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Coordinated regulation of mesenchymal stem cell differentiation on microstructured titanium surfaces by endogenous bone morphogenetic proteins

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ABSTRACT

Human mesenchymal stem cells (MSCs) differentiate into osteoblasts on microstructured titanium (Ti) surfaces without addition of medium supplements, suggesting that surface-dependent endogenous mechanisms are involved. They produce bone morphogenetic proteins (BMPs), which regulate MSC differentiation and bone formation via autocrine/paracrine mechanisms that are modulated by changes in BMP mRNA and protein, receptors, and inhibitors (Noggin, Cerberus, Gremlin 1, and Chordin). We examined expression of BMPs, their receptors and their inhibitors over time and used BMP2-silenced cells to determine how modulating endogenous BMP signaling can affect the process. MSCs were cultured on tissue culture polystyrene or Ti [PT (Ra < 0.4 μm); sandblasted/acid-etched Ti (SLA, Ra = 3.2 μm); or hydrophilic-SLA (modSLA)]. BMP mRNAs and proteins increased by day 4 of culture. Exogenous BMP2 increased differentiation whereas differentiation was decreased in BMP2-silenced cells. Noggin was regulated by day 2 whereas Gremlin 1 and Cerberus were regulated after 6 days. Osteoblastic differentiation increased in cells cultured with blocking antibodies against Noggin, Gremlin 1, and Cerberus. Endogenous BMPs enhance an osteogenic microenvironment whereas exogenous BMPs are inhibitory. Antibody blocking of the BMP2 inhibitor Cerberus resulted in IL-6 and IL-8 levels that were similar to those observed when treating cells with exogenous BMP2, while antibodies targeting the inhibitors Gremlin or Noggin did not. These results suggest that microstructured titanium implants supporting therapeutic stem cells may be treated with appropriately selected agents antagonistic to extracellular BMP inhibitors in order to enhance BMP2 mediated bone repair while avoiding undesirable inflammatory side effects observed with exogenous BMP2 treatment.

Introduction

Bone formation, healing, and regeneration are complex processes orchestrated by osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. This requires a broad array of molecules that regulate cell differentiation, extracellular matrix synthesis, mineral deposition, and bone formation as well as bone remodeling. Several molecules influence osteoblastic differentiation in vitro and bone formation in vivo in both preclinical models and clinically [1]. Among these molecules, bone morphogenetic proteins 2, 4, and 7 (BMP2, BMP4, BMP7) are osteoinductive [2].

BMPs orchestrate several cellular functions such as cell proliferation, differentiation, transformation, and apoptosis in embryonic and adult cells via autocrine/paracrine mechanisms [2]. A complex system exists to ensure that these pleiotropic actions are regulated tightly both spatially and temporally. BMP molecules must dimerize to signal, either as heterodimers or as homodimers [3]. BMP receptors are heterodimers [2], providing additional levels of control with respect to availability of functional subunits. In addition, a number of intracellular proteins transduce the signal to the nucleus [3] and the relative availability of each of these may help modulate the rate and extent of the signaling cascade. Soluble inhibitors such as Noggin, Gremlin1, Cerberus, and Chordin physically bind to BMPs thereby regulating BMP signaling by preventing their dimerization and transduction of downstream events [4].

Recombinant human BMP2 is used clinically to induce bone formation in hard-to-heal fractures and bony defects in dental and orthopedic applications [5,6]. Although it has been demonstrated to increase osteogenesis in lumbar spine fusions [7,8], inflammatory complications including seroma and osteolysis have been reported [9–12]. Titanium (Ti) dental

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implants that were dip-coated with BMP2 at concentrations typically used in spine fusions also caused bone resorption in the mandible [10]. There are a number of possible reasons for this, but stimulatory effects of BMP2 on production of pro-inflammatory mediators may be a contributing factor.

A series of in vitro studies support this hypothesis. Osteoblasts cultured on microtextured Ti substrates produced higher levels of anti-inflammatory cytokines and lower levels of pro-inflammatory cytokines than cells on smooth Ti surfaces [13]. In addition, osteoblasts cultured on microstructured Ti or Ti alloy surfaces produced higher levels of BMP2, BMP4, and BMP7 [14,15], suggesting that osteoblast differentiation on the microtextured surfaces was due to intrinsic production of these osteoinductive proteins. Importantly, production of BMP inhibitors also increased on the microtextured substrates, providing a mechanism for regulating their paracrine action. However, when osteoblasts on microtextured Ti surfaces were treated with exogenous BMP2, production of pro-inflammatory cytokines increased and production of anti-inflammatory cytokines decreased [13]. These results indicate that the intrinsic regulation of endogenous BMP2 signaling afforded by BMP2 inhibitors was insufficient to modulate the inflammatory effects of exogenous BMP2. These same microtextured Ti surface features result in reduced healing time and improved bone-to-implant contact clinically [16], suggesting that modifying paracrine BMP signaling may yield more robust bone formation than application of exogenous BMPs. In vivo studies using RNA interference to knock down the BMP antagonist Noggin [17] showed enhanced bone formation, supporting this hypothesis. Inhibition of endogenous Noggin enhanced osteoblast maturation on microtextured Ti surfaces in vitro [15] and the BMP2 antagonist inhibitor L519 enhanced the osteogenic potential of BMP2 [17]. Thus, modifying paracrine BMP signaling may yield more robust bone formation than application of exogenous BMPs.

The stimulatory effects of microtexture on osteoblast differentiation are observed in cultures of human mesenchymal stem cells (MSCs), even in the absence of exogenous factors or osteogenic media, and this effect of surface microtopography is enhanced on rough hydrophilic Ti surfaces [18]. This raises the question of whether substrate-dependent endogenous BMP signaling is involved. Moreover, knockdown of Chordin has been shown to enhance osteogenic differentiation of MSCs on TCPS [19], suggesting that the effects of Ti surface microtopography on multipotent osteoprogenitor cells may also be modulated by manipulating levels of BMP inhibitors.

The purpose of this study was to examine the role of endogenous BMP in the regulation of peri-implant bone formation by assessing the expression and production of proteins involved in regulation of BMP action in osteoblastic differentiation of human MSCs cultured on microstructured Ti substrates. MSCs were cultured on Ti surfaces with two different surface topographies: a relatively smooth surface (PT) and a complex grit blasted and acid etched (SLA) surface. In addition, the role of surface chemistry was examined by culturing MSCs on Ti with a topography identical to the grit blasted/acid etched surface but with a hydrophilic chemistry (modSLA). Expression and protein levels for a number of proteins involved in BMP signaling, including BMPs and BMP receptor subunits and inhibitors, were determined, as were markers of osteoblastic differentiation, modulators of bone remodeling and angiogenesis, and inflammatory mediators. To assess the specific role of BMP2, MSCs stably silenced for BMP2 were generated. Finally, to determine whether inhibition of BMP2 action is involved in modulating BMP2 action, cells were treated with exogenous Noggin in addition to blocking the effects of BMP inhibitors using specific antibodies.

Materials and methods

Cell culture

Human bone marrow-derived MSCs (Lonza Biosciences, Walkersville, MD) plated at a density of 10,000 cells per cm^2 were cultured in Mesenchymal Stem Cell Growth Media (MSCGM, Lonza Biosciences) at 37 °C, 5% CO_2 and 100% humidity for all experiments.

Titanium disks

Ti disks were prepared from 1 mm thick sheets of grade 2 unalloyed Ti (ASTM F67 "Unalloyed Ti for surgical implant applications") and provided by Institut Straumann AG (Basel, Switzerland). Disks were punched to 15 mm in diameter to fit snugly into the well of a 24-well tissue culture plate. The fabrication method and characterization of the resulting morphology have been reported previously [20,21]. Briefly, smooth Ti surfaces (PT) have a mean peak-to-valley roughness (R_a) of 40 nm. To create SLA surfaces, PT surfaces are grit blasted and acid etched to create topography with craters (100 μm diameter) overlaid with pits (1–3 μm diameter) and coated with spikes (700 nm diameter). The resulting surface has a R_a of 3.2 μm. SLA surfaces were fabricated in a nitrogen environment to prevent exposure to air, and packaged in a sealed glass tube with isotonic saline to minimize exposure to the ambient atmosphere, thus retaining high surface energy, to create the modSLA surface. Disks were sterilized by gamma irradiation at 25 kGy overnight.

Quantification of secreted BMPs

MSCs were plated on tissue culture polystyrene (TCPS) or on Ti disks (PT, SLA, or modSLA). Media were changed 24 h after plating and every 48 h thereafter until cultures reached confluence on TCPS, typically after 7 days of culture. At confluence, cells were incubated with fresh media for 24 h. Secreted osteocalcin, BMP2, BMP4, and BMP7 levels were measured in the collected conditioned media as described below. Results are presented as normalized to total cell number.

BMP mRNA levels

MSCs were plated on TCPS or on Ti disks. For studies at confluence, RNA was isolated from cultures (TRIzol® Reagent, Life Technologies, Carlsbad, CA). RNA was quantified by spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA) and 250 ng RNA reverse transcribed using random primers (High Capacity cDNA Reverse Transcription kit, Life Technologies). For time course studies, RNA was isolated from cells after 2, 4, or 6 days in culture (Ambion RNAqueous®-Micro Kit, Life Technologies) and 125 ng RNA reverse transcribed as above.

Levels of mRNA were quantified with real-time quantitative PCR (StepOnePlus, Life Technologies) using gene-specific primers. Primers (Table 1) were designed using Beacon designer software and synthesized by Eurofins MWG Operon (Huntsville, AL); the primers for Noggin were purchased from Qiagen (QuantiTect Primer Assay, QT00210833). Fluorescence values were related to starting mRNA quantities using known dilutions of MSCs grown on TCPS. mRNA levels for each gene are presented as normalized to mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

BMP2 silencing

MSCs were transduced with shRNA lentiviral particles (NM_001200 TRCN0000058193, Mission®, Sigma Aldrich, St. Louis, MO) to establish stably silenced MSC cultures (shBMP2-MSC). MSCs were plated at 20,000 cells per cm^2 and cultured overnight in MSCGM. Particles were added to the cells at a multiplicity of infection of 5.0 in MSCGM supplemented with 8 mg/ml hexadimethrine bromide (Sigma Aldrich) and incubated for 18 h. After incubation, transduced cells were selected with MSCGM containing 0.25 μg/ml puromycin. Silencing of BMP2 was confirmed using real-time qPCR and ELISA as described. MSCs or shBMP2 MSCs were cultured on TCPS or Ti substrates. Culture medium was changed 24 h after plating and every 48 h thereafter until cultures reached confluence on TCPS. Cells were incubated with fresh MSCGM