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Beta-1 Integrins Mediate Substrate Dependent Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on Osteoblasts

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Abstract

Surface microroughness increases osteoblast differentiation and enhances responses of osteoblasts to $1,25$ -dihydroxyvitamin D_3 [$1\alpha,25(\text{OH})_2\text{D}_3$]. β_1 integrin expression is increased in osteoblasts grown on Ti substrates with rough microarchitecture, and it is regulated by $1\alpha,25(\text{OH})_2\text{D}_3$ in a surface-dependent manner, suggesting that it has a role in mediating osteoblast response. Here, we silenced β_1 expression in MG63 human osteoblast-like cells using small interfering RNA (siRNA) and examined the responses of the β_1 -silenced osteoblasts to surface microtopography and $1\alpha,25(\text{OH})_2\text{D}_3$. MG63 cells were also treated with two different monoclonal antibodies to human β_1 to block ligand binding. β_1 -silenced MG63 cells grown on a tissue culture plastic had reduced alkaline phosphatase activity and levels of osteocalcin, transforming growth factor β_1 , prostaglandin E_2 , and osteoprotegerin in comparison with control cells. Moreover, β_1 -silencing inhibited the effects of surface roughness on these parameters and partially inhibited effects of $1\alpha,25(\text{OH})_2\text{D}_3$. Anti β_1 antibody AIIB2 had no significant effect on cell number and osteocalcin, but decreased alkaline phosphatase; MAB2253Z decreased cell number and alkaline phosphatase and increased osteocalcin in a dose-dependent manner. Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on cell number and alkaline phosphatase were reduced and effects on osteocalcin were increased. These findings indicate that β_1 plays a major and complex role in osteoblastic differentiation modulated by either surface microarchitecture or $1\alpha,25(\text{OH})_2\text{D}_3$. The results also show that β_1 mediates, in part, the synergistic effects of surface roughness and $1\alpha,25(\text{OH})_2\text{D}_3$.

Keywords

$1\alpha, 25(\text{OH})_2\text{D}_3$; β_1 integrin subunit; Ti surface roughness; osteoblasts

INTRODUCTION

We and others have shown that osteoblasts are sensitive to micron scale and submicron scale structural properties of their substrate. These studies, recently reviewed by Bachle and Kohal

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[1], demonstrate that osteoblast proliferation is decreased on surfaces with rougher microtopographies whereas differentiation is enhanced. The in vitro observations are correlated with in vivo assessments of pull-out strength. There is greater bone-to-implant contact when implants with rough microtopographies are used and greater torque is required to remove the implants after bone healing [2–4].

The effects of surface microstructure on osteoblast behavior depend on the actual topography, including the shape of any spikes and the dimensions of any craters. We have used photolithography to fabricate titanium substrates with specific microfeatures and have found that osteoblast differentiation is greater when the cells are cultured in the presence of craters between 30 to 100 micrometers in diameter that are overlaid with a lawn of pits formed by spikes less than 1 micrometer in height. These conditions approximate those found on commercial Ti surfaces that have been sand blasted followed by an acid etch procedure (SLA). The SLA surface has an overall average roughness (Ra) of 4–5 micrometers. Roughness can also be achieved by forming a coating of irregular protrusions using a Ti plasma spraying technique (TPS), resulting in an Ra of 5–7 micrometers. These relatively small differences in surface shape can result in altered surface chemistry, not only because different methods are used for producing them but also because of differences in resulting surface energy. Thus, the response of an osteoblast to its substrate reflects a combination of factors including the physical structure, the chemical environment, and surface energy.

To study the regulation of osteoblast phenotype by surface structural elements, we have taken advantage of the SLA and TPS substrates provided to us by Institut Straumann AG (Basel, Switzerland), as well as of the smooth pretreatment substrate (PT) used as a base for SLA and TPS fabrication. Because these substrates are produced using the same manufacturing conditions used to produce dental implants, quality control is high and the results we obtain are more readily comparable to clinical observations. Using these substrates, we have found that MG63 osteoblast-like cells, normal human osteoblasts (NHOb cells), and fetal rat calvarial cells exhibit similar behaviors on tissue culture polystyrene (plastic) and smooth PT surfaces with respect to proliferation and differentiation [5–7]. In addition, they exhibit a decrease in proliferation and an increase in differentiation when treated with $1\alpha,25(\text{OH})_2\text{D}_3$, that is typical of osteoblasts reported in the literature by many laboratories. $1\alpha,25(\text{OH})_2\text{D}_3$ reduces cell number and stimulates osteocalcin production. When these cells are cultured on SLA and TPS, however, cell number is reduced and the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on proliferation is less. Moreover, differentiation is markedly increased and it is further stimulated by $1\alpha,25(\text{OH})_2\text{D}_3$.

This is also seen for factors related to osteoclast recruitment and activation. Messenger RNA transcripts for osteoprotegerin (OPG) and secreted OPG protein are both increased in MG63 cells cultured on SLA and TPS and are further increased by treatment of the cells with $1\alpha,25(\text{OH})_2\text{D}_3$ [8]. In contrast, there is no change in RANK ligand (RANKL) mRNA or in the levels of soluble RANKL in the conditioned media of these cells.

MECHANISM OF $1\alpha,25(\text{OH})_2\text{D}_3$ ACTION

These observations suggest that the change in phenotype caused by growth on the microrough substrates alters the response of osteoblasts to regulatory factors like $1\alpha,25(\text{OH})_2\text{D}_3$. Osteoblasts grown on tissue culture plastic become more sensitive to $1\alpha,25(\text{OH})_2\text{D}_3$ as they become more differentiated [9–11], but why this is the case is not well understood. The fact that osteoblasts exhibit a more differentiated phenotype when cultured on SLA and TPS provided us with an opportunity to investigate how sensitivity to $1\alpha,25(\text{OH})_2\text{D}_3$ is regulated.

Our results indicate that part of the substrate effect is mediated by prostaglandin. MG63 cells produce low levels of PGE_2 or PGE_1 when cultured on plastic or smooth Ti substrates and this

is unchanged when the cells are treated with $1\alpha,25(\text{OH})_2\text{D}_3$ [12]. However, the amount of both prostanoids is increased when the cells are grown on SLA or TPS and treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ results in a synergistic increase in production [12]. Inhibition of prostanoid synthesis with indomethacin blocks the surface effect, including the response to $1\alpha,25(\text{OH})_2\text{D}_3$. Specific inhibition of constitutive cyclooxygenase-1 (Cox-1) as well as specific inhibition of inducible Cox-2 also reduce the effects of surface roughness and $1\alpha,25(\text{OH})_2\text{D}_3$, indicating that prostaglandin production via both pathways is required.

$1\alpha,25(\text{OH})_2\text{D}_3$ regulates osteoblasts through rapid protein kinase C (PKC) dependent signaling pathways mediated by the $1\alpha,25(\text{OH})_2\text{D}_3$ membrane receptor ERp60 [11] as well as traditional nuclear vitamin D receptor (VDR) mechanisms [13], suggesting that one or both of these may have been affected. We are presently investigating whether either of these receptors has altered expression or if one or more components required for their action is/are changed by growth of the cells on microrough substrates.

ROLE OF INTEGRINS

One hypothesis is that growth on substrates with microrough structural feature alters integrin expression and function. Because integrins signal through some of the same pathways as $1\alpha,25(\text{OH})_2\text{D}_3$, cross talk is possible. We found that mRNA levels for integrin subunits beta-1 (β_1), alpha-2 and alpha-5, but not for alpha-v or beta-3, were affected by substrate roughness and that the effects were specific to each subunit and varied with time in culture [14]. $1\alpha,25(\text{OH})_2\text{D}_3$ modulated levels of β_1 and the effects were greater on the rougher surface. β_1 is able to partner with alpha-2 and alpha-5, making it an excellent candidate for mediating the cross-talk between substrate-dependent integrin signaling and $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent signaling.

We used several methods to investigate the role of the β_1 integrin subunit. Our initial studies used antibodies to the β_1 integrin to block signaling [15], as has been typically done in the literature [16;17]. If antibodies are added to the culture medium together with the cells, they block adhesion. For this reason, MG63 cells were permitted to attach to the substrates and antibodies were added with the culture medium following attachment. Addition of anti β_1 antibody AIIB2 to cells cultured on SLA and TPS had no significant effect on cell number and osteocalcin, but decreased alkaline phosphatase; MAB2253Z decreased cell number and alkaline phosphatase and increased osteocalcin in a dose-dependent manner. Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on cell number and alkaline phosphatase were reduced and effects on osteocalcin were increased.

These results were initially confusing because they indicated that by blocking β_1 integrin function, the cells were actually more differentiated and had an even greater response to $1\alpha,25(\text{OH})_2\text{D}_3$. Related studies in our lab using PT and SLA substrates coated with polyethylene glycol and functionalized with an RGD-containing peptide demonstrated suggested that greater numbers of attachment sites might actually result in a less differentiated cell [18]. The behavior of MG63 cells on coated SLA could be restored to the behavior of these cells on PT and even tissue culture plastic simply by increasing the number of RGD peptides on the surface.

To overcome the limitations of the blocking antibody experimental design, we developed MG63 cell lines that stably expressed siRNA for the beta-1 integrin or a scrambled siRNA as a control [15]. β_1 -silenced MG63 cells grown on a tissue culture plastic had reduced alkaline phosphatase activity and levels of osteocalcin, transforming growth factor β_1 , prostaglandin E_2 , and osteoprotegerin in comparison with control cells. Moreover, β_1 -silencing inhibited the effects of surface roughness on these parameters.

While we anticipated that the response to surface microtopography would be affected, we were surprised to see that β_1 -silencing partially inhibited effects of $1\alpha,25(\text{OH})_2\text{D}_3$. As shown in

Figure 1a, β_1 siRNA reduced the stimulatory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on osteocalcin production on all surfaces. However, even though there was a marked reduction in osteocalcin production in cultures grown on SLA and TPS, the levels of this protein in the conditioned media was still elevated over that seen in cultures grown on plastic or PT. This was more evident when measuring OPG levels in the conditioned medium (Figure 1b). β_1 siRNA reduced the stimulatory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on all surfaces but the surface-dependent effect of $1\alpha,25(\text{OH})_2\text{D}_3$ was only partially reduced on SLA and TPS.

These results support the hypothesis that at least part of the interaction between surface topography and $1\alpha,25(\text{OH})_2\text{D}_3$ on osteoblast differentiation is mediated by cross-talk between the two signaling pathways: one initiated by integrin function and one initiated by $1\alpha,25(\text{OH})_2\text{D}_3$. A potential candidate for this is the ERK1/2 family of mitogen activated protein kinases. Integrin signaling can influence gene expression via ERK activation [5786] and we have shown that blocking activation of ERK1/2 with PD98059 affects osteoblast response to surface microstructure [19]. ERK1/2 also mediates some of the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ signaling via Erp60 [20] and others have shown that ERK1/2 participates in responses to $1\alpha,25(\text{OH})_2\text{D}_3$ via the VDR [21]. Thus, by modifying osteoblast differentiation via changes in substrate topography, resulting changes in ERK1/2 may modify the actions of $1\alpha,25(\text{OH})_2\text{D}_3$ through multiple mechanistic pathways.

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References

1. Bachle M, Kohal RJ. A systematic review of the influence of different titanium surfaces on proliferation, differentiation and protein synthesis of osteoblast-like MG63 cells. *Clin Oral Implants Res* 2004;15:683–692.
2. Albrektsson T, Wennerberg A. Oral implant surfaces: Part 1--review focusing on topographic and chemical properties of different surfaces and in vivo responses to them. *Int J Prosthodont* 2004;17:536–543. [PubMed: 15543910]
3. Cochran DL, Buser D, ten Bruggenkate CM, Weingart D, Taylor TM, Bernard JP, Peters F, Simpson JP. The use of reduced healing times on ITI implants with a sandblasted and acid-etched (SLA) surface: early results from clinical trials on ITI SLA implants *Clin. Oral Implants Res* 2002;13:144–153.
4. Khang W, Feldman S, Hawley CE, Gunsolley J. A multi-center study comparing dual acid-etched and machined-surfaced implants in various bone qualities. *J Periodontol* 2001;72:1384–1390. [PubMed: 11699480]
5. Boyan BD, Bonewald LF, Paschalis EP, Lohmann CH, Rosser J, Cochran DL, Dean DD, Schwartz Z, Boskey AL. Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int* 2002;6:519–529. [PubMed: 12232675]
6. Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J, Dean DD, Cochran DL, Boyan BD. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). *J Biomed Mater Res* 1995;29:389–401. [PubMed: 7542245]
7. Lohmann CH, Tandy EM, Sylvia VL, Hell-Vocke AK, Cochran DL, Dean DD, Boyan BD, Schwartz Z. Response of normal female human osteoblasts (NHOst) to 17beta-estradiol is modulated by implant surface morphology. *J Biomed Mater Res* 2002;62:204–213. [PubMed: 12209940]
8. Lossdorfer S, Schwartz Z, Wang L, Lohmann CH, Wieland M, Cochran DL, Boyan BD. Microrough Implant Surface Topographies Increase Osteogenesis by Reducing Osteoclast Formation and Activity. *J Biomed Mater Res* 2004;70:361–369.
9. Majeska RJ, Rodan GA. The effect of $1,25(\text{OH})_2\text{D}_3$ on alkaline phosphatase in osteoblastic osteosarcoma cells. *J Biol Chem* 1982;257:3362–3365. [PubMed: 6949902]

10. Boyan BD, Schwartz Z, Bonewald LF, Swain LD. Localization of 1,25-(OH)₂D₃ responsive alkaline phosphatase in osteoblast-like cells (ROS 17/2.8, MG 63, and MC 3T3) and growth cartilage cells in culture. *J Biol Chem* 1989;264:11879–11886. [PubMed: 2545688]
11. Boyan BD, Bonewald LF, Sylvia VL, Nemere I, Larsson D, Norman AW, Rosser J, Dean DD, Schwartz Z. Evidence for distinct membrane receptors for 1 α ,25(OH)₂D₃ and 24R,25-(OH)₂D₃ in osteoblasts. *Steroids* 2002;67:235–246. [PubMed: 11856547]
12. Boyan BD, Batzer R, Kieswetter K, Liu Y, Cochran DL, Szmuckler-Moncler S, Dean DD, Schwartz Z. Titanium surface roughness alters responsiveness of MG63 osteoblast-like cells to 1 α ,25-(OH)₂D₃. *J Biomed Mater Res* 1998;39:77–85. [PubMed: 9429099]
13. Markose ER, Stein JL, Stein GS, Lian JB. Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. *Proc Natl Acad Sci USA* 1990;87:1701–1705. [PubMed: 2308930]
14. Raz P, Lohmann CH, Turner J, Wang L, Poythress N, Blanchard C, Boyan BD, Schwartz Z. 1 α ,25(OH)₂D₃ regulation of integrin expression is substrate dependent. *J Biomed Mater Res A* 2004;71:217–225. [PubMed: 15386491]
15. Wang L, Zhao G, Olivares-Navarrete R, Bell BF, Wieland M, Cochran DL, Schwartz Z, Boyan BD. Integrin beta1 silencing in osteoblasts alters substrate-dependent responses to 1,25-dihydroxy vitamin D₃. *Biomaterials* 2006;27:3716–3725. [PubMed: 16569430]
16. Garcia AJ. Get a grip: integrins in cell-biomaterial interactions. *Biomaterials* 2005;26:7525–7529. [PubMed: 16002137]
17. Damsky CH. Extracellular matrix-integrin interactions in osteoblast function and tissue remodeling. *Bone* 1999;25:95–96. [PubMed: 10423030]
18. Tosatti S, Schwartz Z, Campbell C, Cochran DL, VandeVondele S, Hubbell JA, Denzer A, Simpson J, Wieland M, Lohmann CH, Textor M, Boyan BD. RGD-containing Peptide GCRGYGRGDSPG Reduces Enhancement of Osteoblast Differentiation by Poly(L-lysine)-graft-poly(ethylene glycol) Coated Titanium Surfaces. *J Biomed Mat Res* 2004;68A:458–472.
19. Schwartz Z, Lohmann CH, Vocke AK, Sylvia VL, Cochran DL, Dean DD, Boyan BD. Osteoblast response to titanium surface roughness and 1 α ,25-(OH)₂D₃ is mediated through the mitogen activated protein (MAP) kinase pathway. *J Biomed Mater Res* 2001;56:417–426. [PubMed: 11372060]
20. Schwartz Z, Ehland H, Sylvia VL, Larsson D, Hardin RR, Bingham V, Lopez D, Dean DD, Boyan BD. 1 α ,25-dihydroxyvitamin D(3) and 24R,25-dihydroxyvitamin D(3) modulate growth plate chondrocyte physiology via protein kinase C-dependent phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase. *Endocrinology* 2002;143:2775–2786. [PubMed: 12072413]
21. Hughes PJ, Brown G. 1 α ,25-dihydroxyvitamin D₃-mediated stimulation of steroid sulphatase activity in myeloid leukaemic cell lines requires VDRnuc-mediated activation of the RAS/RAF/ERK-MAP kinase signalling pathway. *J Cell Biochem* 2006;98:590–617. [PubMed: 16440327]

Figure 1.

Levels of osteocalcin (A) and OPG (B) in the conditioned media of confluent MG63 cells cultured on tissue culture polystyrene (plastic), smooth Ti, grit blasted/acid etched Ti (SLA), and Ti plasma sprayed surfaces (TPS). Cultures were treated with 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ (1,25) for 24 hours. Values are means + SEM for N = 6 independent cultures per variable. * $p < 0.05$, Ti surface v. plastic surface; # $p < 0.05$, with β_1 siRNA v. without siRNA.