Osteogenic response of human MSCs and osteoblasts to hydrophilic and hydrophobic nanostructured titanium implant surfaces

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Abstract: Microstructured implant surfaces created by grit blasting and acid etching titanium (Ti) support osseointegration. This effect is further enhanced by storing in aqueous solution to retain hydrophilicity, but this also leads to surface nanostructure formation. The purpose of this study was to assess the contributions of nanostructures on the improved osteogenic response of osteoblast lineage cells to hydrophilic microstructured Ti. Human mesenchymal stem cells (MSCs) and normal human osteoblasts (NHOst) were cultured separately on non-nanostructured/hydrophobic (SLA), nanostructured/hydrophilic (modSLA), or nanostructured/hydrophobic (SLAnano) Ti surfaces. XPS showed elevated carbon levels on SLA and SLAnano compared to modSLA. Contact angle measurements indicated only modSLA was hydrophilic. Confocal laser microscopy revealed minor differences in mean surface roughness. SEM showed the presence of nanostructures on modSLA and SLAnano. MSCs and NHOst cells exhibited similar morphology on the substrates and osteoblastic differentiation and maturation were greatest on modSLA. These results suggest that when the appropriate microstructure is present, hydrophilicity may play a greater role in stimulating MSC and NHOst osteoblastic differentiation and maturation than the presence of nanostructures generated during storage in an aqueous environment.

Key Words: titanium, nanostructures, hydrophilic, osteoblast

INTRODUCTION
Successful dental implantation is measured by the degree of osseointegration between the surface of the implant material and bone tissue.1-2 Surface modifications altering implant topography3-4 and energy5-6 on titanium (Ti) and Ti-based alloys7-8 have been heavily emphasized in current research efforts in order to overcome problems associated with osseointegration. Surface roughness at the microlevel enhances osteoblast differentiation and local factor production9-14 by mimicking resorption pit structural features.15 Microscale and submicroscale features are sensed by osteoblast lineage cells via integrin alpha-1, beta-1 (α1β1), and alpha-2, beta-1 (α2β1) signaling.16-19 leading to osteoblast differentiation of mesenchymal stem cells (MSCs)10 and maturation of committed osteoblasts (NHOst cells)19 and osteoblast-like MG63 cells.17 Recent studies have suggested that nanostructured topography provides a surface structure much more analogous to that of natural bone.5,20-24 Nanoscale surface features mimic the extracellular matrix with which cells normally interact, thereby influencing the type, quantity, and adsorbed protein conformation, integrin signaling, and signaling pathways that work together to control cellular adhesion, proliferation, and differentiation.55 However, many studies examining the role of nanotopography have used patterned tissue culture polystyrene (TCPS) as the substrate,26-28 which is very different from materials used clinically such as Ti. Thus, it is difficult to extrapolate the reported findings to implant surface effects.

Hydrophilic (high energy) implant surfaces have also been shown to improve clinical outcomes,5,6 particularly when the surface is microtextured.18 Recently it was shown that when grit blasted/acid etched Ti implants were stored in water or saline to retain their hydrophilic surface post-processing, nanostructures formed on the surface due to reorganization of the outermost oxide layer.29 It is not clear what contribution the nanostructures make to the improved osseointegration seen with hydrophilic, microtextured Ti
implants. Nonetheless, many studies have suggested the importance of a nanostructured surface for facilitating osteoblast differentiation and maturation in vitro. Furthermore, an in vivo rabbit study by Wennerberg et al., which used disc implants, suggests that the presence of nanostructures is beneficial for new bone formation.

The hypothesis of the present study is that nanostructures generated by storage in saline will impact the overall biological response to hydrophilic, microstructured Ti. To do this, we cultured human MSCs and NH0st cells on Ti surfaces that were fabricated using the same methods employed for production of commercially available implants. Ti discs were grit blasted and acid etched, then stored in air (SLA); grit blasted, acid etched, and stored in saline (modSLA); or grit blasted, acid etched, stored in saline to generate nanostructures, then dried, and stored in air (SLAnano). This experimental design enabled comparison of hydrophilicity + nanostructures; hydrophobicity + nanostructures; and hydrophobicity without nanostructures.

MATERIALS AND METHODS

Ti disc preparation

5 mm diameter and 1 mm thick discs were fabricated from Grade 4 Ti and subjected to one of the following modifications.

SLA. Sand-blasted with large grit particulate (250–500 μm corundum) followed by acid etching in a boiling mixture of HCl and H2SO4. Discs were cleaned in HNO3, rinsed in ultrapure water, packed in aluminum foil, and γ-irradiated before use. modSLA. Same initial sandblasting and acid etching procedure as used for SLA samples, but subsequent steps took place under nitrogen to prevent exposure to air: The discs were rinsed, stored in 0.9% NaCl solution, and γ-irradiated before use.

SLAnano. modSLA samples that were removed from 0.9% NaCl solution after aging 5 weeks, a procedure outlined by Wennerberg et al. Once removed from solution, samples were rinsed in ultrapure water, packed in aluminum foil, and γ-irradiated before use.

Surface characterization

Scanning electron microscopy (SEM). Surface topography was qualitatively evaluated using SEM (Ultra60 FE-SEM, Zeiss). Six images at varying magnifications were captured on each of n = 3 discs per surface modification using 5 kV accelerating voltage for a total of 18 images per modification.

X-ray photoelectron spectroscopy (XPS). Chemical composition of the samples (n = 3) was obtained from the sample surfaces by XPS (Thermo K-Alpha XPS, Thermo Fisher Scientific). Spectra were collected using a 400 μm X-ray spot size at three different locations on each individual sample.

Contact angle measurement. Measurements were obtained using a goniometer (CAM 250, Ramé-Hart). Samples (n = 3) were measured in three different locations, and dried with nitrogen between each measurement. A drop volume of 2 μL was used per individual measurement and a contact angle was calculated every 5 for 20 s. The four measurements were then averaged together to obtain one of the three measurements per disc.

Confocal laser microscopy (CLM). Surface roughness of the samples (n = 3) was evaluated using a confocal laser microscope (Lext, Olympus). Three measurements per sample were taken over an area of 644 μm × 644 μm with a 20× objective and a scanning pitch of 50 nm. A cutoff wavelength of 100 μm was used when calculating surface roughness (Ss) and peak-to-valley height (Sp).

Cell culture

Human MSCs (Lonza Biosciences, Walkersville, MD) were cultured in MSC growth medium (MSCGM; Lonza Biosciences). NH0st cells (Lonza Biosciences) were cultured using Dulbecco’s modified Eagle medium (DMEM CellGro®; Mediatech, VA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) and 1% penicillin–streptomycin (Life Technologies). All cells were 4th passage and cultured at 37°C in 5% CO2 and 100% humidity. MSCs and NH0sts were cultured on unmodified/nanotextured tissue culture polystyrene (TCPS), SLA, SLAnano, or modSLA at a density of 10,000 cells/cm² in a 96-well plate. After 24 h, discs were transferred to a new 96-well plate with fresh medium and subsequently fed every 48 h until confluence.

Cell morphology

MSCs and NH0st cells were plated on to SLA, SLAnano, or modSLA at a density of 5,000 cells/cm² in a 96-well plate and media were changed after 24 h. Cells were cultured for 72 h, washed in PBS, and then fixed in 3.7% paraformaldehyde. After a 10 min fixation, surfaces were washed with PBS, and permeabilized with 0.05% Triton X-100 for 10 min. Surfaces were washed again with PBS, and incubated in a staining solution consisting of 0.165 μM Alexa-Fluor 488 phalloidin (Life Technologies) and 2 μg/mL Hoechst 33342 (Life Technologies) to visualize actin filaments and nucleic acids respectively. After staining, surfaces were mounted on glass cover slips using SlowFade (Life Technologies) and images were captured with a Laser Scanning Microscope (Zeiss LSM 710, Carl Zeiss SMT, Cambridge, UK) at an excitation of 488 nm for actin filaments and an excitation of 405 nm for nucleic acids using a 40× objective.

Biological response

At confluence, cells were incubated with fresh media for 24 h. Media were collected and ELISAs were used to measure levels of osteocalcin (Biomedical Technologies, Stoughton, MA), osteoprotegerin (R&D Systems, Minneapolis, MN), vascular endothelial growth factor-A (VEGF, R&D Systems), bone morphogenetic protein-2 (BMP2, PeproTech, Rocky Hill, NJ), interleukin 6 (IL6, R&D Systems), and interleukin 10 (IL10, R&D Systems) following manufacturer’s instructions. Cells attached to surfaces were washed twice...
with 0.2 mL PBS. Three 5 mm discs were combined in a total sample size of six (n = 6). Cells were lysed by sonication at amplitude 40 using an ultrasonicator (Vibra-Cell, Sonics, Newtown, CT).

DNA content in the cell lysate was measured (Quantifluor™ dsDNA system, Promega, Madison, WI) using a fluorosence detector (Synergy H1 Hybrid Reader, BioTek, Winooski, VT) at an excitation of 485 nm and emission of 538 nm. Immunoassay levels were normalized to DNA content. Cell lysate was also analyzed for alkaline phosphatase activity as the release of p-nitrophenol from p-nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO) at a pH of 10.25 and a temperature of 37°C. Absorbance was measured at 405 nm and alkaline phosphatase activity was quantified using a standard curve. Activity was normalized to total protein content in the cell lysates, as determined by bichinchonic acid protein assay kit (Thermo Fisher Scientific).

To quantify mRNA, cells were plated as described above. At confluence on TCPS, cells were incubated with fresh media for 12 h. Samples were harvested using a TRIzol® (Invitrogen, Carlsbad, California) extraction method. Six 5 mm discs were combined in TRIzol® for each individual sample in a total sample size of six (n = 6). RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and then reverse transcribed into 250 ng/μL cDNA. Real-time PCR was performed using a fluorescent dye (Power SYBR Green, Applied Biosystems, Foster City, CA) and StepOnePlus™ Real-Time PCR system (Applied Biosystems) to quantify starting mRNA levels using gene specific primers (Table I). Specific genes were amplified for 40 cycles and levels of mRNA were normalized to GAPDH.

**Statistical analysis**

Data from surface characterization experiments are presented as the mean ± standard error (SE). Data for surface analyses represent three measurements per disc, resulting in an n = 9 per variable. For cell morphology, no quantitative analyses were performed. All cell culture experiments had an n = 6 independent cultures for each variable. Experiments were repeated at least three times to ensure validity of the results. Data from individual experiments were not combined and data shown in the figures are from representative experiments. A one-way analysis of variance was performed followed by a Bonferroni correction to maintain an experiment-wise error rate (α) of 0.05. All statistical analyses were performed using JMP statistical software.

**RESULTS**

**Material characterization**

Low magnification SEM imaging [Fig. 1(A–C)] revealed a similar topography among SLA, SLAnano, and modSLA. Confocal microscopy confirmed these qualitative observations [Fig. 1(G–I)], resulting in no significant differences among the mean surface roughnesses (Sd) or the mean peak-to-valley height (Sz) of the SLA (Sd = 1.65 ± 0.01 μm; Sz = 23.17 ± 0.82 μm), SLAnano (Sd = 1.79 ± 0.03 μm; Sz = 25.55 1 ± 0.80 μm), and modSLA (Sd = 1.74 ± 0.01 μm; Sz = 21.88 ± 0.51 μm). At a higher SEM magnification, no nanostructures were observed on SLA [Fig. 1(D)] while similar needle-like nanostructures were observed on SLAnano [Fig. 1(E)] and modSLA [Fig. 1(F)]. Using ImageJ software (National Institutes of Health, USA), a morphometric analysis was conducted by superimposing a grid composed of 20 evenly spaced squares arranged into a 4 × 5 matrix on to the highest magnification SEM images. The diameter and peak distance to its closest neighbor were obtained for the centermost nanostructure within each square for a total of 60 measurements per material. Differences between SLAnano and modSLA mean nanostructure diameter (11.44 ± 0.38; 10.21 ± 0.34 nm, respectively) and mean nanostructure separation (18.87 ± 0.7; 12.95 ± 0.5 nm, respectively) were observed.

XPS survey spectra displayed titanium (Ti), oxygen (O), and carbon (C) as the main atomic components of the SLA, SLAnano, and modSLA surfaces [Fig. 1(I–L)]. The chemical composition was almost identical between SLA and SLAnano with spectral analyses indicating a high presence of Ti, O, and C. The spectral analysis for modSLA revealed a lower quantity of C compared to that detected on SLA and SLAnano. Measured contact angles are also shown in Figure 1. SLA [Fig. 1(M)] and SLAnano [Fig. 1(N)] had hydrophobic surfaces with contact angles of 126 ± 4° and 132 ± 4°, respectively while modSLA [Fig. 1(O)] had a hydrophilic surface with 0° contact angle.

**Cell morphology**

Figure 2 shows both the stained components of the MSCs and NHØst cells superimposed with the rough metallic surfaces of the SLA, SLAnano, and modSLA (actin—green; DNA—blue). Striations of the actin filaments and the nucleus can be visualized in each of the images. The actin filaments in both cell types adopted a parallel orientation that did not exhibit a preference for any particular

**TABLE I. Human Primers Used in Real-Time PCR Analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGα1</td>
<td>F CACTCGTAAATGCCAAGAAAAAG</td>
</tr>
<tr>
<td></td>
<td>R TAGAACCCCAACACAAAGATGC</td>
</tr>
<tr>
<td>ITGα2</td>
<td>F ACGTCTCAAGGGGAGGAC</td>
</tr>
<tr>
<td></td>
<td>R GGTCAAAGGCTTGTAGG</td>
</tr>
<tr>
<td>ITGα5</td>
<td>F ATCTGTGGCTGCTGCTG</td>
</tr>
<tr>
<td></td>
<td>R AAGTTCCCTGGGTGTCTG</td>
</tr>
<tr>
<td>ITGβ1</td>
<td>F ATTAATTGACATCCACAC</td>
</tr>
<tr>
<td></td>
<td>R TCCCTCTATTACATTCTAC</td>
</tr>
<tr>
<td>BMP4</td>
<td>F ACGTCTAAGGAGGCTAG</td>
</tr>
<tr>
<td></td>
<td>R CGAATCCTAAGAGGATG</td>
</tr>
<tr>
<td>BMP7</td>
<td>F ACGAGGCAACCAGGAGG</td>
</tr>
<tr>
<td></td>
<td>R ACAGATGGCACGCGTGAC</td>
</tr>
<tr>
<td>BMPR1A</td>
<td>F CAAGAGGCACTCCTAAGCAGCAG</td>
</tr>
<tr>
<td></td>
<td>R CAGACCCCTACACAGACCTCTG</td>
</tr>
<tr>
<td>NOG</td>
<td>QuantiTect primer assay, QT00210833</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F GCTCTGCAAGGACCATC</td>
</tr>
<tr>
<td></td>
<td>R TGGTCTACCACCATCTTG</td>
</tr>
</tbody>
</table>

**Statistical analysis**

Data from surface characterization experiments are presented as the mean ± standard error (SE). Data for surface analyses represent three measurements per disc, resulting in an n = 9 per variable. For cell morphology, no quantitative analyses were performed. All cell culture experiments had an n = 6 independent cultures for each variable. Experiments were repeated at least three times to ensure validity of the results. Data from individual experiments were not combined and data shown in the figures are from representative experiments. A one-way analysis of variance was performed followed by a Bonferroni correction to maintain an experiment-wise error rate (α) of 0.05. All statistical analyses were performed using JMP statistical software.
structure. Nuclei and the immediate surrounding actin filaments mostly avoid the valleys (dark areas) of the rough surfaces; however, the more distal actin filaments can be seen spanning valleys. MSCs\textsuperscript{34,35} and NHOst cells\textsuperscript{36} cultured on TCPS exhibited morphologies comparable to those reported in the literature for nonpatterned TCPS.

FIGURE 1. Material characterization of the non-nanostructured/hydrophobic SLA, nanostructured/hydrophobic SLAnano, and nanostructured/ hydrophilic modSLA surface modifications. Substrates were examined by scanning electron microscopy at 20Kx (A–C) and 200Kx (D–F). Surface roughness and morphology were assessed using laser confocal microscopy measured using a 644 \( \mu \text{m} \times 644 \mu \text{m} \) scan size. Topographical images shown were taken with a 60 \( \mu \text{m} \times 60 \mu \text{m} \) to exaggerate surface features (G–I). Surface chemical composition was assessed using XPS with spectra of O, Ti, and C shown (J–L). Surface energy was assessed using contact angle measurements (M–O).
Biological response

DNA content was significantly lower on SLA, SLAnano, and modSLA compared to TCPS for both MSCs [Fig. 3(A)] and NHOst cells [Fig. 3(E)] with modSLA exhibiting significantly lower DNA compared to SLA and SLAnano. Compared to TCPS, MSCs, and NHOst cells cultured on SLA, SLAnano, and modSLA had significantly higher alkaline phosphatase specific activities [Fig. 3(B,F)] and production of osteocalcin [Fig. 3(C,G)] and osteoprotegerin [Fig. 3(D,H)]. Alkaline phosphatase specific activity in the MSC cultures was comparable on the modified surfaces. MSCs cultured on modSLA produced significantly higher osteocalcin and osteoprotegerin compared to SLA and SLAnano with SLAnano producing significantly lower osteocalcin compared to SLA. Alkaline phosphatase specific activity and osteocalcin production by NHOst cells were significantly higher on modSLA compared to SLA and SLAnano with significantly lower alkaline phosphatase specific activity detected on SLAnano compared to SLA. Alkaline phosphatase specific activity and osteocalcin production by NHOst cells were significantly higher on modSLA compared to SLA and SLAnano with significantly lower alkaline phosphatase specific activity detected on SLAnano compared to SLA. No significant differences were detected in NHOst VEGF production among modified surface. NHOst cells cultured on SLAnano produced significantly lower quantities of BMP2 compared to SLA and modSLA, which were not significantly different from each other. IL10 production by NHOst cells was significantly higher on modSLA compared to SLA and SLAnano with a significantly lower production detected on SLAnano compared to SLA. Both MSCs and NHOst cells cultured on modSLA exhibited significantly lower productions of IL6 compared to SLA and SLAnano. No significant differences were detected between MSC IL6 production between SLA and SLAnano while NHOsts produced a significantly lower amount of IL6 on SLAnano compared to SLA.

BMPs, receptor, and antagonist expression are shown in Figure 5. Compared to TCPS, significantly higher mRNA expressions of BMP4, BMP7, BMPR1A, and NOG were detected for MSCs [Fig. 5(A–D)] and NHOst cells [Fig. 5(E–H)] on SLA, SLAnano, and modSLA. MSCs cultured on modSLA had significantly higher expressions of each gene with significantly lower BMP4, BMPR1A, and NOG expressions detected on SLAnano compared to SLA. NHOst cells cultured on modSLA exhibited significantly higher expressions of BMP4, BMP7, and NOG compared to SLA and SLAnano. SLAnano significantly upregulated NHOst BMPR1A expression compared to SLA and modSLA. NHOst NOG expression was significantly greater on SLAnano compared to SLA.

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levels of ITGα1 [Fig. 6(A,E)], ITGα2 [Fig. 6(B,F)], and ITGβ1 [Fig. 6(D,H)] were significantly higher on modified surfaces compared to TCPS for both cell types. Additionally, MSC and NHOrs ITGα5 [Fig. 6(C,G)] expression was significantly lower on modified surfaces compared to TCPS with the exception of MSCs cultured on SLAnano which was significantly greater. MSCs cultured on modSLA exhibited significantly higher mRNA levels of ITGα1, ITGα2, and ITGβ1 compared to SLA and SLAnano with significantly lower mRNA levels of ITGα1 and ITGβ1 on SLAnano compared to SLA. No significant difference was detected in MSC ITGα2 mRNA levels between SLA and SLAnano. Among modified surfaces, NHOrs expression of ITGα1 was not significantly different. NHOrs cultured on modSLA had significantly higher ITGα2 and ITGβ1 mRNA levels compared to SLA and SLAnano. MSCs and NHOrs cultured on SLAnano exhibited significantly higher ITGα5 expression compared to SLA and modSLA. No significant difference was detected between SLA and modSLA MSC ITGα5 expression while NHOrs ITGα5 expression was significantly lower on modSLA compared to SLA.

FIGURE 3. Effects of surface nanoscale features and energy on the maturation of MSCs (A–D) and NHOrs cells (E–H). MSCs and NHOrs cells were plated separately on TCPS, SLA, SLAnano, or modSLA and grown to confluence. At confluence, cells were incubated in fresh media for 24 h, collected, and measured for (A, E) DNA content, (B, F) alkaline phosphatase specific activity, (C, G) osteocalcin, and (D, H) osteoprotegerin. Data shown are the mean ± standard error (SE) of six independent samples. *p < 0.05 vs. TCPS; #p < 0.05 vs. SLA; $p < 0.05 vs. SLAnano.
DISCUSSION

Implant surface properties dictate the resulting molecular events that take place at the cell-material interface. Studies have highlighted the importance of surface properties such as roughness, topography, chemistry, and energy in the modulation of cell proliferation and differentiation.1–8,10,11 In the present study, Ti metal was treated to exhibit either a hydrophilic or hydrophobic nanostructured surface in order to distinguish between the effects these parameters have on osteogenic responses of MSCs and NHÖst cells.

Using a hydrophobic, non-nanostructured surface (SLA) as a control, cells at two different stages in the osteoblast lineage were grown separately on TCPS, SLA, SLAnano, or modSLA and grown to confluence. Subsequent examination of mRNA expression and protein production showed that a hydrophilic nanostructured surface facilitated the greatest increase in osteogenic markers. Interestingly, our data suggest that a hydrophobic nanostructured surface may stunt or delay the osteogenic capacity of MSC and NHÖst cells compared to a hydrophobic non-nanostructured surface.
Although many other cell types are involved in osseointegration and the subsequent remodeling of peri-implant bone, MSCs and NHOst cells are critical for the process of bone mineralization. We used commercially available MSCs and NHOst cells from single donors, which limits the scope of our results since it does not take into account variability inherent to the human population, or the health of the donors themselves. Despite these limitations, our results indicate that the positive osteogenic effects nanostructures have on MSC and NHOst cells are dependent on the inclusion of hydrophilicity.

Scanning electron microscopy confirmed the presence or absence of nanostructures on the modified Ti surfaces. In accordance with previous studies, sand blasting and acid etching techniques generated submicron scale roughness, but did not induce nanostructure formation. Instead, nanostructure formation was dependent upon the reorganization of the oxide layer facilitated by the aqueous NaCl solution. Consistent with this idea, nanostructures with a similar size were present on SLAnano and modSLA but not SLA. The exact process of nanostructure formation is unknown; however, many studies have fabricated nanostructures on metallic surfaces...
using a variety of methods involving changes in temperature, pressure, and chemical treatments. The modification process used in this study also provided a consistent micro-roughness among SLA, SLAnano, and modSLA, allowing for an accurate separation between the effects of the nanostructures and the surface hydrophilicity.

The hydrophilicity of the surfaces was validated using X-ray photoelectron spectroscopy and contact angle measurements. The XPS spectra revealed that the thin Ti oxide layer consists mainly of TiO₂. Additionally, the presence of C(1s) is indicative of aliphatic carbons due to the adsorption of carbon-containing molecules from the air. Exposure to air increased the value of the peak corresponding to the presence of C(1s) carbon which was detected on SLA and SLAnano surfaces. Immediate storage in saline without any exposure to air was an effective method for preventing organic impurity deposition resulting in a very low C(1s) peak on the surface of modSLA. Consistent with the XPS results, contact angle measurements demonstrated SLA and SLAnano to be hydrophobic and modSLA to be super-hydrophilic.

FIGURE 6. Effects of surface nanoscale features and energy on integrin mRNA levels in MSCs and NH0st cells. MSCs and NH0st cells were plated separately on TCPS, SLA, SLAnano, or modSLA and grown to confluence. At confluence, cells were incubated in fresh media for 12 h, collected, and measured mRNA levels for ITGa1, ITGa2, ITGa5, and ITGβ1. *p < 0.05 vs. TCPS; #p < 0.05 vs. SLA; $p < 0.05 vs. SLAnano.
Cytoskeleton staining revealed that SLA, SLAnano, and modSLA surfaces were able to support a normal osteoblastic morphology as demonstrated by the clear, branched morphology adopted by MSCs and NH0st cells. Cytoskeleton organization of the cell is essential for focal adhesion, stress fiber formation, and spreading, and is dependent on the spatial distribution of both micro- and nanostructured topographies. Cell morphology influences gene expression and MSC differentiation relies in part on extracellular mechanical cues inherent to the underlying structure of the cytoskeleton. It has been shown previously that MSC adipogenesis is preceded by round cell morphology while MSC osteoblastogenesis is preceded by a branched cell morphology when cultured on titanium substrates. The branched morphology displayed by MSCs undergoing osteoblastogenesis is similar to that observed in mature osteoblasts grown on titanium substrates. Moreover, the production and expression of osteoblastic markers further support enhanced osteoblastogenesis on the SLA, SLAnano, and modSLA.

A decrease in DNA compared to TCPS was accompanied by an increase of osteoblastic markers of differentiation and maturation on SLA, SLAnano, and modSLA for both cell types. Previous studies have shown that MSCs and NH0st cells grown on microstructured Ti surfaces produce an osteogenic environment facilitating their differentiation and maturation with the highest being produced on modSLA followed by SLA. These include the early marker of osteoblastic differentiation, alkaline phosphatase specific activity; the late marker of osteoblastic differentiation, osteocalcin; the osteoclast inhibitory factor; osteoprotegerin; the angiogenic factor; VEGF; and the osteogenic factor; BMP2. Additionally, modified surfaces had the ability to mitigate the inflammatory response compared to TCPS as indicated by the increased production of the anti-inflammatory cytokine interleukin 10 (IL10) and the decreased production of the pro-inflammatory cytokine interleukin 6 (IL6) in accordance with previous reports. Interestingly, the inflammatory response was highest on SLAnano among modified surfaces. Additionally, MSC osteocalcin, VEGF, BMP2, and NH0st alkaline phosphatase specific activity, osteoprotegerin, and BMP2 on SLAnano were lowest among modified surfaces. Although each modification was able to support a healthy osteoblastic phenotype, it was frequently observed that SLAnano had a comparatively diminished capacity to enhance production of osteoblastic markers.

MSC and NH0st gene expression was less upregulated by SLAnano compared to SLA and modSLA. In general, for both cell types, the expression of BMPs (BMP4, BMP7), receptors (BMPRIA), and antagonists (NOG) was highest on modSLA followed by SLA as seen previously. Additionally, both cell types primarily expressed α5β1 subunits when grown on TCPS, but shifted to the production of α1β1 and α2β1 subunits when grown on rough titanium substrates. A further increase was observed for α2β1 subunits on hydrophilic and nanostructured substrates as previously described. However, removal of the hydrophilicity while retaining nanostructures resulted in fluctuations in osteoblastic gene expression. MSC expression of BMP4, its receptor BMPRIA, and its specific antagonist NOG decreased on SLAnano suggesting a lessened capacity to stimulate an osteogenic environment by nanostructures in the absence of hydrophilicity. SLAnano also altered the expression of BMP complexes in NH0st cells. NOG expression was increased compared to SLA without a similar increase in BMP4; furthermore, BMPRIA expression was highest on SLAnano.

Alterations in the balance of BMPs, their receptors, and antagonist results in altered bone remodeling, a potential outcome indicated by the in vitro gene expression modulated by SLAnano. Integrins are also important for both cell attachment and maintenance of the osteoblastic phenotype, and an altered expression of integrin subunits was observed on SLAnano. Although pro-osteoblastic integrin subunits increased on SLAnano, α1 and β1 were decreased compared to SLA. Moreover, an increase of the nonosteoblastic subunit α5 was observed for both MSCs and NH0st cells cultured on SLAnano with its expression being greater than TCPS. Together, the expression of BMP and integrin complexes suggests that the bone remodeling process is somewhat diminished on a hydrophobic and nanostructured surface. However, this is capable of being recovered with the addition of surface hydrophilicity.

Previous studies analyzing the biological response to a hydrophobic and nanostructured surface determined a favorable effect nanostructures have on osteoblastic differentiation and maturation. This is in contrast to other studies comparing SLA to a hydrophobic, nanostructured surface. Using thermally nanomodified hydrophobic Ti (NMSLA) and Ti-6Al-4V alloy surfaces, it was shown that the osteoblastic differentiation of MG63 cells was enhanced compared to a microrough and hydrophobic SLA. Interestingly, human MSC response to the NMSLA modification suggested that differentiation was suppressed by the superposition of nanostructures. These studies, nanostructures were classified as submicron, reporting a size range from 40 to 200 nm, while the nanostructure size range produced on surfaces used in this study was between 10 and 11 nm. It is possible that osteoblastic differentiation and maturation can be altered by varying the size and shape of the nanostructures, an idea previously suggested.

Our results may be partially explained by a recent study investigating the influence of nanostructures and hydrophilic Ti substrates on fibrinogen and fibronectin protein adsorption and the degree of blood coagulation. It was shown that a hydrophilic and nanostructured surface led to the highest protein adsorption levels and the most pronounced degree of blood coagulation whereas the effects of a hydrophobic nanostructured surface on these outcomes were weak. This suggests that the effects the surface parameters have on early protein adsorption may dictate the rate of MSC osteoblastic differentiation or NH0st maturation. The effect of the nanostructures can be recovered and improved through the addition of the hydrophilicity providing the optimum conditions of osteoblastic differentiation and maturation.
CONCLUSION

Clinical grade Ti was successfully modified to fabricate microrough surfaces exhibiting hydrophobic or hydrophilic nanostructured surfaces. Although modified surfaces were able to support osteoblastic morphology in MSCs, indicated by staining of the cytoskeleton, production and expression of markers of osteoblastic differentiation and maturation suggest that an average nanostructure size of approximately 11 nm may delay the process of osteoblastogenesis. The effect of the nanostructures can be recovered with the addition of hydrophilicity. In order to promote enhanced osteoblastic differentiation of MSCs and maturation of NHØst cells, a nanostructured Ti surface will be dependent on an increased surface hydrophilicity.

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