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Mechanisms regulating increased production of osteoprotegerin by osteoblasts cultured on microstructured titanium surfaces

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ABSTRACT

Osteoblasts grown on microstructured Ti surfaces enhance osteointegration by producing local factors that regulate bone formation as well as bone remodeling, including the RANK ligand decoy receptor osteoprotegerin (OPG). The objective of this study was to explore the mechanism by which surface microstructure and surface energy mediate their stimulatory effects on OPG expression. Titanium disks were manufactured to present different surface morphologies: a smooth pretreatment surface (PT, Ra < 0.2 μm), microstructured sandblasted/acid etched surface (SLA, Ra = 3–4 μm), and a microstructured Ti plasma-sprayed surface (TPS, Ra = 4 μm). Human osteoblast-like MG63 cells were cultured on these substrates and the regulation of OPG production by TGF-β1, PKC, and α2β1 integrin signaling determined. Osteoblasts produced increased amounts of OPG as well as active and latent TGF-β1 and had increased PKC activity when grown on SLA and TPS. Exogenous TGF-β1 increased OPG production in a dose-dependent manner on all surfaces, and this was prevented by adding blocking antibody to the TGF-β type II receptor or by reducing TGF-β1 binding to the receptor by adding exogenous soluble type II receptor. The PKC inhibitor chelerythrine inhibited the production of OPG in a dose-dependent manner, but only in cultures on SLA and TPS. shRNA knockdown of α2 or a double knockdown of α2β1 also reduced OPG, as well as production of TGF-β1. These results indicate that substrate-dependent OPG production is regulated by TGF-β1, PKC, and α2β1 and suggest a mechanism by which α2β1 signaling increases PKC, resulting in TGF-β1 production and TGF-β1 then acts on its receptor to increase transcription of OPG.

1. Introduction

Numerous studies have shown that osteoblasts exhibit a more differentiated phenotype when grown on titanium (Ti) substrates with micron scale roughness than when grown on smooth Ti substrates or on tissue culture polystyrene [1–4]. In addition to producing a collagen-rich extracellular matrix [5], increased alkaline phosphatase activity and elevated levels of osteocalcin [6], osteoblasts produce increased levels of local factors on these surfaces, including prostaglandins E1 and E2 (PGE2) [7] and transforming growth factor beta-1 (TGF-β1) [8,9]. Recent studies have also shown that the more differentiated osteoblasts produce increased levels of factors that stimulate vasculogenesis [10] and factors that decrease osteoclastic activity such as the RANK ligand (RANKL) decoy receptor osteoprotegerin (OPG) [11,12]. These observations indicate that growth on microstructured Ti promotes osteogenesis over resorption during peri-implant bone formation in vivo, and this hypothesis has been supported by preclinical and clinical studies showing increased bone-to-implant contact and increased pull out strength [13,14].

There is an increasing understanding of the mechanisms by which surface microstructure modulates cell response. Integrin signaling clearly plays a major role. Expression of α2 and β1 integrin subunits increases when osteoblasts are grown on microstructured surfaces [15] and silencing of these integrins blocks the substrate-dependent increases in osteoblast differentiation and local factor production [16,17]. Autocrine/paracrine mechanisms also appear to be involved. Treatment with cyclooxygenase (COX) inhibitors to block either COX-1 or COX-2 dependent prostaglandin production reduces the stimulatory effect of microstructured surfaces on osteoblast differentiation [18,19].

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Other studies have implicated protein kinase C (PKC) signaling in the response of osteoblasts to substrate microstructure [9]. Inhibition of PKC activity by chelerythrine results in increased levels of PGE_2 in the conditioned media of MG63 cells, particularly when the cells are cultured on Ti substrates with micron scale and submicron scale features. In addition, PKC signaling is involved in the regulation of TGF-β1 by osteoblasts; inhibition of PKC results in increased levels of the growth factor in the media and this increase is greatest in cultures grown on microstructured Ti substrates.

TGF-β1 may also mediate effects of surface microstructure. TGF-β1 stimulates extracellular matrix synthesis by osteoblasts and increases alkaline phosphatase activity [20], indicating that it promotes osteoblastic differentiation. However, TGF-β1 has also been shown to stimulate osteoprogenitor cell proliferation [21] and to block terminal differentiation of osteoblasts [22]. In addition to its direct effects on osteoblast differentiation, TGF-β1 promotes osteoclast survival [23] but inhibits bone remodeling [8], suggesting that it does so indirectly by stimulating osteoblasts to produce factors like OPG [24,25], which prevent contact-dependent activation of new osteoclasts by binding RANKL on the osteoblast surface [11].

These observations demonstrate that TGF-β1 has pleiotropic effects on osteoblasts due in part to its local concentration and in part to the maturation state of the responding cell. When osteoblasts are cultured on microstructured Ti substrates, they have more TGF-β1 in their conditioned media than osteoblasts cultured on tissue culture polystyrene (TCPS) or smooth Ti, and they incorporate more TGF-β1 into their extracellular matrix on rougher surfaces [9]. The fact that PKC inhibition results in increased media levels of TGF-β1 suggests that PKC may be involved in controlling TGF-β1 availability in the cell layer, thereby modulating TGF-β1-dependent actions on the cell.

The purpose of this study was to determine the mechanisms by which surface structure mediates its effects on OPG production by osteoblasts. We hypothesized that OPG content of the conditioned media is regulated by a mechanism that involves signaling by α2β1 and PKC resulting in production of TGF-β1 and downstream TGF-β1-mediated OPG expression. To test this hypothesis, we used an in vitro model in which MG63 osteoblast-like cells are cultured on two different microstructured Ti surfaces, one that is produced by grit-blasting followed by an acid etch (SLA) and one that results from coating Ti with irregular projections produced by titanium plasma spray (TPS).

2. Methods

2.1. Cell culture model

MG63 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50 μg/ml ascorbic acid. Cells were grown on TCPS or one of three Ti different substrates described in detail previously [6,7] and described briefly below. Ti disks that were 1.5 cm in diameter were fabricated by Institut Straumann AG (Basel, Switzerland) to fit a 24-well culture dish, and resulting in six separate cultures per surface. The pretreatment (PT) Ti disk surface had an overall average roughness of <0.02 μm and was produced by machining the surface to a uniform texture. The PT disks were grit blasted and acid etched to produce a complex topography (SLA) characterized by craters approximately 30–100 μm in diameter overlaid with pits approximately 1–3 μm in diameter, with an overall roughness of $Ra = 3.2$ μm. In addition, PT disks were coated via titanium plasma spray (TPS) to produce a surface with an overall roughness of $Ra = 5.2$ μm that was characterized by irregular projections. Thus, SLA and TPS both had micron scale and submicron scale roughness, but the morphology of the surfaces was very different.

MG63 cells were cultured on TCPS and the three disk surfaces to confluence, at which time they were treated as described below.

2.2. Effect of surface microstructure on OPG production

Initial studies validated the culture model by assessing the effects of surface microstructure on cell number and osteocalcin levels in the conditioned media as described previously [12]. The levels of latent and active TGF-β1 in the conditioned media of the cells were assessed using an immunoassay kit according to the manufacturer's directions (R&D Systems, Minneapolis, MN), as described previously [26]. An aliquot of the conditioned media was removed and assayed without prior acidification to determine the levels of active TGF-β1. Total TGF-β1 was determined following acid activation of a second aliquot of conditioned media. Latent TGF-β1 was assessed by subtracting active growth factor from the total amount. OPG was quantified by immunoassay of the conditioned media as per manufacturer's directions (R&D Systems). Osteocalcin, TGF-β1, and OPG were normalized to cell number.

2.3. Regulation of OPG by TGF-β1

To determine if exogenous TGF-β1 could stimulate OPG production in a surface-dependent manner, confluent cultures were treated for 24 h with media containing 0.22 ng/ml TGF-β1 (R&D Systems). This dose was based on previous work on the effect of TGF-β1 on growth plate chondrocytes, which showed that 0.22 ng/ml stimulated matrix vesicle alkaline phosphatase activity in cultures of hypertrophic cells [9] and on MG63 cells showing that concentrations of TGF-β1 as low as 0.1 ng/ml activated matrix vesicle alkaline phosphatase [26]. Although higher concentrations of TGF-β1 also stimulated alkaline phosphatase activity, they blocked the stimulatory effect of $1\alpha,25(OH)_2D_3$ on osteocalcin production by the MG63 cells, indicating that they had the potential to prevent differentiation on the microstructured substrates. OPG content of the conditioned media was determined as above.

To test the hypothesis that endogenously produced TGF-β1 could stimulate OPG production in an autocrine manner, confluent cultures were treated for 24 h with 2 μg/ml non-specific IgG1 (R&D Systems) or 2 μg/ml anti-TGF-β1 type II receptor antibody (R&D Systems). Alternatively, cells were grown in the presence of 100 μg/ml soluble type II receptor (R&D Systems), which acted as a decoy receptor for the growth factor, effectively reducing its availability for binding to the cell surface type II receptor.

2.4. Role of PKC signaling

TGF-β1 exerts its effects on osteoblasts via PKC signaling [27], in addition to SMAD signaling [28]. Confluent cultures were treated for 24 h with the protein kinase C (PKC) inhibitor chelerythrine (10 μM) (Calbiochem, San Diego, CA), based on studies showing that a 24 h treatment with 10 μM chelerythrine caused a small increase in TGF-β1 and a marked increase in PGE_2 in the conditioned media of cultures of MG63 cells grown on microstructured Ti.

2.5. Requirement for α2β1 integrin signaling

To determine if signaling via the α2 integrin subunit was required for OPG expression, we took advantage of an MG63 cell line that was stably silenced using α2 shRNA [17], as described briefly below. The α2 integrin shRNA targets 21 bases starting at base 3406 of the α2 gene (NM-002203.3). After annealing the oligonucleotides, the fragments were cloned into a pSuppressorNeo vector containing a U6 promoter with a GeneSupressorTM system (IMGENEX Corp., San Diego, CA) following the manufacturer's instructions. MG63 cells were transfected with plasmids containing the α2 shRNA template. Controls included cells treated with empty vector or with a plasmid containing scrambled shRNA. Silencing was assessed by Western blot analysis. MG63-α2, transfected with plasmid P4-1, exhibited a consistent 70% reduction in the α2 integrin subunit. Permanent cell lines were established using 600 mg/ml of the antibiotic G418 (Invitrogen, Carlsbad, CA).

α2 Partners with β1, and β1-silenced MG63 cells exhibited a loss of response to microstructured and high energy Ti surfaces [16]. To verify that α2β1 was the integrin pair required for OPG expression, we constructed a double knockdown cell line. Because the α2 cell line was established using a plasmid with G418 sulfate resistance, we chose to use a lentiviral delivery system with puromycin resistance to silence integrin β1 in these cells. To establish the double knockdown, α2 silenced cells were plated at 20,000 cells/cm^2 and incubated overnight at 37 °C in a 5% CO2 and 100% humidity atmosphere. Lentiviral transduction particles (Sigma–Aldrich) containing shRNA sequences specific to the integrin β1 gene (NM-002211.3) were added to the integrin-α2 silenced cells at 7.5 MOI and incubated for 18 h. After incubation, transduced cells were selected with 0.25 μg/ml of puromycin (Sigma–Aldrich) for 12 days. After puromycin selection, α2β1-silenced cells were fed with either 600 μg/ml of G418 sulfate or 0.25 μg/ml of puromycin every 48 h. α2β1-silenced cells exhibited a 70% reduction in the α2 integrin subunit and a 65% reduction in the β1 integrin subunit, based on real time PCR and Western blot (data not shown).

Wild type and knockdown cells were cultured on TCPS, PT and SLA surfaces for this study. At confluence one half of the cultures were treated for 24 h with 10^{-8} M $1\alpha,25$-dihydroxyvitamin D3 [$1\alpha,25(OH)_2D_3$], which regulates transcription of TGF-β1 [29] and OPG [30]. Control cultures were treated with the $1\alpha,25(OH)_2D_3$ vehicle, which was 0.05% ethanol.