**SPECIAL FOCUS: STRATEGIC DIRECTIONS
IN IMMUNORESPONSIVE BIOMATERIALS IN TISSUE ENGINEERING***

Bone Morphogenetic Protein 2 Alters Osteogenesis and Anti-Inflammatory Profiles of Mesenchymal Stem Cells Induced by Microtextured Titanium *In Vitro*

Sharon L. Hyzy, MS,¹ Rene Olivares-Navarrete, DDS, PhD,¹ Sarah Ortman, BS,² Barbara D. Boyan, PhD,^{1,2} and Zvi Schwartz, DMD, PhD^{1,3}

Objectives: Microtextured titanium (Ti) induces osteoblast differentiation of mesenchymal stem cells (MSCs) in the absence of exogenous osteogenic factors; and high-energy surface modifications speed healing of micro-rough Ti implants. Bone morphogenetic protein 2 (BMP2) is used clinically to improve peri-implant bone formation and osseointegration but can cause inflammation and bone-related complications. In this study, we determined whether BMP2 alters human MSC differentiation, apoptosis, and inflammatory factor production when grown on Ti implants with different surface properties.

Materials and Methods: Human MSCs were cultured on Ti substrates (smooth [PT], sandblasted acid-etched [SLA], hydrophilic-SLA [modSLA]), or tissue culture polystyrene (TCPS). After 7 days, inflammatory mRNAs were measured by polymerase chain reaction array. In addition, 7-day cultures were treated with exogenous BMP2 and osteogenic differentiation and production of local factors, proinflammatory interleukins, and anti-inflammatory interleukins assessed. Finally, osteogenic markers and interleukins were measured in MSCs cultured for 48 h on BMP2 dip-coated SLA and modSLA surfaces.

Results: Expression of interleukins, chemokines, cytokines, and growth factors was affected by surface properties, particularly on modSLA. MSCs on Ti produced fewer resorptive and more osteogenic/anti-inflammatory factors than cells on TCPS. Addition of 100 ng/mL BMP2 not only increased differentiation but also increased proinflammatory and decreased anti-inflammatory/antiresorptive factors. Two hundred nanograms per milliliter BMP2 abolished osteogenesis and dramatically increased pro-osteoclastogenic factors. MSCs cultured on BMP2-dip-coated disks produced similar proinflammatory profiles with inhibited osteogenic differentiation and had increased apoptotic markers at the highest doses.

Conclusions: MSCs underwent osteogenesis and regulated inflammatory cytokines on microtextured Ti. Exogenous BMP2 inhibited MSC differentiation and stimulated a dose-dependent proinflammatory and apoptotic response. Use of BMP2 with microtextured metal implants may increase inflammation and possibly delay bone formation dependent on dose, suggesting that application of BMP2 clinically during implant insertion may need to be reevaluated.

Keywords: stem cells, interleukins, titanium, bone morphogenetic protein 2, microtexture

Introduction

TITANIUM (Ti) is used as an implant biomaterial in dentistry and orthopedics due to its excellent biocompatibility, corrosion resistance, and physical and mechanical properties.¹ Successful osseointegration of these implants is determined by

the interactions of the host with the implant surface. Because earlier integration decreases the time from implant collocation to load, surface properties such as roughness,^{2,3} chemistry,⁴⁻⁶ and/or energy^{7,8} have been used to stimulate osteoblast maturation and local factor production. We and others have shown that multipotent mesenchymal stem cells (MSCs) can undergo

¹Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, Virginia.

²Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia.

³Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas.

*This article is part of a special focus issue on Strategic Directions in Immunoresponsive Biomaterials in Tissue Engineering. An additional article can be found in Tissue Engineering Part B, volume 23, number 5.

osteoblastic differentiation *in vitro* on micron-/submicron-scale rough Ti surfaces in the absence of exogenous osteogenic factors.^{9,10} High surface energy, achieved by preventing atmospheric hydrocarbon adsorption on these same rough surfaces following processing, further stimulates MSC differentiation *in vitro* and increases peri-implant bone formation and decreases healing time clinically.^{4,11,12}

The first physiological event after implant collocation is the formation of the blood clot on the implant followed by an inflammatory response. The inflammatory response affects osseointegration by changing the profile of inflammatory cytokines, chemokines, and interleukins produced. Proinflammatory molecules extend the inflammatory response, recruiting monocytes, macrophages, and neutrophils to the wound site, activating osteoclasts and bone resorption, and delaying bone formation. Anti-inflammatory proteins temper this response, decreasing acute inflammation and speeding healing. MSCs secrete immunomodulatory factors *in vivo*,¹³ but whether they secrete these factors while in contact with a biomaterial surface is not well understood.

Exogenous factors are used during bone regeneration and have recently been used in addition to biomaterials to enhance bone regeneration around the material. Bone morphogenetic protein 2 (BMP2), a member of the transforming growth factor- β (TGF- β) superfamily,^{14,15} induces bone and cartilage formation *in vivo*¹⁴ and plays a critical role in bone healing.¹⁶ Recombinant human BMP2 is used to induce bone regeneration in several dental and orthopedic applications and is used clinically in combination with biomaterials to improve peri-implant bone formation and osseointegration. Supraphysiological doses of BMP2, either delivered as a bolus or coated on Ti implants, have been reported to induce inflammation, bone resorption, and ectopic bone formation around the implant site.^{17–19} While MSCs may respond predictably to surface cues, the addition of BMP2 to the local environment may change the inflammatory and cell differentiation context, in which the implant resides.

Therefore, the aims of our study were to (1) determine if MSCs modulate inflammatory molecules in response to surface microstructure; and (2) determine how BMP2 presence affects the differentiation, apoptosis, and inflammatory microenvironment created by MSCs.

Materials and Methods

Disk preparation and characterization

Ti disks (15 mm diameter, 1 mm thick) were prepared from sheets of grade 2 commercially pure Ti (Institut Straumann AG, Basel, Switzerland). The fabrication methods used to create smooth pretreatment (PT) disks, rough sandblasted acid-etched (SLA) disks, and hydrophilic SLA (modSLA) disks and resulting characterization have been reported previously.^{20–22} In brief, disks were washed with acetone and then processed in a solution of 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid for 30 s at 55°C to produce pretreatment Ti (PT) disks with a mean peak to valley roughness (R_a) of $R_a < 0.4 \mu\text{m}$. PT disks were sandblasted with large corundum grit (0.25–0.50 μm) at 5 bar and then acid-etched in a solution of hydrochloric and sulfuric acids heated above 100°C for several minutes to produce SLA disks ($R_a = 3.2 \mu\text{m}$) with a water contact angle of $128 \pm 3^\circ$. Hydrophilic SLA disks (modSLA) were prepared in the same

manner as SLA, but were rinsed under nitrogen to control oxide layer formation and stored in an isotonic saline solution, decreasing surface hydrocarbon contamination by more than 50% and resulting in a contact angle of 0° .²¹ All disks were sterilized by gamma irradiation before use.

Cell culture

Human bone marrow-derived MSCs (Lonza, Walkersville, MD) were grown in MSC Growth Medium (MSCGM; Lonza). MSCs were plated on Ti substrates at a density of 10,000 cells/cm² and cultured at 37°C and 100% humidity. Cells cultured on tissue culture polystyrene (TCPS) served as control.

MSC inflammatory profile

The immune context produced by MSCs on microtextured Ti surfaces was examined by polymerase chain reaction (PCR) array. When cultures reached confluence on TCPS, all cells were incubated with fresh media for 12 h. RNA was isolated using TRIzol[®] (Life Technologies, Carlsbad, CA). RT² Profiler[™] PCR Array Human Inflammatory Response PCR Array (Qiagen, Inc., Valencia, CA) was performed according to the manufacturer's instructions. In brief, 1 μg of mRNA was treated with DNase I and reverse transcribed to cDNA. cDNA was combined with RT² SYBR[®] Green quantitative polymerase chain reaction (qPCR) Master Mix (Qiagen, Inc.) into the PCR array, each well of which contained primers for 1 of 84 genes of interest, 5 housekeeping genes, or 7 assay controls. PCR reactions were performed on an Applied Biosystems StepOnePlus real-time PCR machine (Life Technologies) following manufacturer's instructions. Raw data for these genes were analyzed by a web-based Array Analysis Software (Qiagen, Inc.) using $\Delta\Delta C_T$ normalization of raw data to the five housekeeping genes. Data represent the mean $\pm$ standard error of four independent cultures per surface. Genes with at least a twofold upregulation or a twofold downregulation in comparison to cells cultured on TCPS were identified as statistically significant.

Cell response to BMP2 treatment

To determine how rhBMP2 affected osteogenic differentiation and interleukin (IL) production of MSCs, cells were plated as described on TCPS or Ti substrates. Cells were fed daily with culture media supplemented with 0, 100, or 200 ng/mL BMP2. After 7 days of culture, osteogenic differentiation (alkaline phosphatase specific activity, osteocalcin), local factor production (osteoprotegerin [OPG], vascular endothelial growth factor-A [VEGF]), and interleukin production (IL6, IL8, IL10) were measured. Cells were released from TCPS and Ti surfaces using two sequential incubations with 0.25% trypsin for 10 min at 37°C; this procedure ensured complete removal of the cells from the Ti surfaces.³ Cells were counted using a cell counter (Z2 Particle Counter; Beckman Coulter, Fullerton, CA). Cellular alkaline phosphatase specific activity (orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1) was measured in cell lysates at pH 10.2 as the release of *p*-nitrophenol from *p*-nitrophenyl phosphate, and results were normalized to the protein content of the cell lysates. Secreted osteocalcin was measured using a commercially available radioimmunoassay

kit (Human Osteocalcin RIA Kit; Biomedical Technologies, MA). OPG, VEGF, and IL6, IL8, and IL10 were assayed in the conditioned medium by ELISA (R&D Systems DuoSet, Minneapolis, MN) and were normalized to the total cell number.

Response to BMP2 dip-coated implants

To determine whether changing the presentation of BMP2 affected osteogenesis or IL expression, SLA and modSLA substrates were dip coated^{23,24} in 1 µg/mL BMP2 or 2 µg/mL BMP2 of recombinant human BMP2. The excess protein was blotted and substrates were dried in a biosafety cabinet for 24 h. MSCs were cultured on coated surfaces or uncoated controls for 48 h. RNA was isolated and mRNA analyzed as described above. MSCs grown on TCPS were used to generate a standard curve for each gene of interest, and values for each unknown sample were extrapolated. Messenger RNA was measured for interleukins (*IL1B*, *IL6*, *IL8*, *IL10*) and markers of osteoblastogenesis (osteocalcin [*OCN*], *OPG*, and runt-related transcription factor 2 [*RUNX2*]) using gene-specific primers (Eurofins Operon, Huntsville, AL) (Table 1). mRNA data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Table 1).

Statistical analysis

Data represent the mean ± standard error of six individual cultures per variable. Data were first subjected to analysis of variance followed by Student's *t*-test with Bonferroni's correction for multiple comparisons. Values of *p* < 0.05 were considered significant.

Results

MSCs produced immunomodulatory chemokines, cytokines, and growth factors in response to the biomaterial substrates. Of the 84 genes examined, 65 genes were positive ($C_T < \text{cycle } 35$). Nineteen were more than fivefold different on

at least one surface in comparison with TCPS (the remaining are listed in Table 2). *CSF2* was increased 100% on PT and SLA compared with TCPS, while *CSF3* was increased 170-fold on modSLA compared with PT or SLA (Fig. 1A). *FIGF* and *NODAL* were upregulated in a roughness-dependent and surface-energy-dependent manner, with the highest regulation on modSLA (Fig. 1A). *VEGFA* was upregulated by surface roughness and increased PT < SLA < modSLA (Fig. 1A). *TNFSF4* and *TNFSF11* were downregulated on rough Ti surfaces in comparison with smooth (Fig. 1B). MSCs cultured on PT upregulated *TNFRSF11B* (7.4-fold), further increasing mRNA levels on rough SLA (85.5-fold increase) and high-energy/rough modSLA (106.7-fold increase).

mRNAs of proinflammatory *IL1A*, *IL1B*, and *IL6* were downregulated on high-energy, rough modSLA surfaces, but were unchanged on PT and SLA in comparison with TCPS (Fig. 1C). *IL8* mRNAs were similar on smooth TCPS and PT surfaces, but the downregulation seen on SLA was further enhanced on modSLA. Anti-inflammatory *IL1RN* was downregulated by culture on PT (−11.7-fold) but was upregulated on SLA (11.8-fold) and modSLA (66.7-fold) surfaces (Fig. 1D). MSCs on modSLA surfaces upregulated *IL10*, *IL22*, and *IL24* only in comparison with TCPS. *IL23A* was downregulated on high-energy, rough modSLA surfaces, but was unchanged on PT and SLA compared with TCPS.

MSC differentiation was sensitive to surface microstructure and hydrophilicity. Cell number significantly decreased on all of the Ti substrates when compared with TCPS. This reduction was greatest on microrough surfaces (SLA) and hydrophilic surfaces (modSLA) (Fig. 2A). Cell number was lower in MSCs treated with BMP2 and decreased in a dose-dependent manner. Alkaline phosphatase specific activity, an early marker of osteogenic differentiation, increased twofold on Ti surfaces when compared with TCPS, with additional increases on SLA and modSLA surfaces. Cells on TCPS produce ~200 pg/mL BMP2, while cells on modSLA produce up to nine times that amount.²⁵

TABLE 1. PRIMER SEQUENCES USED IN REAL-TIME QPCR ANALYSIS

BAX	F	AAA GTA GAA AAG GGC GAC AAC C	NM_001291428
	R	GAC GAA CTG GAC AGT AAC ATG G	
BCL2	F	CTG TTT GAT TTC TCC TGG CTG TCT C	NM_000633
	R	TCT ACT GCT TTA GTG AAC CTT TTG C	
IL1B	F	TGG CAG AAA GGG AAC AGA AAG G	NM_000576
	R	AAC AAA AGG GCT GGG GAT TGG	
IL6	F	GCA GAA AAC AAC CTG AAC CTT C	NM_000600
	R	ACC TCA AAC TCC AAA AGA CCA G	
IL8	F	GAC ATA CTC CCA AAC CTT TCC AC	NM_000584
	R	AAA CCT CTC CAC AAC CCT CTG	
IL10	F	TTA TCT TGT CTC TGG GCT TGG	NM_000572
	R	GAA TGA AGT GGT TGG GGA ATG	
OCN	F	GTG ACG AGT TGG CTG ACC	NM_199173
	R	TGG AGA GGA GCA GAA CTG G	
OPG	F	ACC ACT ACT ACA CAG ACA GC	NM_002546
	R	CAA GCA GAA CTC TAT CTC AAG G	
RUNX2	F	GTC TCA CTG CCT CTC ACT TG	NM_001024630
	R	CAC ACA TCT CCT CCC TTC TG	
GAPDH	F	GCT CTC CAG AAC ATC ATC C	NM_002046.3
	R	TGC TTC ACC ACC TTC TTG	

TABLE 2. mRNA WITH LESS THAN FIVEFOLD REGULATION FROM TISSUE CULTURE POLYSTYRENE IN POLYMERASE CHAIN REACTION ARRAY ANALYSIS

Gene	PT		SLA		modSLA	
	Mean	SEM	Mean	SEM	Mean	SEM
CNTF	-1	0.121	-1.1	0.309	-1.1	0.184
CSF1	-1.7	0.127	-1.2	0.075	-4	0.053
LEFTY2	-2.3	0.158	-2.1	0.451	2.4	0.125
LIF	2	0.169	1.9	0.355	1.3	0.134
OSM	2.6	0.204	1.8	0.24	2.4	0.218
PDGFA	1.1	0.053	1.2	0.105	-1.3	0.173
TGFA	1.2	0.127	-1.4	0.193	-1.2	0.137
THPO	3.7	0.212	1.6	0.729	-1.2	0.131
IFNA1	1.1	0.079	3.4	0.257	1.3	0.167
IFNA2	-1.2	0.071	1.1	0.054	1.4	0.103
IFNA4	1.4	0.194	1.8	0.064	2.4	0.118
IFNA5	1.1	0.077	-1.1	0.068	1.1	0.077
IFNB1	1.4	0.094	1.8	0.024	2.4	0.118
IFNG	-2.3	0.12	-1.8	0.076	-1.4	0.062
CD40LG	4.2	0.314	4.9	0.35	4.1	0.242
CD70	1.4	0.094	1.8	0.14	2.4	0.118
FASLG	1.1	0.042	1.4	0.088	2.2	0.195
LTA	1.2	0.022	2.1	0.186	1.3	0.083
LTB	1.8	0.044	2.1	0.115	1.8	0.116
TNF	1.3	0.067	1.7	0.031	2.2	0.202
TNFSF8	1.8	0.026	2.2	0.231	2.4	0.102
TNFSF10	-2.0	0.124	-1.5	0.039	1.2	0.027
TNFSF12	1.0	0.069	-1.3	0.095	-2.8	0.184
TNFSF13	1.3	0.085	1.3	0.062	-1.1	0.081
TNFSF13B	-1.3	0.081	-1.6	0.079	-1.2	0.069
TNFSF14	1.4	0.102	1.5	0.067	-1.7	0.103
IL2	1.4	0.094	1.8	0.124	2.4	0.218
IL3	1.5	0.132	-1	0.082	-1.1	0.063
IL4	2.8	0.114	-2.9	0.025	2.1	0.189
IL5	-2.7	0.115	-2.1	0.04	-1.6	0.069
IL7	-1.7	0.074	-1.9	0.055	-3.3	0.073
IL9	-3.7	0.13	-2.9	0.045	-2.2	0.078
IL12A	1.2	0.054	-1.3	0.048	-1.7	0.167
IL12B	1.6	0.139	2.1	0.176	-1.2	0.068
IL13	-2.2	0.172	-1.9	0.161	2	0.047
TXLNA	-1.1	0.073	-1.4	0.138	-2.4	0.123
IL15	-1.1	0.076	-2.1	0.134	-3.2	0.123
IL17A	-1.6	0.067	-1.2	0.058	1.0	0.1
IL17B	1.0	0.108	-2.0	0.125	-4.7	0.037
IL17C	-1.0	0.03	-3.2	0.253	1.2	0.128
IL18	1.1	0.106	-1.1	0.08	-1.5	0.074
IL19	1.4	0.094	1.8	0.124	2.4	0.118
IL20	1.4	0.094	1.8	0.124	2.4	0.118
IL21	-1.9	0.138	-1.4	0.082	-1.1	0.043
IL25	1.4	0.133	1.8	0.124	2.4	0.118
IL27	-1.2	0.119	-1.8	0.111	-1.2	0.097

Genes with more than twofold change from tissue culture polystyrene are indicated in bold.

Addition of 100 ng/mL BMP2 increased differentiation, but MSCs cultured with 200 ng/mL BMP2 produced less alkaline phosphatase (Fig. 2B). OCN, a later marker of osteoblastic differentiation, was altered in a similar manner (Fig. 2C). Secreted OPG was higher in MSCs grown on rough SLA and modSLA surfaces, but exogenous BMP2 decreased levels in a dose-dependent manner (Fig. 2D). VEGF was higher on rough surfaces than on smooth; addition of 100 ng/mL BMP2 had no effect on these levels, but VEGF decreased on Ti in cells treated with 200 ng/mL BMP2 (Fig. 2E). Proinflammatory interleukins IL6 and IL8 decreased in MSCs

cultured on rough surfaces, but the addition of BMP2 dramatically increased their production particularly on modSLA (Fig. 3A, B). Anti-inflammatory IL10, which increases as MSCs undergo osteogenesis on microtextured Ti substrates, was decreased by BMP2 in a dose-dependent manner (Fig. 3C).

MSCs cultured on 1 µg/mL BMP2-coated SLA and modSLA surfaces had the same production of OCN as on uncoated surfaces (Fig. 4A), but OCN was lower in cells cultured on 2 µg/mL BMP2-coated surfaces. MSCs cultured on 1 µg/mL BMP2-coated SLA surfaces had similar RUNX2 as cells on

FIG. 1. PCR array analysis of inflammatory mediators produced by MSCs on microstructured Ti surfaces. MSCs were cultured on TCPS or Ti surfaces. At confluence, real-time PCR array analysis was performed examining mRNA for inflammatory mediators (A), TNF superfamily (B), proinflammatory interleukins (C), and anti-inflammatory interleukins and cytokines (D). The red line indicates a twofold increase from TCPS. MSC, mesenchymal stem cell; PCR, polymerase chain reaction; TCPS, tissue culture polystyrene; Ti, titanium. Color images available online at www.liebertpub.com/tea

control, but *RUNX2* was lower in cells grown on 2 μ g/mL BMP2-coated surfaces (Fig. 4B). MSCs on BMP2-coated modSLA surfaces decreased *RUNX2* in a dose-dependent manner (Fig. 4B). MSCs cultured on BMP2-coated SLA surfaces decreased *OPG* mRNA in a dose-dependent manner; levels of *OPG* were reduced on coated modSLA surfaces compared with control (Fig. 4C). MSCs cultured on 1 μ g/mL BMP2-coated SLA and modSLA surfaces had lower production of *IL10* than on the control surfaces. This production decreased further when the MSCs were grown on 2 μ g/mL BMP2-coated SLA and modSLA (Fig. 4D). MSCs cultured on 1 μ g/mL BMP2-coated SLA and modSLA surfaces upregulated *IL1B*, *IL6*, *IL8* compared with uncoated controls, but production was decreased in cells grown on 2 μ g/mL BMP2-coated surfaces (Fig. 4E–G). Culture on BMP2-coated surfaces increased the apoptotic *BAX/BCL2* ratio in MSCs in a dose-dependent manner (Fig. 4H).

Discussion

In this study, we examined the role of surface features on the inflammatory microenvironment created by MSCs and

how BMP2 alters MSC differentiation and microenvironment. Our results show that Ti surface properties such as roughness and energy affected the production of secreted factors relevant to osseointegration. Moreover, surface topography and energy affected mRNA and protein levels of important pro- and anti-inflammatory mediators. This response was altered in response to treatment or coating with BMP2, which increased the production of proinflammatory mediators and osteoclastogenic factors.

The inflammatory response is one important event in osseointegration. Proteins adsorbed on the implant surface and inflammatory cells modulate this initial response, but it is not clear whether progenitor cells or MSCs actively participate in this process. Different reports have shown that MSCs are immunomodulatory, reducing the inflammatory response and resolving the inflammatory microenvironment.²⁶ In addition, MSCs undergo spontaneous osteoblastic differentiation when grown on microstructured Ti surfaces,¹⁰ increasing levels of essential proteins related to bone and blood vessel formation. In this study, MSCs on Ti underwent osteogenic differentiation in response to surface roughness and surface energy as we saw previously, but our

FIG. 2. Modulation of osteogenesis and local factors by MSCs on microtextured Ti after BMP2 treatment. MSCs were cultured on TCPS, PT, SLA, or modSLA substrates in culture media (control) or media containing 100 ng/mL or 200 ng/mL BMP2. Markers of osteogenesis [cell number (A), alkaline phosphatase specific activity (B), secreted osteocalcin (C)] and secreted local factors [osteoprotegerin (D), VEGF (E)] in MSCs were measured after 7 days in culture. * $p < 0.05$ vs. TCPS; # $p < 0.05$ vs. control; \$ $p < 0.05$ vs. 100 ng/mL BMP2. BMP2, bone morphogenetic protein 2; VEGF, vascular endothelial growth factor.

results demonstrate that surface characteristics differentially affect the levels of pro- and anti-inflammatory interleukins.

The role of inflammatory cytokines in the regulation of bone repair and remodeling has long been recognized.^{27–30} In this study, we were interested in control of resorption by cells on the biomaterial surface to achieve net new bone formation during osseointegration. IL1, IL6, and RANK expression decreases as OPG expression increases,³¹ which correlates well with our results that proinflammatory interleukins decrease and OPG increases on rough, hydrophilic surfaces.

Several studies have shown the effect of MSCs in inflammatory microenvironments. They have reported an attenuation or modulation of inflammatory events in response to MSC delivery or presence.^{32–35} Our results demonstrate that MSCs can induce an anti-inflammatory, osteogenic, and angiogenic microenvironment in response to biomaterial

surface properties. MSCs modulated the production of an inflammatory microenvironment in response to biomaterial surface properties, as we noted previously when MSCs were cultured on titanium–aluminum–vanadium alloy (Ti-6Al-4V) and polyether ether ketone.³⁶ In the present study, we were able to differentiate effects of surface topography and chemistry. IL6 and IL8 decreased when MSCs were grown on hydrophilic microstructured surfaces while anti-inflammatory IL10 was increased by surface roughness, with the highest levels measured in hydrophilic-microstructured surfaces. This corroborates results of our earlier work that demonstrated that osteoblast-like cells cultured on microstructured Ti surfaces that induced osteoblast maturation produced more anti-inflammatory interleukins and less proinflammatory ILs than cells on smooth Ti,³⁷ an effect also seen on Ti-6Al-4V.³⁶

FIG. 3. Modulation of interleukin production by MSCs on microtextured Ti after BMP2 treatment. MSCs were cultured on TCPS, PT, SLA, or modSLA substrates in culture media (control) or media containing 100 ng/mL or 200 ng/mL BMP2. Levels of secreted IL6 (A), IL8 (B), and IL10 (C) produced by MSCs were measured after 7 days in culture. * $p < 0.05$ vs. TCPS; # $p < 0.05$ vs. control; \$ $p < 0.05$ vs. 100 ng/mL BMP2.

FIG. 4. Modulation of inflammatory cytokines and osteogenic markers in MSCs cultured on dip-coated Ti substrates. Levels of interleukin and osteogenic mRNA in MSCs cultured on SLA or modSLA surfaces dip coated in 0, 1, or 2 µg/mL BMP2. mRNA for osteogenic markers [OCN (A), RUNX2 (B), OPG (C)], interleukins [IL10 (D), IL1B (E), IL6 (F), IL8 (G)], or apoptosis [BAX/BCL2 (H)] were measured in MSCs cultured on BMP2-dip-coated Ti disks for 48 h. * $p < 0.05$ vs. SLA; # $p < 0.05$ vs. control.

BMP2 has been found to improve healing in the repair of nonunion fractures, to increase bone in spinal fusions and to increase bone before dental implant collocation.³⁸ Our study tested whether BMP2 treatment or coating could improve osteoblastic differentiation, increasing the osteogenic microenvironment without affecting the inflammatory environment. The dose we chose for these applications was 10^3 times more than endogenous levels produced by cells on these surfaces, but are 10^3 lower in magnitude than the 1.5 mg/mL used clinically. Although BMP2 has been demonstrated to induce bone formation,^{39,40} it can also cause seroma or bone related complications clinically.^{17,41} Our results provide insight into why this might be the case. We showed that BMP2 treatment at the lower dose tested in this study increased markers of osteoblastic differentiation of MSCs cultured on smooth or rough surfaces. However, the same treatment also increased levels of proinflammatory IL6 and IL8 and decreased anti-inflammatory IL10 on microstructured Ti surfaces. In addition, treatment with the higher BMP2 concentrations decreased alkaline phosphatase activity, osteocalcin, OPG, VEGF, and IL10 levels, and dramatically increased IL6 and IL8 levels on microstructured surfaces. Because BMPs are morphogens, the dose delivered is critical in determining cell response. In previous studies, we found that treating cells with 50 ng/mL BMP2 increased alkaline phosphatase and osteocalcin, but this effect was smaller than for cells, in which the BMP inhibitor Noggin was silenced.^{25,42}

BMP2 has also been used to coat Ti dental implants with the aim of speeding up bone formation, but those results demonstrated that BMP2 caused bone resorption ranging from minimal to massive areas of bone loss.¹⁹ Since dip coating is the most convenient way for clinicians to apply BMP2 to implants in the clinic, we examined inflammation, differentiation, and apoptosis using this application method. We previously reported that exogenous treatment with BMP2 increased levels of important proinflammatory interleukins in osteoblasts and that TAB/TAK activation mediated this response.³⁷ In addition, other reports have shown that exogenous treatment with BMP2 increased secreted IL6, resulting in an increase in the proinflammatory microenvironment.⁴³ However, when more BMP2 was added, there was a significant decrease in IL6 levels due to the dose-specific effects of BMP2.⁴⁴ It is important to note that MSC response to BMP2 was different on smooth surfaces than rough surfaces, indicating that surface properties such as roughness, chemistry, and energy can affect the cellular response to growth factors and other important biological molecules. This finding is in agreement with our previous findings that surface microstructure affects osteoblast response to osteotropic hormones such as $1\alpha,25$ -dihydroxyvitaminD₃ and 17 β -estradiol.⁴⁵

Conclusion

In addition to differentiating, MSCs regulated inflammatory cytokines on microtextured Ti. Exogenous BMP2 inhibited MSC differentiation and stimulated a dose-dependent proinflammatory and apoptotic response when grown on microtextured surfaces. MSCs grown on SLA and modSLA surfaces coated with low amounts of BMP2 increased proinflammatory interleukins. MSCs grown on high-BMP2

coatings showed the lowest mRNA levels of most genes analyzed. The results reveal that addition of BMP2 or coating implants with BMP2 affects osteogenesis and increases the proinflammatory response, which may negatively influence the implant osseointegration process and the eventual implant success. These data suggest that the use of BMP2 in concert with implants may need to be reevaluated.

Acknowledgments

This study was supported by grant 798_2011 from the ITI Foundation for the Promotion of Implantology, Basel, Switzerland. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR052102. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funding sources played no role in study design; collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the article for publication. Institut Straumann AG (Basel, Switzerland) provided Ti disks as a gift.

Disclosure Statement

S.L.H. and S.O. have no competing financial interests to disclose. B.D.B. and Z.S. are unpaid consultants for Institut Straumann AG. R.O.-N., B.D.B., and Z.S. have grant funds from Institut Straumann AG. Z.S. is a paid consultant for AB Dental (Ashdod, Israel). B.D.B. is a paid consultant for Titan Spine LLC (Mequon, WI).

References

- Mantripragada, V.P., Lecka-Czernik, B., Ebraheim, N.A., and Jayasuriya, A.C. An overview of recent advances in designing orthopedic and craniofacial implants. *J Biomed Mater Res A* **101**, 3349, 2013.
- Boyan, B.D., Bonewald, L.F., Paschalis, E.P., Lohmann, C.H., Rosser, J., Cochran, D.L., Dean, D.D., Schwartz, Z., and Boskey, A.L. Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int* **71**, 519, 2002.
- Martin, J.Y., Schwartz, Z., Hummert, T.W., Schraub, D.M., Simpson, J., Lankford, J., Jr., Dean, D.D., Cochran, D.L., and Boyan, B.D. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). *J Biomed Mater Res* **29**, 389, 1995.
- Buser, D., Brogini, N., Wieland, M., Schenk, R.K., Denzer, A.J., Cochran, D.L., Hoffmann, B., Lussi, A., and Steinemann, S.G. Enhanced bone apposition to a chemically modified SLA titanium surface. *J Dent Res* **83**, 529, 2004.
- Liu, X., Lim, J.Y., Donahue, H.J., Dhurjati, R., Mastro, A.M., and Vogler, E.A. Influence of substratum surface chemistry/energy and topography on the human fetal osteoblastic cell line hFOB 1.19: phenotypic and genotypic responses observed in vitro. *Biomaterials* **28**, 4535, 2007.
- Sul, Y.T., Johansson, C., Wennerberg, P., Cho, L.R., Chang, B.S., and Albrektsson, P. Optimum surface properties of oxidized implants for reinforcement of osseointegration: surface chemistry, oxide thickness, porosity,

- roughness, and crystal structure. *Int J Oral Maxillofac Implants* **20**, 349, 2005.
7. Gittens, R.A., Olivares-Navarrete, R., McLachlan, T., Cai, Y., Hyzy, S.L., Schneider, J.M., Schwartz, Z., Sandhage, K.H., and Boyan, B.D. Differential responses of osteoblast lineage cells to nanotopographically-modified, micro-roughened titanium-aluminum-vanadium alloy surfaces. *Biomaterials* **33**, 8986, 2012.
 8. Gittens, R.A., Scheideler, L., Rupp, F., Hyzy, S.L., Geis-Gerstorfer, J., Schwartz, Z., and Boyan, B.D. A review on the wettability of dental implant surfaces II: biological and clinical aspects. *Acta Biomater* **10**, 2907, 2014.
 9. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315, 2006.
 10. Olivares-Navarrete, R., Hyzy, S.L., Hutton, D.L., Erdman, C.P., Wieland, M., Boyan, B.D., and Schwartz, Z. Direct and indirect effects of microstructured titanium substrates on the induction of mesenchymal stem cell differentiation towards the osteoblast lineage. *Biomaterials* **31**, 2728, 2010.
 11. Boyan, B.D., Lossdorfer, S., Wang, L., Zhao, G., Lohmann, C.H., Cochran, D.L., and Schwartz, Z. Osteoblasts generate an osteogenic microenvironment when grown on surfaces with rough microtopographies. *Eur Cell Mater* **6**, 22, 2003.
 12. Sela, J., Gross, U.M., Kohavi, D., Shani, J., Dean, D.D., Boyan, B.D., and Schwartz, Z. Primary mineralization at the surfaces of implants. *Crit Rev Oral Biol Med* **11**, 423, 2000.
 13. Bernardo, M.E., and Fibbe, W.E. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* **13**, 392, 2013.
 14. Hogan, B.L. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* **10**, 1580, 1996.
 15. Wagner, D.O., Sieber, C., Bhushan, R., Borgermann, J.H., Graf, D., and Knaus, P. BMPs: from bone to body morphogenetic proteins. *Sci Signal* **3**, mr1, 2010.
 16. Tsuji, K., Bandyopadhyay, A., Harfe, B.D., Cox, K., Kakar, S., Gerstenfeld, L., Einhorn, T., Tabin, C.J., and Rosen, V. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet* **38**, 1424, 2006.
 17. Smoljanovic, T., Bojanic, I., and Delimar, D. Adverse effects of posterior lumbar interbody fusion using rhBMP-2. *Eur Spine J* **18**, 920, 2009.
 18. Lee, K.B., Taghavi, C.E., Murray, S.S., Song, K.J., Keorochana, G., and Wang, J.C. BMP induced inflammation: a comparison of rhBMP-7 and rhBMP-2. *J Orthop Res* **30**, 1985, 2012.
 19. Leknes, K.N., Yang, J., Qahash, M., Polimeni, G., Susin, C., and Wikesjo, U.M. Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2: radiographic observations. *Clin Oral Implants Res* **19**, 1027, 2008.
 20. Olivares-Navarrete, R., Hyzy, S.L., Park, J.H., Dunn, G.R., Haithcock, D.A., Wasilewski, C.E., Boyan, B.D., and Schwartz, Z. Mediation of osteogenic differentiation of human mesenchymal stem cells on titanium surfaces by a Wnt-integrin feedback loop. *Biomaterials* **32**, 6399, 2011.
 21. Zhao, G., Raines, A.L., Wieland, M., Schwartz, Z., and Boyan, B.D. Requirement for both micron- and submicron scale structure for synergistic responses of osteoblasts to substrate surface energy and topography. *Biomaterials* **28**, 2821, 2007.
 22. Rupp, F., Scheideler, L., Olshanska, N., deWild, M., Wieland, M., and Geis-Gerstorfer, J. Enhancing surface free energy and hydrophilicity through chemical modification of microstructured titanium implant surfaces. *J Biomed Mater Res A* **76A**, 323, 2006.
 23. Wikesjo, U.M., Xiropaidis, A.V., Qahash, M., Lim, W.H., Sorensen, R.G., Rohrer, M.D., Wozney, J.M., and Hall, J. Bone formation at recombinant human bone morphogenetic protein-2-coated titanium implants in the posterior mandible (Type II bone) in dogs. *J Clin Periodontol* **35**, 985, 2008.
 24. Wikesjo, U.M., Qahash, M., Polimeni, G., Susin, C., Shanaman, R.H., Rohrer, M.D., Wozney, J.M., and Hall, J. Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2: histologic observations. *J Clin Periodontol* **35**, 1001, 2008.
 25. Olivares-Navarrete, R., Hyzy, S.L., Haithcock, D.A., Cundiff, C.A., Schwartz, Z., and Boyan, B.D. Coordinated regulation of mesenchymal stem cell differentiation on microstructured titanium surfaces by endogenous bone morphogenetic proteins. *Bone* **73**, 208, 2015.
 26. Stagg, J., and Galipeau, J. Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. *Curr Mol Med* **13**, 856, 2013.
 27. McGuire-Goldring, M.K., Murphy, G., Gowen, M., Meats, J.E., Ebsworth, N.M., Poll, C., Reynolds, J.J., and Russell, R.G. Effects of retinol and dexamethasone on cytokine-mediated control of metalloproteinases and their inhibitors by human articular chondrocytes and synovial cells in culture. *Biochim Biophys Acta* **763**, 129, 1983.
 28. Gowen, M., Meikle, M.C., and Reynolds, J.J. Stimulation of bone resorption in vitro by a non-prostanoid factor released by human monocytes in culture. *Biochim Biophys Acta* **762**, 471, 1983.
 29. Kimble, R.B., Vannice, J.L., Bloedow, D.C., Thompson, R.C., Hopfer, W., Kung, V.T., Brownfield, C., and Pacifici, R. Interleukin-1 receptor antagonist decreases bone loss and bone resorption in ovariectomized rats. *J Clin Invest* **93**, 1959, 1994.
 30. Kimble, R.B., Bain, S., and Pacifici, R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. *J Bone Miner Res* **12**, 935, 1997.
 31. Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., and Einhorn, T.A. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* **88**, 873, 2003.
 32. Meier, R.P., Muller, Y.D., Morel, P., Gonelle-Gispert, C., and Buhler, L.H. Transplantation of mesenchymal stem cells for the treatment of liver diseases, is there enough evidence? *Stem Cell Res* **11**, 1348, 2013.
 33. Eckert, M.A., Vu, Q., Xie, K., Yu, J., Liao, W., Cramer, S.C., and Zhao, W. Evidence for high translational potential of mesenchymal stromal cell therapy to improve recovery from ischemic stroke. *J Cereb Blood Flow Metab* **33**, 1322, 2013.
 34. English, K., and Wood, K.J. Mesenchymal stromal cells in transplantation rejection and tolerance. *Cold Spring Harb Perspect Med* **3**, a015560, 2013.

35. Wang, L., Zhao, Y., and Shi, S. Interplay between mesenchymal stem cells and lymphocytes: implications for immunotherapy and tissue regeneration. *J Dent Res* **91**, 1003, 2012.
36. Olivares-Navarrete, R., Hyzy, S.L., Slosar, P.J., Schneider, J.M., Schwartz, Z., and Boyan, B.D. Implant materials generate different peri-implant inflammatory factors: poly-ether-ether-ketone promotes fibrosis and microtextured titanium promotes osteogenic factors. *Spine (Phila Pa 1976)* **40**, 399, 2015.
37. Hyzy, S.L., Olivares-Navarrete, R., Hutton, D.L., Tan, C., Boyan, B.D., and Schwartz, Z. Microstructured titanium regulates interleukin production by osteoblasts, an effect modulated by exogenous BMP-2. *Acta Biomater* **9**, 5821, 2013.
38. McKay, W.F., Peckham, S.M., and Badura, J.M. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). *Int Orthop* **31**, 729, 2007.
39. Wikesjo, U.M., Lim, W.H., Thomson, R.C., Cook, A.D., Wozney, J.M., and Hardwick, W.R. Periodontal repair in dogs: evaluation of a bioabsorbable space-providing macroporous membrane with recombinant human bone morphogenetic protein-2. *J Periodontol* **74**, 635, 2003.
40. Wikesjo, U.M., Guglielmoni, P., Promsudthi, A., Cho, K.S., Trombelli, L., Selvig, K.A., Jin, L., and Wozney, J.M. Periodontal repair in dogs: effect of rhBMP-2 concentration on regeneration of alveolar bone and periodontal attachment. *J Clin Periodontol* **26**, 392, 1999.
41. Lewandowski, K.U., Nanson, C., and Calderon, R. Vertebral osteolysis after posterior interbody lumbar fusion with recombinant human bone morphogenetic protein 2: a report of five cases. *Spine J* **7**, 609, 2007.
42. Olivares-Navarrete, R., Hyzy, S.L., Pan, Q., Dunn, G., Williams, J.K., Schwartz, Z., and Boyan, B.D. Osteoblast maturation on microtextured titanium involves paracrine regulation of bone morphogenetic protein signaling. *J Biomed Mater Res A* **103**, 1721, 2015.
43. Okamoto, T., Shibata, M., Tsuboi, S., Nakagaki, H., Fukuta, O., Kusabe, Y., and Inukai, J. Remineralization of primary tooth enamel from individuals with Down syndrome. *J Dent Child (Chic)* **78**, 43, 2011.
44. Hyzy, S.L., Olivares-Navarrete, R., Schwartz, Z., and Boyan, B.D. BMP2 induces osteoblast apoptosis in a maturation state and noggin-dependent manner. *J Cell Biochem* **113**, 3236, 2012.
45. Olivares-Navarrete, R., Hyzy, S.L., Chaudhri, R.A., Zhao, G., Boyan, B.D., and Schwartz, Z. Sex dependent regulation of osteoblast response to implant surface properties by systemic hormones. *Biol Sex Differ* **1**, 4, 2010.

Address correspondence to:

Barbara D. Boyan, PhD

School of Engineering

Virginia Commonwealth University

601 West Main Street

Suite 331

Richmond, VA 23284

E-mail: bboyan@vcu.edu

Received: January 3, 2017

Accepted: March 15, 2017

Online Publication Date: May 19, 2017