Res 2002;13:86–93.
12. Bowers K, Keller JC, Randolf B, Wick D, Michaels C. Optimization of surface micromorphology for enhanced osteoblast responses in vitro. Int J Oral Maxillofac Implants 1992;7:302–310.
13. Keller J, Stanford CM, Wightman JP, Draughn RA, Zaharias R. Characterization of titanium implant surfaces III. J Biomed Mater Res 1994;28:939–946.
14. Rosa A, Beloti MM. Effect of cpTi surface roughness on human bone marrow cell attachment, proliferation, and differentiation. Braz Dent J 2003;14:16–21.
15. Kieswetter K, Schwartz, Z, Dean, DD, Boyan, BD. The role of implant surface characteristics in the healing of bone. Crit Rev Oral Biol Med 1996;7:329–345.
16. Stanford C, Keller JC, Solursh M. Bone cell expression on titanium surfaces is altered by sterilization treatments. J Dent Res 1994;73:1061–1071.
17. Martin J, Schwartz Z, Hummert TW, et al. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). J Biomed Mater Res 1995;29:389–401.
18. Hatano K, Inoue H, Kojo T, et al. Effect of surface roughness on proliferation and alkaline phosphatase expression of rat calvarial cells cultured on polystyrene. Bone 1999;25:439–445.
19. Wennerberg A, Albrektsson T, Andersson B. Bone tissue response to commercially pure titanium implants blasted with fine and coarse particles of aluminum oxide. Int J Oral Maxillofac Implants 1996;11:38–45.
20. Deyneka-Dupriez N, Kocdemir B, Herr U, Fecht HJ, Wilke HJ, Claes L. Interfacial shear strength of titanium implants in bone is significantly improved by surface topographies with high pit density and microroughness. J Biomed Mater Res B Appl Biomater 2007;82:305–312.
21. Yang Y, Cavin R, Ong JL. Protein adsorption on titanium surfaces and their effect on osteoblast attachment. J Biomed Mater Res 2003;67A:344–349.
22. Yang Y, Bumgardner JD, Cavin R, Carnes DL, Ong JL. Osteoblast precursor cell attachment on heat-treated calcium phosphate coatings. J Dent Res 2003;82:449–453.
23. Yang Y, Glover, R, Ong, JL. Fibronectin adsorption on titanium surfaces and its effect on osteoblast precursor cell attachment. Colloids Surfaces B: Interfaces. 2003;30:291–297.
24. Park S, Yang Y, Kim K, Ong JL. Osteoblast precursor cell response to SLA and RBM titanium surfaces [abstract]. J Dent Res 2004;A083.

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