Effect of Titanium Surface Roughness on Human Osteoblast Proliferation and Gene Expression In Vitro

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Purpose: *Cell proliferation and extracellular matrix formation are primary events in bone formation. At the dental implant-tissue interface, implant surface roughness modulates osteoblast functions. The aim of the present in vitro study was to investigate the effect of varying surface roughness of titanium implant material on cell proliferation and mRNA expression of specific markers of osteoblast phenotype.* ***Materials and Methods:*** *Primary cultures of osteoblasts derived from human mandibular bone were cultured on titanium surfaces. Three titanium surfaces were studied: machined titanium, micro-sandblasted titanium, and macro-sandblasted titanium (average surface roughnesses of 0.5 and 3 μm, respectively). Cell morphology was estimated by scanning electron microscope analysis and cell proliferation by measuring the amount of ^{3}H-thymidine incorporation into DNA. mRNA expression of osteonectin, osteopontin, bone sialoprotein (BSP), and Runx2, which are markers of osteoblastic phenotype, were determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis.* ***Results:*** *Human osteoblasts cultured on machined titanium spread more and were flatter than cells cultured on rough titanium. All blasted surfaces showed significantly higher DNA synthesis than the machined surfaces. Osteonectin mRNA expression was similar on all surfaces. Other mRNA transcripts were increased in osteoblasts cultured on rough titanium surfaces, particularly the macro-sandblasted surface.* ***Conclusions:*** *An average surface roughness of 3 μm (macro-sandblasted titanium) is more suitable than an average surface roughness of 0.5 μm (micro-sandblasted titanium) in favoring osteoblast differentiation in vitro.* Int J Oral Maxillofac Implants 2006;21:719–725

Key words: *differentiation, human osteoblasts, proliferation, surface roughness, titanium implants*

Bone formation induced by osteoblast-like cells at the implant-tissue interface is a complex process involving a sequence of cellular functions such as attachment, migration and proliferation, expression of markers of osteoblast phenotype, and bone matrix mineralization. Systemic hormones and local factors regulate these events.

Implant surface features influence bone formation and maintenance at the interface and play a major role in vivo osseointegration.[1] In vivo and in vitro studies show surface topography affects cellular biological functions.[2,3] Reactions to topographical cues contribute to cell attachment, repair, and regeneration. To understand interactions at the dental implant surface, osteoblast cell cultures have been used to evaluate the effects of machined and rough surfaces on cell behavior and metabolism.[4]

Titanium implants with machined surfaces have been used longer than any other type of implant.[5] With its excellent biocompatibility, titanium permits good tissue integration, but concerns regarding the use of these surfaces in low-density bone have promoted new surface treatments of implants. Reports of the effects of titanium surface modifications on osteoblast differentiation and metabolism are somewhat contradictory. Some in vivo and in vitro studies

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suggest that surface roughness, cellular attachment, and osteoblast activity are directly correlated.[6] Initial stability is more likely to be achieved with rough-surfaced implants, and bone-to-implant interface shear strength correlates positively with surface roughness.[7,8] However, Anselme and associates[9] reported that cellular proliferation decreased as surface roughness increased, while Mustafa and colleagues[6] demonstrated that proliferation and differentiation were enhanced by surface roughness. This suggests the titanium surface not only regulates bone growth but also osteoblast differentiation by modulating the expression of key osteoblast genes in osteogenesis.[10]

For the current study, the authors tested the hypothesis that the microtopography of roughened implant surfaces affects cell proliferation and Runx2 type II gene expression as well as transforming growth factor β_2 ($TGF\beta_2$) production. $TGF\beta_2$ is a potent regulator of osteoblast proliferation and differentiation and of extracellular matrix production.[11,12] The transcription factor Runx2 type II is essential for bone formation and osteoblast differentiation.[13] It increases gene expression of osteocalcin, osteopontin, alkaline phosphatase, and collagen type I and is upregulated by $TGF\beta$.[14] The present study investigated the effects of titanium with varying degrees of surface roughness on human mandibular bone cells and evaluated the impact of these surfaces on cell morphology, cell proliferation, and osteoblast differentiation.

MATERIALS AND METHODS

Materials

Grade 5 titanium alloy disks measuring 25 mm in diameter and 5 mm in thickness were prepared by the standard turning process.[6] The titanium surfaces were turned using a cutting tool. The cutting speed was approximately 30 m/min, and it was kept constant from the periphery to the center of the disks by varying the rotational speed of the titanium bar.

The surfaces were then blasted with Al_2O_3 particles of different grain sizes to produce 2 different surface roughnesses. The air pressure was 2.5 bar during the micro-sandblasting process and 3 bar during the macro-sandblasting. A total of 90 disks were produced and classified into 3 groups according to surface topography:

- 30 disks were left as machined, ie, turned surface (control)
- 30 disks were blasted with Al_2O_3 particles with a grain size of 50 μm (micro-sandblasted surface)
- 30 disks were blasted with Al_2O_3 particles with a grain size of 350 μm (macro-sandblasted surface)

After surface preparation, all disks were decontaminated as described by Mustafa and coworkers.[6] Briefly, disks were cleaned ultrasonically for 10 minutes in alkaline detergent, put in a nitric acid (HNO_3) bath 6.5% diluted for 1 hour, cleaned ultrasonically again for 10 minutes in alkaline detergent, rinsed in running water, put in a thermal disinfector at 90°C for 10 minutes, and then sterilized using the gamma ray method.

Surface Roughness Characterization

The titanium disks were analyzed qualitatively using a Quanta 200 scanning electron microscope (SEM; Fei, Hillsboro, OR) as described by Mustafa and associates.[6] Surface roughness was determined quantitatively using a surface roughness detector model SJ-201 (Mitutoyo, Kawasaki, Japan). This surface roughness detector takes a series of readings by means of a diamond point. Segments 12.5 mm in width were scanned in a series of samples, as recommended by EN-ISO 13565 (DIN 4776). Roughness was defined as the mean of the peak-valley distance on surface irregularities. Micro-sandblasted titanium surfaces had an average roughness of 0.5 μm; macro-sandblasted surfaces had an average roughness of 3 μm.

Cell Cultures

After the protocol was approved by the University of Perugia Review Board, human bone cells were obtained from jaw fragments taken from 4 young, healthy, human subjects during orthodontic surgical extractions. Human jaw fragments were cultured in sterile polystyrene Falcon flasks (Becton Dickinson, Lincoln Park, NJ) using Eagle's minimal essential medium (MEM, Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, UK), antibiotics and amphotericin B (Gibco). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. Subcultures were obtained 20 to 30 days later. All tests were performed at the fourth or fifth subculture. Human bone cells were identified as previously described.[15]

Cultures on Titanium Substrates

Human bone cells were collected and seeded at a density of 1×10^6 cells/mL in 9 cm^2 wells containing sterile titanium disks (machined titanium, macro-sandblasted, or micro-sandblasted titanium). After 24 hours (subconfluent cultures) or 48 hours (confluent cultures) in MEM supplemented with 10% FCS, the disks were transferred to new wells containing 3 mL of MEM and observed with an SEM for cellular morphological analysis and used for ^{3}H-thymidine incorporation, $TGF\beta_2$ assay, and RNA extraction.

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Morphological Analysis

Subconfluent human bone cells cultured on sterile titanium disks were maintained for 24 hours in MEM. Cells were then fixed in 1.5% glutaraldehyde in phosphate buffer solution (PBS) for 15 minutes at room temperature, washed 3 times in PBS, and dehydrated stepwise in a series of ethanols. For SEM examination, after critical point drying using the freon method, samples were coated with gold-palladium (60:40) by vacuum evaporation on a moving stage and viewed under a 501 Philips SEM (Philips, Eindhoven, Holland). The acceleration voltage was 15 KV. Resolution power was 3.4 nm.

^{3}H-thymidine Incorporation

Human osteoblasts, at a density of 1×10^6 cells/mL, were cultured on sterile titanium disks for 24 and 48 hours in MEM containing 1 μCi/mL of ^{3}H-thymidine (Amersham International, Little Chalfont, England; s.a. 13.4 Ci/mmol). After incubation, the medium was discarded and cells were solubilized in 0.5 mol/L sodium hydroxide (NaOH). An aliquot of cell lysate was precipitated with 10% trichloroacetic acid (TCA) (30 minutes at 4°C), filtered into glass fiber 0.45 μm Millipore filters (Millipore, Milan, Italy), and washed with cold 1% TCA. The filters holding the acid-insoluble fraction were dried and counted in 10 mL of InstaGel scintillation fluid (Packard, Meriden, CT) in a Packard 2425 scintillation counter (TCA insoluble fraction). Results were expressed as cpm/mg protein.[16]

Preparation of Conditioned Media

Confluent bone cells cultured on sterile titanium dishes were washed with saline solution and cultured for 12 hours in serum-free MEM. This medium was discarded to avoid contamination by seric factors. Cells were cultured for the next 48 hours in MEM alone. After the addition of phenylmethylsulphonylfluoride, conditioned medium (CM) was collected, centrifuged for 10 minutes at 350 *g* to remove cell debris, dialyzed, lyophilized, and used for the TGFβ_2 assay.[17]

TGFβ_2 Assay

Enzyme-linked immunosorbent assay (ELISA) analysis (R&D Systems, Minneapolis, MN) of the CM was carried out according to the manufacturer's instructions. A standard curve was run to determine TGFβ_2 concentration.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Confluent bone cells, cultured for 12 days on titanium disks in the presence of MEM plus 10% FCS, were lysed by adding 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted and quantified by reading the optical density at 260 nm on a BioPhotometer (Eppendorf, Hamburg, Germany). Two micrograms of total RNA were reverse transcribed to cDNA for 60 minutes at 37°C using 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) per reaction with an oligo-dT primer (Invitrogen) in a final volume of 25 μL. After RT, 2 μL of the cDNA was amplified with 25 polymerase chain reaction (PCR) cycles. Primer sequences used for glyceraldehyde phosphate dehydrogenase (GAPDH), bone sialoprotein (BSP), osteonectin, osteopontin and Runx2 (type II) are: sense 5′-AGC CGC ATC TTC TTT TGC GTC-3′ and antisense 5′-GCA TGG ACT GTG GTC ATG AGT-3′ for GAPDH (used as an internal control for each set of conditions); sense 5′-ATT GAA AAC GAA AGC GAA G-3′ and antisense 5′-ATC ATA GCC ATC GTA GCC TTG T-3′ for BSP; sense 5′-TCT CTC TTT AAC CCT CCC C-3′ and antisense 5′-CCG ATT CAC CAA CTC CAC T-3′ for osteonectin; sense 5′-AAG CGA GGA GTT GAA TGG-3′ and antisense 5′-GGA AAG TTC CTG ACT ATC-3′ for osteopontin; and sense 5′-ATG CTT CAT TCG CCT CAC AAA C-3′ and antisense 5′-AGT CCC TCC TTT TTT TTT CAG-3′ for Runx2 (type II). These primers yielded the following products: 587 base pair (bp) for GAPDH; 450 bp for BSP; 853 bp for osteonectin; 220 bp for osteopontin; and 412, 246, and 212 bp for Runx2 (type II). All primers were obtained from Invitrogen. PCR was performed using a thermal cycler (Hybaid; Promega, Madison, WI) under the following conditions: denaturation program (94°C, 2 minutes), amplification and quantification program (94°C, 15″; 58°C for GAPDH and Runx2, 56°C for osteonectin, 51°C for osteopontin, and 49°C for BSP, 20″; 72°C, 30″; followed by 72°C for 5 minutes). Equivalent aliquots of each amplification reaction were electrophoresed on a 1% agarose gel in 1× tris-acetate-EDTA (TAE) buffer containing ethidium bromide and photographed under ultraviolet light. A 100 bp DNA ladder was used as standard (Invitrogen) to confirm the sizes of the fragments. Gels were analyzed by computerized scanning densitometry. The absolute counts, obtained by densitometric analysis, were normalized to GAPDH mRNA levels.

Protein Determination

Cells were collected and lysed, and protein concentrations were determined by Lowry assay using aliquots of cell lysate.[18]

Statistical Analysis

Results in the tables are the means ± standard deviation (SD) of 3 separate experiments, performed in quadruplicate. The results presented in the figures are the means ± SDs of 4 separate experiments. Statistical analysis was performed by analysis of variance (ANOVA) followed by the Scheffé test. Differences were considered significant at $P < .05$.

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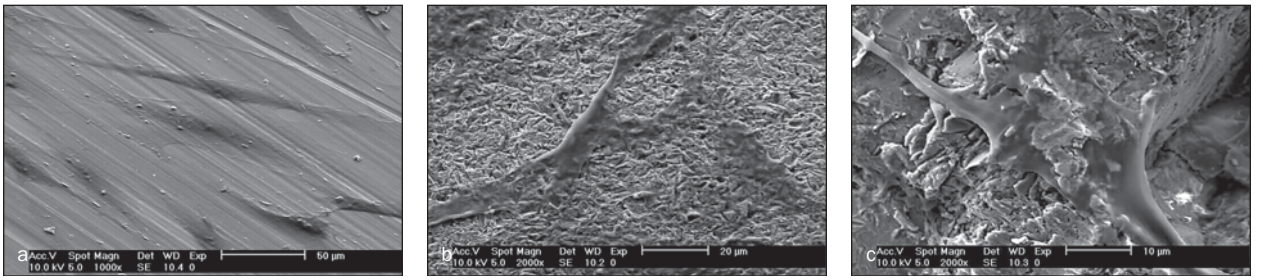

Fig 1 SEM micrograph of human mandibular osteoblasts cultured for 24 hours in MEM on *(a)* machined titanium (original magnification ×1,000); *(b)* micro-sandblasted titanium (original magnification ×2,000), and *(c)* macro-sandblasted titanium surface (original magnification ×2,000).

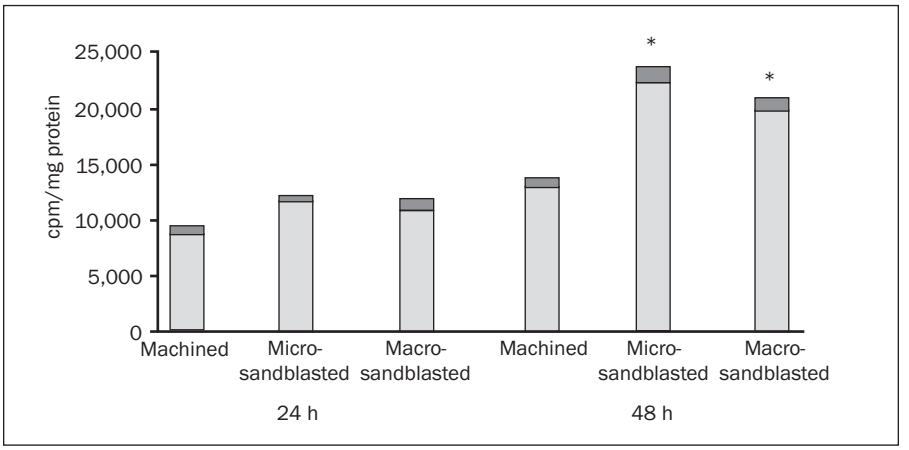

Fig 2 ^{3}H-thymidine incorporation in human mandibular osteoblasts cultured on disks of machined titanium (control), micro-sandblasted titanium, and macro-sandblasted titanium for 24 and 48 hours in MEM. Values are expressed as the means ± SDs of 3 separate experiments carried out in quadruplicate. Data were analyzed by ANOVA. *Significant difference compared with machined titanium (F test; significant at 99% level).

RESULTS

Scanning Electronic Microscopy

On machined titanium, SEM images show that the cells were irregularly triangular or elongated in shape. They were primarily oriented along the grooves and appeared flattened, with some long protoplasmic processes that were well attached to the substrate (Fig 1a). The thinness of cells reveals underlying titanium surface irregularities and cytoskeletal elements.

Osteoblasts cultured on micro-sandblasted titanium disks also adhered well to the substrate, although, because of surface irregularities, the cells were not as thin as the cells cultured on machined titanium (Fig 1b). Osteoblasts raised above the substrate emitted protoplasmic processes to facilitate anchorage.

On macro-sandblasted titanium disks, which were more irregular in surface than the micro-sandblasted disks, osteoblasts did not adhere homogeneously to the substrate. Osteoblasts formed cytoplasmic bridges of varying thickness, which were suspended above depressions in the substrate (Fig 1c).

Cell Proliferation

The effect of machined and rough titanium surfaces on human osteoblasts was evaluated by measuring DNA synthesis after 24 and 48 hours of in vitro maintenance in the presence of ^{3}H-thymidine. The results are summarized in Fig 2. Compared to osteoblasts grown on machined titanium, significant increases in the level of radioactivity incorporated into DNA were seen in osteoblasts grown on micro-sandblasted titanium (+33% after 24 hours and +72% after 48 hours of in vitro maintenance) and in osteoblasts grown on macro-sandblasted titanium (+24% after 24 hours and +53% after 48 hours of in vitro maintenance). No significant differences emerged between micro- and macro- sandblasted titanium surfaces.

TGFβ_2 Assay

TGFβ_2 secretion, assayed using an ELISA kit, was 6.3 pg/micrograms protein in CM from human bone cells cultured on machined titanium (Fig 3). A small increase was observed in bone cells cultured on micro-sandblasted surface (1.19-fold), and a significant increase was observed in those cultured on the macro-sandblasted titanium surface (2.4-fold).

RT-PCR analysis

Human osteoblasts were cultured for 12 days on machined and rough titanium disks and mRNA levels of osteonectin, osteopontin, Runx2 type II, and BSP were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). GAPDH mRNA levels served as controls. Relative densitometric units were normalized to GAPDH mRNA levels.

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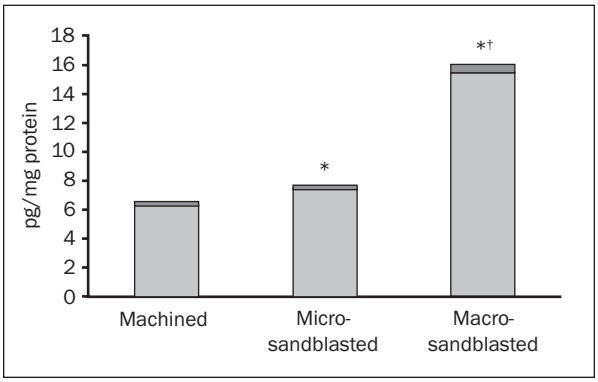

Fig 3 TGFβ_2 concentration in CM from human bone cells cultured on disks of machined titanium (control), micro-sandblasted titanium, and macro-sandblasted titanium for 24 hours, as quantified by an ELISA kit. Means ± SDs of 3 determinations, each in quadruplicate. The statistical analysis was performed with ANOVA. *Significant difference compared with machined titanium (F test; significant at 99%). †Significant difference compared with micro-sandblasted titanium (F test; significant at 99%).

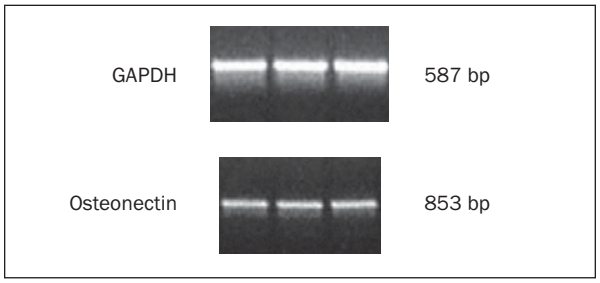

Fig 4 Expression of osteonectin mRNA in osteoblasts cultured on machined, micro-sandblasted, and macro-sandblasted titanium surfaces. Equal aliquots of total mRNA were analyzed by RT-PCR technique. Lane 1: osteoblasts cultured on machined titanium; lane 2: osteoblasts cultured on micro-sandblasted titanium; lane 3: osteoblasts cultured on macro-sandblasted titanium.

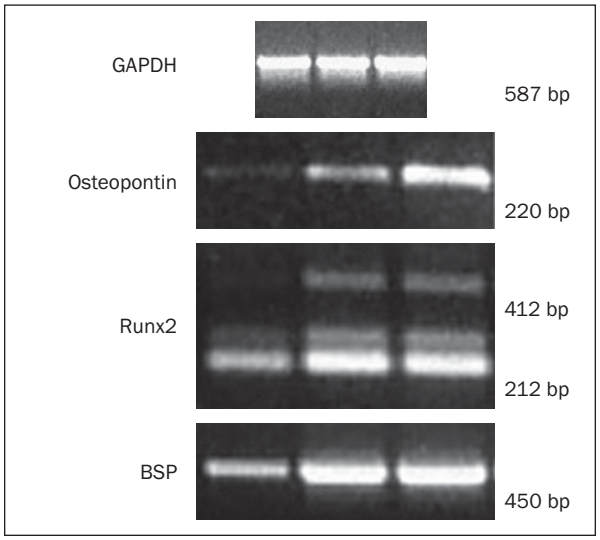

Fig 5 Expression of osteopontin, Runx2, and BSP mRNA in osteoblasts cultured on machined, micro-sandblasted, and macro-sandblasted titanium surfaces. Equal aliquots of total mRNA were analyzed by RT-PCR technique. Lane 1: osteoblasts cultured on machined titanium; lane 2: osteoblasts cultured on micro-sandblasted titanium; lane 3: osteoblasts cultured on macro-sandblasted titanium.

No differences in osteonectin mRNA expression were observed (Table 1; Fig 4). Osteopontin and BPS steady-state levels were higher (about 1.6- to 2.6-fold) in osteoblasts grown on rough surfaces than in those grown on machined surfaces (Table 1; Fig 5). Moreover, the mRNA osteopontin transcripts were significantly higher on macro- than on micro- sandblasted titanium surfaces, while BSP mRNA transcripts were similar on the two rough titanium surfaces.

Runx2 showed 2 bands. One band showed unspliced 412-bp mRNA, which completely retained intron-1; the other was due to an alternative splicing variant with a relative molecular weight of 212 bp. The micro-sandblasted titanium surface increased the 412-bp band 1.6-fold and the 212-pb band 1.4-fold. The macro-sandblasted surface increased the 2 bands 1.7- and 1.4-fold, respectively, compared with the machined titanium surface.

DISCUSSION

Tests assessing cell response to titanium surfaces make a major contribution to knowledge about osteogenesis and subsequent osseointegration. Several in vivo and in vitro studies have demonstrated that rough surfaces have a positive effect on cellular activity and implant survival compared with machined titanium surfaces.[1,6,19–22]

Table 1 Expression of Osteonectin, Osteopontin, Runx2, and BSP mRNA

	Machined titanium	Micro-sandblasted titanium	Macro-sandblased titanium
Osteonectin	75 ± 4.2	73 ± 3.4	68 ± 2.9
Osteopontin	39 ± 2.6	67 ± 3.6*	103 ± 6.8*†
Runx2			
412	41 ± 4.3	65 ± 4.1*	69 ± 5.2*
212	99 ± 7.8	135 ± 8.5*	138 ± 6.9*
BSP	76 ± 4.8	121 ± 7.1*	130 ± 7.8*

Means ± SD determined from 4 experiments analyzed by scanning densitometry and assuming as 100% the value of GAPDH mRNA. Data were analyzed by ANOVA.
*Significant difference compared with machined titanium (F test; significant at 99%).
†Significant difference compared with micro-sandblasted titanium (F test; significant at 95%).

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In the present study, human osteoblasts were cultured on machined titanium, micro-sandblasted titanium surfaces, and macro-sandblasted titanium surfaces, and cell morphology, cell proliferation, $TGF\beta_2$ secretion, as well as mRNA expression of several markers of osteoblast phenotype, were investigated. After 24 hours of in vitro culture, cell morphology was modified. The limited cell spreading and flattening of osteoblasts cultured on the rough titanium surfaces facilitated proliferation and, in fact, proliferation was significantly greater than in cells cultured on machined titanium surfaces. Osteoblasts distinguish 1 surface from another through variations in integrin expression, and greater integrin-mediated cell binding may be one of the mechanisms leading to greater bone integration.[23,24] Enhanced cell proliferation was not correlated with osteoblast dedifferentiation, as shown by increases in several markers that are typical of well-differentiated osteoblasts.[8]

Osseointegration involves bone remodeling at the implant surface. High concentrations of $TGF\beta_1$ and $TGF\beta_2$ can be extracted from mineralized bone matrix, and both isoforms are synthesized by osteoblasts and osteoclasts in vivo.[11,25] $TGF\beta_2$, in particular, increases osteoblast and osteoclast activity, with a consequent increase in bone turnover and remodeling.[12] In the light of these data, $TGF\beta_2$ secretion was evaluated. Results showed that surface topography alters cell production of $TGF\beta_2$, which was greater in the cells grown on sandblasted titanium, particularly in those grown on macro-sandblasted titanium. The increase in $TGF\beta_2$, a positive regulator of bone remodeling in vivo, may accelerate bone repair by coordinating osteoblast and osteoclast activities.

$TGF\beta_2$ upregulates expression of Runx2, a runt-related transcription factor-2 that is necessary for osteogenesis and the maintenance of osteoblast phenotype.[12,26] Three major isoforms of Runx2, designated as type I (starting with the sequence MRIPV), type II (starting with the sequence MASNS), and type III (starting with the sequence MLHSPH), have been identified.[13,27] Type I transcript is constitutively expressed in nonosseous mesenchymal tissue and in osteoblast progenitor cells. Type II, expressed during osteoblast differentiation, regulates mRNA transcripts of several osteoblastic genes, including alpha1(I) collagen, osteopontin, BSP, and osteocalcin.[28] Type III has been isolated from mouse osteoblasts but has not been found in human osteoblasts.[27,29,30] In the light of these data Runx2 type II was explored. Osteoblasts cultured on titanium surfaces showed 2 bands,[28,31] a minor band of 412 bp and a major band of 212 bp, and both bands were expressed more on rough than on machined titanium. These alternative splice variants in the Runx2 type II gene could potentially have different biologic effects, and the role of each in regulating osteoblast activity remains to be defined.

Differentiated osteoblasts are responsible for the synthesis and secretion of specific bone proteins, 90% of which are composed of type I collagen and 10% of which are noncollagenous. The most important are BSP, osteopontin, and osteonectin. BSP is associated with the collagenous matrix. As its expression begins in the differentiation stage before the onset of mineralization and continues into mineralization,[32] BSP might function as an epitactic nucleator of hydroxyapatite formation in bone. Osteopontin, a glycoprotein involved in the early organization of osteogenic tissue, is associated with the organic matrix prior to mineralization.[33] Osteonectin is a cystine-rich protein found in osteoblasts when most of the matrix has undergone mineralization.[33] Under the current experimental conditions, osteoblasts cultured on rough titanium had higher levels of BSP and osteopontin, but not osteonectin, indicating a more mature osteoblast phenotype. Interestingly, expression was greater on the macro-sandblasted titanium than on the micro-sandblasted titanium. These increases in osteopontin and BSP gene expression suggest that the Runx2 signaling cascade, and not just the Runx2 gene itself, is influenced by the microtopography of the implant surface.

CONCLUSIONS

The present in vitro study showed that the microtopography of rough and machined implant surface alters expression of osteoblast phenotype markers. Compared with a machined titanium surface, micro- and macro-sandblasted titanium surfaces increased secretion of $TGF\beta_2$, (a growth factor involved in osteoblast proliferation and differentiation), expression of Runx2 type II, mRNA (which regulates expression of osteoblast genes that are key players in mineralized phenotype development), BSP, and osteopontin, but not osteonectin.

Since BSP and osteopontin are highly expressed in the early stage of bone maturation, whereas osteonectin is mostly expressed late in osteogenesis, these findings concur in providing evidence of early-stage osteoblast differentiation on rough surfaces. Moreover, the results indicate that the macro-sandblasted titanium surface facilitated increased expression of BSPs and growth factors more than the micro-sandblasted surface.

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