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Superposition of nanostructures on microrough titanium–aluminum–vanadium alloy surfaces results in an altered integrin expression profile in osteoblasts

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Abstract

Recent studies of new surface modifications that superimpose well-defined nanostructures on microrough implants, thereby mimicking the hierarchical complexity of native bone, report synergistically enhanced osteoblast maturation and local factor production at the protein level compared to growth on surfaces that are smooth, nanorough, or microrough. Whether the complex micro/nanorough surfaces enhance the osteogenic response by triggering similar patterns of integrin receptors and their associated signaling pathways as with well-established microrough surfaces, is not well understood. Human osteoblasts (hOBs) were cultured until confluent for gene expression studies on tissue culture polystyrene (TCPS) or on titanium alloy (Ti6Al4V) disks with different surface topographies: smooth, nanorough, microrough, and micro/nanorough surfaces. mRNA expression of osteogenesis-related markers such as osteocalcin (BGLAP) and bone sialoprotein (BSP), bone morphogenetic protein 2 (BMP2), BMP4, noggin (NOG) and gremlin 1 (GREM1) were all higher on microrough and micro/nanorough surfaces, with few differences between them, compared to smooth and nanorough groups. Interestingly, expression of integrins $\alpha 1$ and $\beta 2$, which interact primarily with collagens and laminin and have been commonly associated with osteoblast differentiation on microrough Ti and Ti6Al4V, were expressed at lower levels on micro/nanorough surfaces compared to microrough ones. Conversely, the αv subunit, which binds ligands such as vitronectin, osteopontin, and bone sialoprotein among others, had

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Declaration of interest

The Smooth and Rough specimens were provided by Titan Spine LLC. RAG attendance at the 11th ICCBMT Conference 2013 to present these results was covered by Titan Spine LLC. BDB is a consultant for Titan Spine LLC.

higher expression on micro/nanorough surfaces concomitantly with regulation of the $\beta 3$ mRNA levels on nanomodified surfaces. These results suggest that the maturation of osteoblasts on micro/nanorough surfaces may be occurring through different integrin engagement than those established for microrough-only surfaces.

Keywords

Bone; integrin gene expression; metallic implants; nanostructures; osseointegration; surface properties

Introduction

The development of new surface modifications for titanium-based implants has continuously improved the clinical outcomes in dental and orthopaedic applications and extended the coverage for populations of compromised patients. Additionally, the development of hierarchically-structured surfaces that more closely mimic the natural environment of bone at the micro-, submicro- and nanoscale levels, has provided insight into the complexity of the biological response that is required to activate osteogenic pathways that, in turn, will result in enhanced bone formation leading to appropriate osseointegration (1,2).

Surface microroughness of titanium-based implants can enhance the osteogenic response of osteoblast lineage cells without the need for exogenous induction factors, and this response has been thoroughly evaluated in terms of protein and mRNA levels (3,4). In recent years, several studies have been published reporting the beneficial effects of adding nanostructures (which generally refers to features with at least one of their dimensions being less than 100 nm) to microrough implants *in vivo* (2). *In vitro* studies looking to explain such a positive outcome of combined micro/nanorough surfaces have found synergistic differentiation of osteoblasts focusing mainly on the protein production of osteogenic markers and other local factors (1). Few studies have looked at the underlying mechanisms eliciting the osteogenic response.

Integrins are receptors on the surface of osteoblasts that interact with the extracellular matrix and transfer both structural and chemical information of the environment to the cell. A direct link exists between specific integrin subunits and different cell stages, such as proliferation and differentiation (5). Although initial studies in the literature reported that integrin $\alpha 5 \beta 1$ was believed to control osteoblast attachment and differentiation, it was later revealed that the $\alpha 5$ subunit was mainly involved in regulating attachment without affecting differentiation on microrough titanium and titanium alloy (Ti6Al4V) surfaces (4). Instead, integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ were directly involved in osteoblast differentiation (6) and were upregulated on microrough surfaces (4).

Fundamental understanding of the interactions between osteoblasts, their integrins, and implant surfaces has allowed the design of new biomaterials to promote osteoblast differentiation (7). However, whether micro/nanorough surfaces enhance the osteogenic response by triggering similar patterns of integrin receptors and their associated signaling pathways, as with well-established microrough surfaces, is not well understood. In this

study, we evaluate osteogenic marker and integrin gene expression of human osteoblasts on microrough and micro/nanorough surfaces, as well as on microsmooth controls. Our results show that the osteogenic response by osteoblasts on micro/nanorough surfaces may involve an alternate pattern of integrin gene expression.

Methods

Specimen production and surface modification

Rods of titanium alloy (ASTM F136 wrought Ti6Al4V extra low interstitials [ELI] alloy for surgical implant applications) with a diameter of 15 mm were cut into 1.5 mm thick disks and either machined to create control specimens with a relatively smooth surface (referred to herein as Smooth specimens) or double-acid-etched (proprietary process, Titan Spine LLC, Mequon, WI) to produce a microrough surface (referred to herein as Rough specimens). These disk specimens were provided by Titan Spine LLC. Some of the smooth and microrough specimens were further processed with a simple oxidation treatment to superpose nanostructures on the surface, as described previously (1), to yield nanorough or micro/nanorough specimens (referred to herein as nSmooth and nRough specimens, respectively). All nanomodified and unmodified disks were ultrasonically cleaned in detergent (Micro-90; International Products Corporation, Burlington, NJ) and ultrapure water (Advantage A10; Millipore, Billerica, MA), followed by autoclave sterilization (Model 2540E; Tuttnauer, Hauppauge, NY) for 20 min at 121 °C and 15 pounds per square inch before cell culture studies.

The different surfaces have been thoroughly characterized previously (8). In brief, confocal laser microscopy (CLM) was used to evaluate the initial surface microroughness of the specimens (Smooth, $S_a = 0.27 \pm 0.01 \mu\text{m}$; Rough, $S_a = 2.20 \pm 0.42 \mu\text{m}$; nSmooth, $S_a = 0.42 \pm 0.01 \mu\text{m}$; nRough, $S_a = 2.08 \pm 0.27 \mu\text{m}$). Atomic force microscopy (AFM) analysis revealed that the nanomodification increased the average nanoscale surface roughness (Smooth, $S_a = 6.1 \pm 4.3 \text{ nm}$ versus nSmooth, $S_a = 17.0 \pm 4.5 \text{ nm}$) without greatly affecting the microroughness or the surface wettability. The elemental composition of all groups was similar but the nanomodification changed the percentage of these elements at the surface, as well as the crystal structure from an amorphous oxide layer (in the case of unmodified Smooth and Rough specimens) to one containing a combination of anatase and rutile TiO_2 (in the case of nanomodified nSmooth and nRough specimens).

Cell culture model

Primary human osteoblasts (hOBs) were isolated from vertebral bone of a 17-year-old male that was collected under Institutional Review Board approval from Children's Healthcare of Atlanta and Georgia Institute of Technology, as described previously (9). hOBs were allowed to migrate from the bone fragments to the culture plate and, at confluence, the cells were further passaged for experiments and were cultured in Dulbecco's modified Eagle medium (DMEM; Corning cellgro®, Manassas, VA) containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Life Technologies). Cells were cultured at 37 °C with 5% CO_2 and 100% humidity. Cells from the sixth passage or lower were used. Osteoblasts were cultured on tissue culture polystyrene (TCPS) or on

the different Ti alloy surfaces (Smooth, nSmooth, Rough, nRough) at a seeding density of 10 000 cells/cm². Cells were fed 24 h after plating and then every 48 h until confluence, as evaluated on the TCPS substrates. At confluence, cells were incubated with fresh medium for 12 h and harvested for mRNA expression levels.

Gene expression studies

RNA was isolated using TRIzol[®] (Life Technologies) following the manufacturer's protocol. Equal amounts of 500 ng RNA for each sample were reverse transcribed into cDNA (High Capacity cDNA Kit, Life Technologies). The resulting cDNA was used in real-time PCR reactions with *Power SYBR[®] Green* (Life Technologies). mRNA quantities for osteogenic genes BGLAP, BSP, BMP2, BMP4, GREM1 and NOG, and integrin subunits α 1, α 2, α 5, α v, β 1, and β 3 were calculated using standard curves generated from known dilutions of hOBs and normalized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Gene specific primers (Table 1) were designed using Beacon Designer Software (PREMIER Biosoft, Palo Alto, CA) and synthesized by Eurofins MWG Operon (Huntsville, AL). mRNA levels for the genes of interest were determined by real-time quantitative polymerase chain reaction (qPCR), as described previously (4).

Statistical analysis

Data from experiments examining cellular gene expression are presented as mean $\pm$ standard error (SE) for six individual cultures per variable ($n = 6$). All experiments were independently repeated at least twice to ensure the validity of the observations, and the results from one of the experiments are presented. Data were evaluated by analysis of variance, and significant differences between groups were determined using Bonferroni's modification of Student's *t*-test. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of osteogenic markers, local factors

The superposition of nanostructures on smooth and microrough TiAlV alloy surfaces influenced gene expression of different osteogenic markers; that is, gene expression was sensitive to the morphology of the substrate. mRNA levels for osteocalcin (BGLAP) and bone sialoprotein (BSP) were higher on all TiAlV surfaces compared to TCPS (Figure 1). In addition, BGLAP and BSP mRNA levels were similar between Rough (microrough only) and nRough (micro/nanorough) surfaces, which were significantly higher than for Smooth and nSmooth (nanorough only) surfaces. mRNA levels for BMP2 and BMP4, as well as their inhibitors noggin (NOG) and gremlin 1 (GREM1), were all higher on Rough and nRough surfaces compared to Smooth and nSmooth groups. Only BMP2 and NOG levels were higher on nSmooth compared to its Smooth control, with highest NOG expression on nRough surfaces compared to all other groups.

Differential profile of integrin gene expression on micro/nanorough surfaces

The profile of integrin gene expression changed on micro/nanorough surfaces (Figure 2). Expression of integrin subunits α 1, β 2 (both bind to collagen I and laminin), α v (binds to

vitronectin, osteopontin and bone sialoprotein among other ligands) and $\beta 1$ (partner of $\alpha 1$, $\alpha 2$, and other α subunits) was higher and expression of $\alpha 5$ (binds to fibronectin) was lower on Rough and nRough surfaces compared to Smooth and nSmooth groups. Integrin $\beta 3$ (partner of αv) was only regulated on nanomodified surfaces (nSmooth, nRough specimens), with the highest levels found on the nSmooth group. nSmooth surfaces also had lower expression of mRNAs for $\alpha 2$ and considerably higher expression of mRNAs for αv integrin subunits, compared to unmodified Smooth controls. Interestingly, mRNAs for integrins $\alpha 1$ and $\alpha 2$ expressed at lower levels on nRough surfaces compared to their Rough counterparts. Conversely, expression of mRNA for αv was significantly higher on nRough surfaces concomitantly with regulation of the $\beta 3$ mRNA levels on nanomodified surfaces (nSmooth, nRough specimens).

Discussion

Integrins play an important role in the process of osteogenic differentiation by interacting with the proteins in the surrounding extracellular matrix (ECM) and activating diverse signaling pathways (5). Previous work from our group had shown that osteoblast differentiation and local factor production on microrough titanium surfaces was dependent on the expression of integrin $\alpha 2\beta 1$ (4). $\alpha 2\beta 1$ and $\alpha 1\beta 1$ bind to motifs in collagen type I and laminin, and have been implicated in osteoblast differentiation and maturation (6). In the present study, we showed that microrough only surfaces (Rough specimens) promote the highest expression of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$, while the combined micro/nanorough surfaces (nRough specimens) do so to a lesser extent. Instead, micro/nanorough surfaces promote the expression of αv and $\beta 3$ subunits and enhance the osteogenic response of human osteoblasts.

In a previous study by our group, osteoblasts were shown to mature synergistically on the same micro/nanostructured surfaces in terms of higher production of osteogenic proteins and other paracrine growth factors (8). The increase in levels of osteoblast differentiation markers and local factors suggested this response on micro/nanostructured surfaces happened through a different gene expression pattern than that of microrough-only surfaces. Interestingly, no differences were evident in the present work at the mRNA level for the osteogenesis-related markers BGLAP and BSP. Correlations between the levels of transcribed mRNA and translated protein have been hard to establish in cellular systems because of the diverse regulatory mechanisms controlling the post-transcriptional and post-translational machineries (10), which might help explain the differences between our mRNA results and the protein levels found in our previous study (8). However, an enhancement was clear on microrough surfaces, with or without nanomodification, compared to microsmooth ones, in agreement with the literature (3).

Gene expression of osteogenic factors BMP2 and BMP4, and their inhibitors NOG and GREM1, was also similar between Rough and nRough surfaces. NOG had the highest levels on micro/nanorough surfaces compared to all other groups. BMP2 is produced endogenously by osteoblasts, if given the right surface properties, and can strongly influence bone formation but at the same time elicit deleterious effects including inflammation and ectopic bone formation (11). Thus, a concomitant production of its potent antagonist NOG is

required to orchestrate normal bone development and to regulate osteoblast differentiation and apoptosis (11). The higher NOG expression on the nRough surfaces suggests that a stronger osteogenic environment may be promoted on these surfaces, which is supported by our previous results at the protein level (8).

The enhanced osteoblastic differentiation promoted by the combined micro/nanorough surfaces was particular in that mRNA levels of different integrin subunits presented an alternate pattern of expression than on microrough surfaces, which could explain the results. Expression of integrins $\alpha 1$ and $\alpha 2$ on nRough surfaces was higher compared to Smooth and nSmooth controls, but to a lesser extent than on Rough surfaces. Other studies have shown a direct involvement of these integrin subunits coupled to integrin $\beta 1$ in osteoblast differentiation and maturation on microstructured surfaces, in contrast to other subunits like $\alpha 5$, which has been linked to cell attachment (4) and, in this case, was down-regulated on the Rough and nRough surfaces. Surprisingly, the integrin subunit with the highest up-regulation on nRough surfaces was integrin αv . This seemed to be an additive effect caused by the nanomodification, because a similar relative increase was found on the nSmooth surfaces compared to their unmodified Smooth controls. It is worth noting that the only surfaces that saw any change in the expression of $\beta 3$, a partner of αv , were the nanostructured (nSmooth, nRough specimens) surfaces, while Rough surfaces remained at the same $\beta 3$ level as for Smooth and TCPS surfaces. Information on the modulation of integrin $\alpha v \beta 3$ by implant surface properties is scarce and we believe $\beta 3$ may serve as a sensitive marker for the osteogenic capability of our nanomodification, considering that the integrin $\alpha v \beta 3$ has been related to osteogenic differentiation (6,12).

Conclusions

In the present study, we evaluated osteoblast gene expression of osteogenic markers and integrins on TiAlV surfaces with different micro- and nanomodified topographies. mRNA levels of osteogenic markers were similar between microrough and micro/nanorough surfaces except for NOG expression, which was significantly enhanced on the latter suggesting a stronger osteogenic environment on these surfaces. At the same time, the nanomodification triggered a different integrin expression profile than the unmodified controls. The combined micro/nanorough surfaces exhibited the highest mRNA levels of integrin αv with a concomitant regulation of the $\beta 3$ subunit on the nanomodified surfaces, suggesting that this integrin heterodimer is important for osteogenic differentiation and bone formation on nanostructured surfaces.

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Figure 1.

Human osteoblast mRNA levels of different osteogenic markers when grown on TiAlV surfaces with different topographies. Osteoblasts were grown on TCPS, smooth TiAlV (Smooth), rough TiAlV (Rough), or nanomodified surfaces (nSmooth, nRough) for 7 d and mRNA levels measured by real-time PCR ($n = 6$ independent cultures/variable). *Refers to a statistically-significant p value below 0.05 versus TCPS; #refers to a statistically-significant p value below 0.05 versus Smooth; \$refers to a statistically-significant p value below 0.05 versus nSmooth; @refers to a statistically-significant p value below 0.05 versus Rough.

Figure 2.

Human osteoblast mRNA levels of different integrin subunits when grown on TiAlV surfaces with different topographies. Osteoblasts were grown on TCPS, smooth TiAlV (Smooth), rough TiAlV (Rough), or nanommodified surfaces (nSmooth, nRough) for 7 d and mRNA levels measured by real-time PCR ($n = 6$ independent cultures/variable). *Refers to a statistically-significant p value below 0.05 versus TCPS; #refers to a statistically-significant p value below 0.05 versus Smooth; \$refers to a statistically-significant p value below 0.05 versus nSmooth; @refers to a statistically-significant p value below 0.05 versus Rough.

Table 1

Primer sequences used for real-time PCR analysis of gene expression.

Gene	Primer	Sequence	Accession number
GAPDH	F	GCT CTC CAG AAC ATC ATC C	NM_002046.3
	R	GCG AGC ACA GGA AGA AGC	
BGLAP	F	GTG ACG AGT TGG CTG ACC	NM_199173
	R	TGG AGA GGA GCA GAA CTG G	
BSP	F	AAC CAC CAC CGT TGA ATA C	NM_004967
	R	CCA TCA TAG CCA TCG TAG C	
BMP2	F	GCG TGA AAA GAG AGA CTG C	NM_001200
	R	CCA TTG AAA GAG CGT CCA C	
BMP4	F	ACG GTG GGA AAC TTT TGA TGT G	NM_130850
	R	CGA GTC TGA TGG AGG TGA GTC	
GREM1	F	GCA GGG TGG GTG AAC TTT ATT G	NM_013372
	R	AGG AGG CTG AGA AGA TAC AAG G	
NOG	Hs_NOG_1_SG QuantiTect primer assay, QT00210833		NM_005450
ITGA1	F	CACTCGTAAATGCCAAGAAAAG	NM_181501.1
	R	TAGAACCCAACACAAAGATGC	
ITGA2	F	AGTTTTCCGATGCTCCTATGAA	NM_002203
	R	CTTCATGCAGAAGTCAGGTGAG	
ITGA5	F	ATC TGT GTG CCT GAC CTG	NM_002205
	R	GGT CAA AGG CTT GTT TAG G	
ITGAV	F	GTTGCTACTGGCTGTTTGG	NM_002210.2
	R	CTGCTCCCTTCTTGTTCCTC	
ITGB1	F	ATT ACT CAG ATC CAA CCA C	NM_002211
	R	CTGCTCCCTTCTTGTTCCTC	
ITGB3	F	AAT GCC ACC TGC CTC AAC	NM_000212
	R	TCC TCC TCA TTT CAT TCA TC	