Published in final edited form as:

Biomaterials. 2009 July ; 30(20): 3390–3396. doi:10.1016/j.biomaterials.2009.03.047.

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<0.2μm), microstructured sandblasted/acid etched surface (SLA, Ra=3–4μm), and a microstructured Ti plasma-sprayed surface (TPS, Ra=4μm). 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.

Keywords

Osteoblast; TGF-β1; Osteoprotegerin; Titanium; Microtopography

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

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Ti substrates or on tissue culture polystyrene [1-4]. In addition to producing a collagenrich 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- β 1) [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 α 2 and β 1 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].

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 PGE₂ 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- β 1 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- β 1 may also mediate effects of surface microstructure. TGF- β 1 stimulates extracellular matrix synthesis by osteoblasts and increases alkaline phosphatase activity [20], indicating that it promotes osteoblastic differentiation. However, TGF- β 1 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- β 1 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- β 1 has pleiotropic effects on osteoblasts due in part to its local concentration and in part to maturation state of the responding cell/ When osteoblasts are cultured on microstructured Ti substrates, they have more TGF- β 1 in their conditioned media than osteoblasts cultured on tissue culture polystyrene (TCPS) or smooth Ti, and they incorporate more TGF- β 1 into their extracellular matrix on rougher surfaces [9]. The fact that PKC inhibition results in increased media levels of TGF- β 1 suggests that PKC may be involved in controlling TGF- β 1 availability in the cell layer, thereby modulating TGF- β 1-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 α 2 β 1 and PKC resulting in production of TGF- β 1 and downstream TGF- β 1-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).

METHODS

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 to 100 µm in diameter overlaid with pits approximately 1-3 µm in diameter, with an overall roughness of $R_a = 3.2$ µm. In addition, PT disks were coated via titanium plasma spray (TPS) to produce a surface with an overall roughness of $R_a = 5.2$ 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.

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 [26]. 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.

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 hours 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 [27]. Although higher concentrations of TGF-β1 also stimulated alkaline phosphatase activity, they blocked the stimulatory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ 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 hours with 2 µg/ml nonspecific 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.

Role of PKC Signaling

TGF- β 1 exerts its effects on osteoblasts via PKC-signaling [28], in addition to SMAD signaling [29]. Confluent cultures were treated for 24 hours with the protein kinase C (PKC) inhibitor chelerythrine (10 μ M) (Calbiochem, San Diego, CA), based on studies showing that a 24 hour treatment with 10 μ M chelerythrine caused a small increase in TGF- β 1 and a marked increase in PGE₂ in the conditioned media of cultures of MG63 cells grown on microstructured Ti.

Requirement for α 2 β 1 Integrin Signaling

To determine if signaling via the α 2 integrin subunit was required for OPG expression, we took advantage of an MG63 cell line that was stably silenced using α 2 shRNA [17], as described briefly below. The α 2 integrin shRNA targets 21 bases starting at base 3406 of the α 2 gene (NM-002203.3). After annealing the oligonucleotides, the fragments were cloned into a pSuppressorNeo vector containing a U6 promoter with a GeneSuppressorTM system (IMGENEX Corp., San Diego, CA) following the manufacturer's instructions. MG63 cells were transfected with plasmids containing the α 2 shRNA template. Controls included cells treated with empty vector or with a plasmid containing scrambled shRNA. Silencing was assessed by Western blot analysis. MG63- α 2, transfected with plasmid P4-1, exhibited a consistent 70% reduction in the α 2 integrin subunit. Permanent cell lines were established using 600 mg/ml of the antibiotic G418 (Invitrogen, Carlsbad, CA).

α 2 partners with β 1, and β 1-silenced MG63 cells exhibited a loss of response to microstructured and high energy Ti surfaces [16]. To verify that α 2 β 1 was the integrin pair required for OPG expression, we constructed a double knockdown cell line. Because the α 2 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 β 1 in these cells. To establish the double knockdown, α 2 silenced cells were plated at 20,000 cells/cm² 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 β 1 gene (NM-002211.3) were added to the integrin- α 2 silenced cells at 7.5 MOI and incubated for 18 hours. After incubation, transduced cells were selected with 0.25 μ g/mL of puromycin (Sigma-Aldrich) for 12 days. After puromycin selection, α 2 β 1 silenced cells were fed with either 600 μ g/mL of G418 sulfate or 0.25 μ g/mL of puromycin every 48 hours. α 2 β 1 silenced cells exhibited a 70% reduction in the α 2 integrin subunit and a 65% reduction in the β 1 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 hours with 10⁻⁸ M 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], which regulates transcription of TGF- β 1 [30] and OPG [31]. Control cultures were treated with the 1 α ,25(OH)₂D₃ vehicle, which was 0.05% ethanol.

RESULTS

The osteoblast cultures used in these studies exhibited the expected decrease in cell number and increase in osteocalcin when grown on microstructured Ti substrates (Fig 1a,b). Levels of latent and active TGF- β 1 were increased in the conditioned media of cultures grown on the SLA and TPS disks, both of which had rough microtopographies (Fig 1c). Moreover, the relative amount of active growth factor increased in the conditioned media of cells grown on these surfaces.

OPG was produced in greater amounts on all Ti substrates compared to TCPS (Fig 2a). The amount of OPG in the media was greatest in cultures grown on SLA and TPS in most experiments (Fig 2b,c). Treatment with exogenous TGF- β 1 caused a 20 to 40% increase in OPG levels on all substrates, independent of surface properties (Fig 2a). The autocrine effect of TGF- β 1 on OPG production was mediated by the TGF- β type II receptor. Treatment with soluble type II receptor reduced OPG production by 50% on all surfaces, including TCPS (Fig 2b). Whereas IgG1 had no effect on OPG production, antibodies to TGF- β 1 type II receptor reduced OPG production on all substrates (Fig 2c). There was a 50% reduction on TCPS and PT and approximately 60% on SLA, but the inhibitory effect of antibody treatment was less robust in cultures grown on TPS.

Inhibition of PKC activity also reduced OPG production (Fig 3). PKC specific activity was reduced by approximately 50% in cultures grown on SLA. The effect on TPS was less robust, causing a 33% reduction in production.

The effects of surface microstructure on OPG were mediated by α 2 β 1 integrin signaling. In MG63 cells silenced for α 2, there was a decrease in active TGF- β 1 only on the SLA surface compared to wild type cells (Fig 4a) and this was also the case for MG63 cells silenced for both α 2 and β 1 (Fig 4b). Similarly, OPG was reduced in MG63 cells grown on SLA silenced for α 2 alone (Fig 4c) and for α 2 β 1 (Fig 4d), when compared to wild type MG63 cells. The stimulatory effect of 1 α ,25(OH) $_2$ D $_3$ on TGF- β 1 (Fig 4a,b) and OPG (Fig 4c,d) was lost in the knockdown cells on all surfaces.

DISCUSSION

The results of this study show that osteoblasts produce increased levels of active TGF- β 1 when grown on microstructured substrates like SLA and TPS and that it acts as an autocrine regulatory factor with respect to production of OPG. Whereas MG63 cells grown on TCPS and PT produced latent TGF- β 1, levels of active growth factor were almost undetectable. In contrast, latent TGF- β 1 was increased by more than 100% in cultures grown on SLA and TPS and the cells also produced significant levels of active growth factor. The amount of TGF- β 1 produced on SLA and TPS was 0.4 ng/ml, comparable to the concentration of growth factor shown to induce chondrocyte differentiation [9] as well as osteoblast differentiation (0.16-10ng/ml) [32]. Addition of exogenous TGF- β 1 increased OPG production on all surfaces to a similar extent, demonstrating that if active growth factor had been present in the TCPS and PT cultures, the cells could have responded to it. Further evidence that TGF- β 1 acted in an autocrine manner is provided by the observation that antibodies to the TGF- β type II receptor prevented the stimulatory effects of SLA and TPS on OPG production as did the addition of soluble type II receptor to the cultures. Moreover, treatment of the cells with non-specific IgG1 had no effect on OPG production. These results support studies showing that OPG is regulated by TGF- β 1 in other models [33].

Both the antibody and the soluble receptor reduced OPG production by cells grown on TCPS and PT, indicating that baseline levels of OPG were also mediated by TGF- β 1 in the culture medium. We did not use charcoal stripped fetal bovine serum for these studies to ensure growth on SLA and TPS and the cells were treated with fresh culture medium containing the antibodies and soluble receptor; thus, all cultures received a low dose of TGF- β but because of the experimental design, all cultures in a given experiment experienced the same artifact.

Our results indicate that OPG is also regulated by α 2 β 1 integrin signaling. Knockdown of α 2 alone or α 2 plus β 1 resulted in a reduction in OPG levels in the conditioned medium but only in cultures grown on SLA and TPS. The α 2 β 1 integrin pair is required for osteoblast differentiation on microstructured Ti, based on antibody blocking studies [34] and studies in

which $\alpha 2$ and $\beta 1$ were silenced independently [16,17]. The present study suggests that the effects of $\alpha 2\beta 1$ on OPG involved TGF- $\beta 1$ at least to some extent. Both the single knockdown and double knockdown cells exhibited reduced levels of active TGF- $\beta 1$ in their conditioned media.

Interestingly, $1\alpha,25(\text{OH})_2\text{D}_3$ increased active TGF- $\beta 1$ in the media of WT cells. Previous studies indicated that $1\alpha,25(\text{OH})_2\text{D}_3$ did not alter the levels of latent TGF- $\beta 1$ produced by MG63 cells on TCPS or PT [32], although $1\alpha,25(\text{OH})_2\text{D}_3$ has been shown to activate latent growth factor [35]. This suggests that it caused the increase in the active form by inducing activation of existing latent TGF- $\beta 1$. In the present study, the $\alpha 2$ and $\alpha 2\beta 1$ silenced cells failed to exhibit $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent increases in TGF- $\beta 1$ or in $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent OPG, demonstrating that the response of the cells to systemic factors was also mediated by this integrin.

PKC signaling was also involved in the mechanism by which surface microstructure regulated OPG production. In the present study, inhibition of PKC with chelerythrine caused a reduction in OPG levels that was particularly evident in cells cultured on SLA and TPS. Interestingly, previous studies showed that a 24-hour treatment with chelerythrine increased TGF- $\beta 1$ levels in the conditioned media of MG63 cells by 100% to approximately to 16 ng/ml, although it was not determined if this increase was due latent or active growth factor [9]. Usually the active TGF- $\beta 1$ is 10% of the total growth factor produced by MG63 cells on microstructured Ti, so the level of active TGF- $\beta 1$ in this previous study should be 1.6ng/ml. Whereas low levels of TGF- $\beta 1$ stimulate differentiation of osteoblasts [36] and hypertrophic chondrocytes [9], higher levels block terminal differentiation of the cells. Given that OPG expression is a feature of well differentiated osteoblasts [37,38], it is possible that the higher levels of TGF- $\beta 1$ produced by the chelerythrine-treated cells were sufficient to inhibit OPG synthesis.

OPG production by osteoblasts during osteointegration is an important step since an increase in OPG with no change in soluble RANKL prevents bone resorption, resulting in net peri-implant bone formation, including in regions not in immediate contact with the implant. The present study indicates that the production of OPG depends on implant roughness, although the precise morphology of the microstructure may not be a critical factor, at least for this parameter. No differences were found between cells cultured on SLA and cells cultured on TPS. The mechanism by which OPG levels are controlled involves activation of the $\alpha 2\beta 1$ integrin receptor, which by activating phospholipase C (PLC), can lead to activation of PKC [39] (Fig 5). Changes in PKC can modulate the production and release of latent and active TGF- $\beta 1$. Active TGF- $\beta 1$ can then bind its cognate receptor to stimulate OPG expression, either via SMAD activation [29] or via PKC dependent signaling [28]. The time course used in our study involving a 24 hour treatment was sufficient for TGF- $\beta 1$ -dependent PKC expression to occur. Thus, PKC inhibition may have resulted in decreased OPG directly through this pathway as well as by blocking terminal differentiation of the osteoblasts.

Other studies in our lab have shown that PKA also mediates the effects of surface microstructure [40,41] and chelerythrine treatment stimulates PGE2 release on SLA and TPS [9]. However, specific inhibition of PKA did not affect TGF- $\beta 1$ levels in MG63 cells cultured on SLA and TPS, suggesting that the increase PGE2, which acts via PKA [40,41], was not involved in the effects of TGF- $\beta 1$ on OPG. This does not rule out an alternate mechanism involving PGE2 and PKA, however.

CONCLUSIONS

This study indicates that the increased levels of OPG in the media of osteoblasts grown on microstructured Ti surfaces are due to autocrine action of TGF- $\beta 1$ released by the cells in

response to $\alpha 2\beta 1$ signaling. Evidence for this was inhibition of the surface effects by blocking TGF- β binding to the TGF- β type II receptor with antibodies and by addition of soluble type II receptor to the media. Cells with reduced expression of the $\alpha 2$ subunit of the $\alpha 2\beta 1$ integrin exhibited reduced levels of OPG in their media. This suggested that $\alpha 2\beta 1$ signaling was responsible since $\alpha 2$ partners exclusively with $\beta 1$ and this hypothesis was confirmed using cells that were silenced for both the $\alpha 2$ and $\beta 1$ subunits. Moreover, the surface-dependent increase in the stimulatory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on TGF- $\beta 1$ and OPG was lost in the knockdown cells. Inhibition of PKC, which is involved in mediating the response of MG63 cells to surface microstructure, also decreased OPG levels, implicating the PKC signaling pathway in the mechanism.

ACKNOWLEDGEMENTS

The authors thank Institut Straumann AG (Basel, Switzerland) for providing PT, SLA and TPS disks for this study. The work was supported by NIH AR052102 and the ITI Foundation.

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Figure 1.

Effect of surface microstructure on TGF-β1 production by osteoblast-like cells. MG63 osteoblast-like cells were cultured on tissue culture polystyrene (TCPS) and Ti substrates: pretreatment (PT), grit-blasted and acid etched (SLA), or Ti plasma-sprayed (TPS). At confluence cell number was determined (A) and osteocalcin (B) and latent and active TGF-β1 (C) in the conditioned media measured by immunoassay. Data are means $\pm$ SEM for N=6 independent cultures per variable. The results are from a representative experiment, all with comparable results. * $p < 0.05$, Ti surface v. TCPS; • $p < 0.05$, SLA or TPS v. PT.

Figure 2.

Regulation of OPG production by TGF-β1. Confluent cultures of MG63 cells grown on TCPS, PT, SLA or TPS substrates were treated for 24 hours with 0.22 ng/ml TGF-β1 (A) and OPG content of the conditioned media measured by immunoassay. Alternatively, confluent cultures were treated with soluble TGF-β type II receptor (B) or with antibodies to the type II receptor (C). Data are means ± SEM for N=6 independent cultures per variable. The results are from a representative experiment, all with comparable results. *p<0.05, Ti surface v. TCPS; •p<0.05, SLA or TPS v. PT; #p<0.05, with type II receptor block v. without receptor block for each surface.

Figure 3.

Regulation of OPG production by PKC. Confluent cultures of MG63 cells grown on TCPS, PT, SLA or TPS substrates were treated for 24 hours with 10 μ M chelerythrine to inhibit PKC activity. OPG was measured in the conditioned media of the cells. Data are means $\pm$ SEM for N=6 independent cultures per variable. The results are from a representative experiment, all with comparable results. *p<0.05, Ti surface v. TCPS; •p<0.05, SLA or TPS v. PT; #p<0.05, with chelerythrine v. without chelerythrine for each surface.

Figure 4.

Role of $\alpha 2, \beta 1$ integrin signaling in substrate-dependent production of TGF- $\beta 1$ and OPG. Confluent cultures of MG63 cells stably silenced for $\alpha 2$ (A,C) or $\alpha 2, \beta 1$ (B,D) grown on TCPS, PT or SLA substrates were treated for 24 hours with $1\alpha, 25(\text{OH})_2\text{D}_3$. Active TGF- $\beta 1$ and OPG were measured in the conditioned media of the cells. Data are means $\pm$ SEM for N=6 independent cultures per variable. The results are from a representative experiment, all with comparable results. * $p < 0.05$, Ti surface v. TCPS; • $p < 0.05$, with $1\alpha, 25(\text{OH})_2\text{D}_3$ v. without $1\alpha, 25(\text{OH})_2\text{D}_3$; \$ $p < 0.05$, v. WT cells for each surface; # $p < 0.05$, v. WT + 1,25 for each surface.

Figure 5.

Mechanisms regulating substrate mediated OPG production. Osteoblasts interact with their substrate via $\alpha 2, \beta 1$. Activation of the integrin results in phospholipase C (PLC) activation, which acts on phosphatidylinositol to yield phosphoinositol-4,5-bisphosphate (PIP₂) and diacylglycerol (DAG). PIP₂ is phosphorylated, producing inositol-1,4,5-trisphosphate, which binds its receptor on the smooth endoplasmic reticulum, releasing Ca²⁺ into the cytoplasm where it binds protein kinase C (PKC). DAG recruits PKC to the membrane, initiating a phosphorylation cascade involving Raf, Mek, and ERK1/2 MAP kinase, which enters the nucleus and phosphorylates the transcription factor AP-1, resulting in transcription of the TGF- β 1 gene. TGF- β 1 mRNA moves to the cytoplasm and is transcribed, producing TGF- β 1, which is secreted into the extracellular matrix. TGF- β 1 binds its cognate receptor (TGF- β R), which then activates SARA and SMAD2/3. SMAD2/3 binds to SMAD4, enters the nucleus and initiates transcription of the OPG gene. PGE₂ is also produced in response to substrate microstructure and binds its cognate EP receptor. This activates a PKA-dependent phosphorylation cascade, which also can stimulate OPG transcription. OPG mRNA moves to the cytoplasm and is transcribed to yield OPG protein, which is then released into the extracellular space.