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The role of phospholipase D in osteoblast response to titanium surface microstructure

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Abstract

Biomaterial surface properties such as microtopography and energy can change cellular responses at the cell-implant interface. Phospholipase D (PLD) is required for the differentiation of osteoblast-like MG63 cells on machined and grit-blasted titanium surfaces. Here, we determined if PLD is also required on microstructured/high-energy substrates and the mechanism involved. shRNAs for human PLD1 and PLD2 were used to silence MG63 cells. Wild-type and PLD1 or PLD1/2 silenced cells were cultured on smooth-pretreatment surfaces (PT); grit-blasted, acid-etched surfaces (SLA); and SLA surfaces modified to have higher surface energy (modSLA). PLD was inhibited with ethanol or activated with 24,25-dihydroxyvitamin-D_3 [24R,25$(OH)_2D_3$]. As surface roughness/energy increased, PLD mRNA and activity increased, cell number decreased, osteocalcin and osteoprotegerin increased, and protein kinase C (PKC) and alkaline phosphatase specific activities increased. Ethanol inhibited PLD and reduced surface effects on these parameters. There was no effect on these parameters after knockdown of PLD1, but PLD1/2 double knockdown had effects comparable to PLD inhibition. 24R,25$(OH)_2D_3$ increased PLD activity and the production of osteocalcin and osteoprotegerin, but decreased cell number on the rough/high-energy surfaces. These results confirm that surface roughness/energy-induced PLD activity is required for osteoblast differentiation and that PLD2 is the main isoform involved in this pathway. PLD is activated by 24R,25$(OH)_2D_3$ in a surface-dependent manner and inhibition of PLD reduces the effects of surface microstructure/energy on PKC, suggesting that PLD mediates the stimulatory effect of microstructured/high-energy surfaces via PKC-dependent signaling.

Keywords

phospholipase D; osteoblast differentiation; titanium surface microstructure and surface energy; vitamin D metabolites; mechanism of cell surface interaction

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INTRODUCTION

Biomaterial surface properties such as chemical composition, microtopography, and energy can change cellular responses at the cell-implant interface.[1–16] Studies using titanium (Ti) as a model to study osteoblast/substrate interactions show that osteoblast-like cells attach more readily to implant surfaces with micron-scale and submicron scale roughness.[3] Proliferation is decreased and osteocalcin (OCN) production is increased on microstructured Ti surfaces,[17,18] indicating a more differentiated phenotype. Moreover, osteoblasts produce greater levels of factors associated with bone formation, vascularization, and inhibition of bone resorption.[6,19,20] *In vitro* results using these cell culture models are positively correlated with preclinical and clinical studies showing improved bone-to-implant contact and greater pull-out strength for dental and orthopedic implants.[21–24]

Previous studies have shown that osteoblast differentiation on microstructured Ti involves prostaglandin production via cyclooxygenase 1 and 2[25] and signaling via protein kinase C (PKC),[12] as well as signaling by the $\alpha 2\beta 1$ integrin.[11,13,26] Recently, it was shown that MG63 cells exhibit increased phospholipase D (PLD) activity on sandblasted Ti surfaces and inhibition of PLD by 2,3-diphosphoglyceric acid reduced alkaline phosphatase activity and OCN production,[27] indicating that PLD also plays a role.

There are two different mammalian isoforms of PLD, PLD1 and PLD2. These isoforms share ~50% homology, but they are regulated and localized differently in the cell. *In vitro*, PLD2 has a higher basal activity than PLD1, but overall cellular activity of PLD is low. PLD1 is activated by PKCα and GTPases such as RhoA, RacI, Cdc42, and ADP-ribosylation factor (ARF), whereas PLD2 is not.[28–38] Four PLD1 splice variants, PLD1a, 1b, 1c, and 1d, have been identified according to the Universal Protein Resource database (UniProt), of which PLD1a and PLD1b have been the most studied. PLD1b is 38 amino acids shorter than PLD1a, but there has been no evidence of any functional or regulatory differences between the variants.[34,35,39] PLD2 has three splice variants, PLD2a, 2b, and 2c according to the UniProt database. PLD2b is a splice variant that lacks 11 amino acids in its C-terminus compared to PLD2a, but is still functional.[39]

The main function of PLD is to hydrolyze membrane phosphatidylcholine (PC) to generate the precursor signaling molecule phosphatidic acid (PA) and choline.[40] PA can also be converted to lipid second messengers, such as lysophosphatidic acid (LPA) and diacylglycerol (DAG), which mediate the functional role of PLD. PA can be metabolized into DAG by PA phosphatase, which recruits PKC to the membrane to activate it.[28,35,41] This raises the possibility that changes in PLD activity might mediate the effects of surface microstructure on osteoblasts via PKC or that PKC mediates the effects of the surface on PLD.

Surface energy is another surface property that impacts the response of osteoblasts to their substrate. The oxide layer that forms spontaneously over the Ti surfaces is highly hydrophilic and thus exhibits a high surface energy. It is thought that the resulting increased wettability improves the interaction between the biological environment and the implant surface via changes in protein adsorption, regulating cell adhesion. In addition, the —OH and —O^{-2} groups in the oxide layer are important for hydroxyapatite formation and thus could enhance osteointegration.[14,15] *In vitro* studies have shown that surface energy can enhance osteoblast differentiation and maturation, and that this effect is additive and in some cases synergistic with the effects of surface microstructure.[2,14,15] Whether the same mechanisms mediate the effects of microstructure and surface energy on osteoblasts is not known.

The objective of this study was to determine whether microstructured/high energy surfaces increase osteoblast differentiation via a PLD-dependent mechanism. To accomplish this, we examined PLD expression and activity in MG63 cells grown on microstructured Ti substrates with hydrophobic and hydrophilic surfaces and we determined which PLD isoform was responsible for the effects on osteoblast differentiation. PLD activity was inhibited using ethanol and the contributions of specific isoforms determined using RNA interference. We examined the hypothesis that PLD mediates the effects of the surface via PKC. In addition, we took advantage of a previous observation showing that PLD-dependent PKC can be activated by 24R,25$(OH)_2D_3$ in growth plate chondrocytes[42,43] and used 24R,25$(OH)_2D_3$ to stimulate PLD activity in the MG63 cells grown on microstructured/high energy surfaces.

The significance of this *in vitro* study has the potential to help elucidate the signaling events occurring at the cell-implant interface and thus help to further understand the mechanism involved in osseointegration. A better understanding of how the cells are responding to the surfaces will allow researchers to possibly tailor implants through surface modification by physical means or coatings with growth factors/hormones or polymers, to induce these responses.

MATERIALS AND METHODS

Cell culture and Ti surfaces

MG63 human osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD). MG63 cells have been shown to be comparable to immature osteoblasts.[1,7,12] MG63 cells were cultured in 24-well plates on tissue culture polystyrene (TCPS) and on three types of 15 mm diameter Ti disks. Smooth pretreatment surfaces (PT) had a mean peak to valley roughness (R_a) of 0.2 μm and acid-etched/sand-blasted surfaces (SLA) had an average roughness of 3.2 μm. In addition, SLA surfaces were fabricated so that they retained high surface energy without altering the SLA microstructure (modSLA). These disks were provided by Institut Straumann AG (Basel, Switzerland) and were previously characterized.[15,44]

MG63 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2 and 100% humidity. Cells were plated at a density of 10,000 cells/cm^2 for all surfaces. The media were replaced at 24 h and then every 48 h until the cells reached confluence on TCPS. Primary alcohols such as ethanol are known PLD inhibitors.[28,35,45–49] Because 24R,25$(OH)_2D_3$ is suspended in ethanol, in order to use it as an activator of PLD activity, it was necessary to remove ethanol by evaporation and then resuspend 24R,25$(OH)_2D_3$ in *tert*-

butanol, a commonly used alcohol control that does not inhibit PLD activity or cause cell toxicity.[46–50] At confluence, the cells were treated with experimental media containing 0.01% vehicle (0.01% *tert*-butanol), or 24R,25$(OH)_2D_3$ (Biomol International, L.P., Plymouth Meeting, PA) at 10^{-8} and 10^{-7} *M* for 24 h.

Conditioned media were collected and the cells were released from the surfaces by two sequential incubations with 0.25% trypsin-EDTA for 10 min at 37°C, to ensure removal of the cells from the Ti substrates. Cells were counted using an automatic cell counter (Z1 cell and particle counter, Beckman Coulter, Fullerton, CA). The cells were pelleted by centrifugation and then resuspended with the PLD reaction buffer from Invitrogen's Amplex® Red PLD assay kit (Carlsbad, CA), containing 50 m*M* Tris-HCl, 5 m*M* $CaCl_2$, and pH at 8.0. The cells were then lysed by sonication for 5 s for four times while on ice and saved for further biochemical analysis.

Biochemical assays

Two osteoblast differentiation markers were assessed in these studies, alkaline phosphatase and osteocalcin. Alkaline phosphatase specific activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2.[51] Osteocalcin content in the conditioned media was measured using a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA). The amount of osteoprotegerin (OPG) in the conditioned media was measured using an enzyme-linked immunosorbent assay (ELISA) kit (DY805 Osteoprotegerin DuoSet, R&D Systems, Minneapolis, MN).

PKC activity in the cell lysates was measured using a commercially available assay kit that detects the amount of radiolabeled phosphate transferred to a peptide specific for PKC (PKC Biotrak Enzyme Assay System, GE Healthcare Life Sciences, Pittsburgh, PA). PLD activity in the cell lysates was measured using a fluorescence assay that detects the release of choline from phosphatidylcholine (Amplex® Red PLD Assay Kit, Invitrogen, Carlsbad, CA). In addition, a PLD standard was made with PLD from *Streptomyces chromofuscus* (Sigma-Aldrich, St. Louis, MO) to assess the activity in the samples.

The manufacturer's instructions were followed for all assays. The levels of osteocalcin and osteoprotegerin in the conditioned media were normalized by cell number, while alkaline phosphatase, PKC, and PLD activity in the cell lysates were normalized by protein content. Protein content was determined in the cell lysates using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL).

Real-time PCR analysis of PLD expression

To determine if there was a change in the expression level of PLD1a, 1b, 2a, and 2b, the mRNA was quantified by real-time PCR. MG63 cells were cultured on the Ti disks in the same manner as above. RNA was extracted using Qiagen's RNeasy kit (Valencia, CA). Three disks of each type were combined to form one sample.

One microgram of RNA was reversed transcribed with 1 μ*M* each of the antisense primers in a 7 μL volume using the Qiagen Omniscript RT kit (Valencia, CA). Part of the control cDNA reaction was purified using the Qiagen Qia-quick PCR purification kit (Valencia, CA) to make a standard (a series of dilutions) for real-time PCR; this was designed to be the calibrator so that PLD gene expression levels of the unknown samples could be compared after normalization by their respective GAPDH expression levels.

The PLD1a, 1b, 2a, and 2b primer sequences were based on sequences from GenBank accession numbers NM_002662, AB209907, NM_002663, and AF038441, respectively. In addition to obtaining a single peak in the melt curve for each of these primers (indicating a single PCR product) for the real-time PCR optimization, reverse transcription PCR was also done and the products were run on agarose gels to ensure that a single band at the correct size was obtained. Table I shows the primer sequences for real-time PCR using SYBR green incorporation (iQ SYBR Green Supermix, Biorad Laboratories, Hercules, CA) in a Biorad iCycler iQ real time system. No additional $MgCl_2$ was added to the real-time PCR amplification reactions; the 1× SYBR Green Supermix contained 3 m*M* $MgCl_2$.

PLD1 and PLD2 shRNAs

To determine if there was a specific PLD isoform involved in the signaling at the cell-implant interface, three small hairpin RNAs for each PLD isoform were tested to determine the best overall knockdown of gene and protein expression. PLD1 shRNA sequences were based on the GenBank accession number NM_002662 PLD1 sequence (Table II). PLD2 shRNA sequences were based on the Gen-Bank accession number NM_002663 PLD2 sequence (Table II). These sequences were put into lentivirus vectors containing the puromycin resistance gene and were packaged in Mission® shRNA Lentiviral Particles, which were purchased from Sigma-Aldrich (St. Louis, MO). The shRNA containing lentiviral particles were transduced in MG63 cells to produce stable PLD1 and PLD2 shRNA cell lines through puromycin selection.

Reverse transcription PCR (RT-PCR), Western blotting, and PLD activity were used to assess the effectiveness of the knockdown of each shRNA clone. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and was subjected to RT-PCR with human PLD1a/b, PLD2a/b, and GAPDH primers. PLD1a/b and PLD2a/b primer sequences and RT-PCR conditions were from Di Fulvio et al.[52] One microgram of RNA was reversed transcribed with 1 μ*M* each of the antisense primers in a 7 μL volume using the Qiagen Omniscript RT kit. The PCR reaction was composed of 1× PCR buffer B (Fisher Scientific, Pittsburgh, PA), 0.2 m*M* dNTPs (Qiagen, Valencia, CA), 1 m*M* $MgCl_2$ (Fisher Scientific), 0.625 units of Taq polymerase (Qiagen), and 1 μ*M* each of the reverse and forward primers in a reaction volume of 25 μL. PCR amplification was carried out in a Biorad iCycler (Hercules, CA). PCR products for PLD1a/b were run on 2% agarose gel, while PCR products for PLD2a/b were run on 3% agarose gel to get better separation of the PLD2a and 2b splice variants. Density measurements of the PCR product bands were measured to determine the percent of control for each PLD shRNA clone (after normalization by their respective GAPDH densities).

For Western blotting, whole cell lysates (WCL) were harvested from cultures of PLD shRNA transduced cells that were grown to confluence similarly to the control nontransduced MG63 cells except with the addition of 0.25 μg/mL puromycin to maintain selection. A lysis solution composed of 20 m*M* Tris-HCl, 150 m*M* NaCl, 5 m*M* EDTA, 1% Nonidet P-40, and pH of 7.5, was used to lyse the cells. The cells were further lysed by sonication for 15 s for two times in 1 min intervals while on ice. Protein content was determined using the BCA protein assay. Fifty micrograms of protein of each WCL clone were run on 4 20% Tris-HEPES-SDS polyacrylamide gels (NuSep, Lawrenceville, GA) were transferred to nitrocellulose using Invitrogen's iblot transfer system (Carlsbad, CA). The blots were probed with PLD1 (Sigma-Aldrich), PLD2 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Millipore, MA) antibodies. Secondary antibodies conjugated with horseradish peroxidase were purchased from Biorad Laboratories (Hercules, CA) and incubated with the blots for the chemiluminescent reaction. Blots were developed using the SuperSignal West Pico Chemiluminescent System (Thermo Fisher Scientific, Rockford, IL).

For PLD activity, cells were cultured to confluence similarly as for the Western blot and harvested using 0.25% trypsin-EDTA to release the cells. Cells were pelleted by centrifugation and then resuspended with the PLD reaction buffer from Invitrogen's Amplex® Red PLD assay kit. The cells were then lysed by sonication for 5 s for four times while on ice. The Amplex® Red PLD assay kit was used to determine the PLD activity in the cell lysates, with the addition of a PLD standard made with PLD from *Streptomyces chromofuscus* to assess the activity in the samples.

After determination of the best PLD1 and PLD2 shRNAs, the transduced cell lines and the control non-transduced MG63 cells were cultured on the Ti disks in the same manner as previously stated and subjected to the same biochemical assays.

Statistical analysis

The data presented here are from one of two separate sets of experiments, both of which yielded comparable observations. For any given experiment, each data point represents the mean ± standard error of six individual cultures or six separate samples. Data were first analyzed by analysis of variance; when statistical differences were detected, the Bonferroni modification of Student's *t*-test was used. p-Values < 0.05 were considered to be significant.

RESULTS

Effect of microstructured and high-energy Ti surfaces on PLD

Cell number decreased [Fig. 1(A)] and osteocalcin [Fig. 1(B)] increased in cultures grown on SLA in comparison with cells grown on TCPS or smooth Ti. These effects were further increased when MG63 cells were cultured on modSLA. PLD specific activity exhibited a comparable substrate-dependent response [Fig. 1(C)]. Activity was increased by 100% on SLA and by more than 200% on modSLA. Similarly, PKC activity was substrate dependent [Fig. 1(D)], but significant increases in activity were observed on modSLA only.

Overall, PLD expression reflected a similar trend as the PLD activity, although the splice variants were differentially expressed. PLD1a was elevated on SLA and to a much greater extent on modSLA [Fig. 2(A)]. PLD1b mRNAs were increased on all Ti surfaces, but the greatest increase was in cultures grown on modSLA [Fig. 2(B)]. Similarly, PLD2a mRNAs were elevated on all Ti substrates, but there were no differences as a function of microstructure or surface energy [Fig. 2(C)]. Finally, PLD2b was increased only on SLA and modSLA and this increase was comparable on both surfaces [Fig. 2(D)].

Requirement for PLD in osteoblast response

Treatment of confluent cultures of MG63 cells with the PLD inhibitor ethanol had no effect on PLD activity in cultures grown on TCPS, but it reduced PLD activity on all Ti substrates to levels below those seen on the plastic substrates [Fig. 3(A)]. PLD did not mediate the inhibitory effect of surface roughness or surface energy on cell number, since inhibition of enzyme activity with ethanol had no effect [Fig. 3(B)]. However, PLD was involved in the substrate-dependent expression of a differentiated phenotype and the effects were substrate specific. Ethanol treatment reduced alkaline phosphatase activity on TCPS and PT to a comparable extent [Fig. 3(C)]. Ethanol reduced alkaline phosphatase in cell layer lysates grown on SLA to levels below those seen in cells grown on TCPS. The PLD inhibitor reduced the synergistic increase in alkaline phosphatase seen on modSLA surfaces, but the inhibitory effect was only to levels seen in untreated cultures on SLA. Similarly, ethanol blocked the increase in osteocalcin production due to growth on SLA and caused a partial reduction in osteocalcin production by cells grown on modSLA [Fig. 3(D)]. This was the case for osteoprotegerin as well [Fig. 3(E)]. PKC activity was increased in MG63 cells grown on PT in the presence of ethanol, but ethanol had no effect on PKC in cells grown on SLA [Fig. 3(F)]. In contrast, ethanol completely blocked the synergistic effects of surface microstructure and surface energy on PKC activity in cells grown on modSLA.

PLD1 and PLD2 shRNA

Stably transfected cell lines were established that exhibited >70% knockdown in PLD1 expression [Fig. 4(A)]. Three PLD1 shRNA clones (A1, A2, and A3) were screened and they expressed 26%, 22%, and 32% of the control (nontransduced MG63 cells) PLD1a mRNA expression, respectively. They expressed 46%, 39%, and 50% of the control PLD1b mRNA expression, respectively. PLD2a and PLD2b expression for clones A1 and A2 were similar to the control. However, A3 had an 80% increase in PLD2a and a 65% increase in PLD2b expression compared to the control. The Western blot for the PLD1 clones correlated with the RT-PCR results, with the exceptions of a slight reduction in band intensity for PLD2 protein expression in A1 and a large reduction in band intensity for PLD2 protein expression in A2 [Fig. 4(B)]. HeLa WCLs were used as a positive control for PLD1 and PLD2. A1, A2, and A3 clones were significantly lower than the PLD activity in the control [Fig. 4(C)].

The PLD2 shRNA clones G1, G2, and G3 were 34%, 30%, and 65% of the control PLD1a mRNA expression, respectively, and were 50%, 53%, and 62% of the control PLD1b mRNA expression, respectively [Fig. 4(D)]. G1, G2, and G3 clones were 79%, 30%, and 56% of the control PLD2a mRNA expression, respectively, and were 69%, 26%, and 34% of the control PLD2b mRNA expression, respectively. The Western blot for the PLD2-silenced cells correlated with the RT-PCR results, with the exceptions of a reduction in band intensity for PLD2 protein expression in G1 and an increase in band intensity for PLD2 protein expression in G3 [Fig. 4(E)]. G1, G2, and G3-silenced cells had significantly lower PLD activity than in the control cells [Fig. 4(F)].

Based on these results, A3 and G2 were selected to knockdown PLD1 and PLD1/2 expression, respectively. Only cells transduced with PLD1/2 shRNA exhibited decreased PLD activity and the effect of the shRNA was to reduce this activity to a comparable level in all cultures, including those grown on TCPS [Fig. 5(A)]. PLD1-silenced cells exhibited a small decrease in cell number on TCPS, but PLD1 silencing did not affect cell number on the Ti substrates [Fig. 5(B)]. In contrast, PLD1/2 shRNA caused an increase in cell number on all Ti substrates. In cultures grown on PT and SLA, silencing PLD1/2 restored cell number to levels seen on TCPS, but in cells grown on modSLA, restoration of cell number was only partial.

PLD silencing also affected osteoblast differentiation. PLD1-silenced cells exhibited a 20–25% decrease in alkaline phosphatase activity on all substrates, but PLD1/2-silenced cells exhibited a 75% reduction in enzyme activity [Fig. 5(C)]. Only PLD1/2-silenced cells had decreased osteocalcin [Fig. 5(D)] and decreased osteoprotegerin [Fig. 5(E)]. PLD1-shRNA reduced PKC activity in cells grown on TCPS, SLA, and modSLA [Fig. 5(F)]. In contrast, PLD1/2-shRNA reduced PKC on PT, SLA, and mod-SLA but did not affect enzyme activity on TCPS.

Effect of PLD activation by 24R,25$(OH)_2D_3$

24R,25$(OH)_2D_3$ decreased PLD activity on PT, but had no effect on PLD on SLA, and caused an increase in PLD activity on modSLA [Fig. 6(A)]. The inhibitory effect on PT was only at the highest concentration of 10^{-7} *M* and the stimulatory effect on modSLA was achieved at 10^{-8} *M*. 24R,25$(OH)_2D_3$ reduced cell number in cultures grown on SLA by 50% and caused a similar percent reduction in cultures grown on modSLA [Fig. 6(B)]. The decrease in cell number was correlated with a corresponding increase in osteocalcin [Fig. 6(C)] and osteoprotegerin [Fig. 6(D)].

DISCUSSION

This study shows that PLD plays an important role in mediating the response of osteoblasts to surface microstructure and to surface energy. Both PLD1 and PLD2 are involved, but they participate in a differential manner depending on the substrate and on the outcome being measured. Finally, these results show that PLD signaling is responsible for substrate-dependent changes in PKC signaling and that 24R,25$(OH)_2D_3$ can be a regulator of this pathway.

MG63 cells expressed four PLD isoforms/variants, PLD1a, PLD1b, PLD2a, and PLD2b, based on mRNA and Western blot. Expression of all of the variants was increased on the rougher and higher energy surfaces. However, the expression of PLD1a and PLD1b was significantly higher on modSLA compared to the SLA surface, while the expression of PLD2a and PLD2b was not sensitive to surface energy and was equivalent to the expression level on the microstructured SLA surface. This observation suggested the possibility that PLD1 mediated the effects of surface energy, but experiments using specific shRNAs for PLD1 did not support this hypothesis.

We successfully silenced PLD1 expression without affecting PLD2 expression and activity, but we were not able to achieve effective silencing of PLD2 without affecting PLD1. The PLD2 shRNA clones had a suppressive effect on PLD1 expression. One possible explanation for the PLD1 suppression is sequence homology. The PLD2 shRNA sequences were aligned to the PLD1 sequence (GenBank accession number NM_002662) using the free shareware program BioEdit Sequence Alignment Editor (Carlsbad, CA). The alignment revealed a similarity in sequence between the PLD2 shRNAs and PLD1 sequence. However, since the G2 clone knocked down PLD1 and PLD2 expression effectively, this provided a double knockdown of both major PLD isoforms. Moreover, we were able to determine which PLD isoform was mediating the surface effect because we also had a PLD1-specific shRNA. By comparing results examining the responses of PLD1-silenced cells to those of the PLD1/2-silenced cells, we were able to assess the contribution of PLD2 to osteoblast response.

Our results confirmed previous studies using machined and grit-blasted Ti substrates, which reported an increase in PLD activity in MG63 cells grown on rough surfaces over that seen in osteoblast cultures grown on TCPS.[27] The earlier study showed that PLD was required for the increase in osteoblast differentiation observed on the machined and grit-blasted substrates since inhibition of the enzyme blocked the effect, and determined that PLD1 was responsible. This study extends these observations to show that PLD1 is also required for osteoblast differentiation on microstructured surfaces produced by acid-etched, grit-blasted Ti surfaces and, importantly, shows that PLD2 is involved in the synergistic response to high surface energy on the microstructured surface.

PLD did not mediate all effects of substrate microstructure or surface energy, nor were those responses in which PLD did play a role regulated by the enzyme to a similar extent. Even though treatment with ethanol reduced PLD activity in cultures grown on Ti to levels below those seen on TCPS, PLD inhibition had no effect on cell number, either on smooth or rough substrates. Alkaline phosphatase activity was reduced on all substrates, suggesting that PLD mediated early events in osteoblast differentiation. This hypothesis was supported by the observation that PLD-inhibition only reduced osteocalcin and osteoprotegerin in cultures grown on SLA and modSLA. Moreover, the reduction was comparable on both surfaces and was only partial. This indicates that PLD was only required for the response to surface roughness and not for the response to surface energy, at least with respect to these two osteoblast proteins.

Although PLD1-shRNA reduced PLD1 mRNA and activity by 70% in cells grown on TCPS, the PLD1-shRNA silenced MG63 cells did not exhibit reduced PLD activity when they were grown on the Ti substrates. In contrast, the PLD1/2-silenced cells had PLD activity comparable to cells grown on TCPS, indicating that PLD2 was the isoform responsive to surface energy. One possible explanation for why there was no reduction in PLD activity in the PLD1-shRNA cells on the control surfaces is that the cells may have produced more PLD2 to compensate for the PLD1 knockdown, which is supported by our mRNA expression results (in our screening process) where there was an 80% and 65% increase in PLD2a and PLD2b expression, respectively, in the PLD1 shRNA clone A3 (the clone used in this surface study).

The combined knockdown of PLD1/2 caused a partial increase in cell number on the SLA and modSLA substrates and reduced osteocalcin and osteoprotegerin on all Ti substrates, supporting the hypothesis that PLD2 was responsible for these surface-dependent effects. That PLD2 was the main isoform involved in the surface roughness and energy induced PLD activity is somewhat contradictory to the mRNA expression results, where we saw that PLD1a and PLD1b expression was sensitive to surface energy, while PLD2a and PLD2b expression was not. It is possible that not all of the mRNA was translated into protein that was functional, so these results do not necessarily have to correlate. Moreover, the results of PLD1/2 knockdown confirmed the PLD inhibitor results.

The fact that alkaline phosphatase activity was regulated by PLD1 and to a greater extent by PLD1/2, suggests that each isoform mediated a separate signaling pathway. Our results indicate that PLD differentially regulates PKC in a substrate-dependent manner. Inhibition of PLD stimulated PKC activity in cells grown on the smooth PT surface to levels comparable to those seen in cells grown on the SLA surface, while reducing the synergistic increase on the modSLA substrate to levels seen on the SLA surface. Based on the assumption that the surface energy effect was mediated by PLD2, it is likely that PLD2 is responsible for the marked increase in PKC seen in cells grown on modSLA. PLD1 also acts via PKC, however, based on the small, but significant decrease in PKC activity observed in PLD1-silenced cells grown on TCPS, SLA, and modSLA. Interestingly, PLD1 has been shown to be regulated by PKCα,[53] suggesting that PKC may act at numerous stages during osteoblastic differentiation and that more than one isoform of PKC may be involved. In this study, we did not determine which PKC isoform was stimulated by surface energy or which isoform was sensitive to PLD1 or PLD2 activation.

24R,25$(OH)_2D_3$ caused an increase in PLD activity in cells grown on modSLA and this was associated with decreased cell number and increased osteocalcin and osteoprotegerin, indicating increased osteoblastic differentiation. Previously, we showed that 24R,25$(OH)_2D_3$ decreases cell proliferation and stimulates alkaline phosphatase in growth plate chondrocytes via PLD2-dependent PKCα signaling.[42,43,54–56] These observations suggest that 24R,25$(OH)_2D_3$ may act this way in osteoblasts as well and that PLD activated PKCα at least to some extent. The stimulatory effect of 24R,25$(OH)_2D_3$ on PLD, osteocalcin, and osteoprotegerin as well as the inhibitory effect on cell number were comparable on SLA and modSLA, indicating that it mediated response to microstructure rather than surface energy.

As noted in previous studies, the number of cells on the microstructured surfaces was less than on TCPS or PT substrates, due to differences in attachment and differences in growth. On the smoother substrates, MG63 cells are flattened and well-spread whereas on the SLA and modSLA surfaces, they assume a more rounded morphology and interact with neighboring cells through cytoplasmic extensions. Time in culture based on confluence on TCPS was used to standardize the cultures, but it is possible that the cells were not all at the same state of osteoblastic differentiation at the time they were treated with 24R,25$(OH)_2D_3$ and this is reflected in the data. The results in this study have been normalized to cell number or to protein content of the culture to permit assessment of the behavior of the cells that were present on the substrate. It is unlikely that the reduction in cell number seen in cultures treated with 24R,25$(OH)_2D_3$ was due to cell death because this vitamin D metabolite has been shown to be antiapoptotic in other systems.[57] Instead, $24R,25(OH)_2D_3$ acted by inhibiting proliferation and stimulating differentiation of the cells on the rougher surfaces.

In summary, we showed that PLD is involved in the regulation of osteoblast differentiation on rough and high energy Ti surfaces. In general, the higher surface energy Ti surface (modSLA) caused a significantly larger cell response than the other surfaces, thus showing the importance of surface chemistry. Surface microstructure and energy-induced PLD activity was positively correlated with osteoblast differentiation. Moreover, inhibition of PLD partially blocked this effect, indicating a role for PLD in the process, but also suggesting that there may be other pathways regulating osteoblast differentiation on the rough and high energy surfaces. The requirement for PLD was also demonstrated by double knockdown of PLD1 and PLD2. Furthermore, we showed that PKC is regulated by surface roughness and energy-induced PLD, suggesting that PKC is downstream from PLD, and indicating that PLD2 is the main isoform involved in this pathway. We further confirmed that the main PLD isoform involved in this pathway was PLD2 with PLD isoform specific shRNAs. We showed that PLD is activated by the vitamin D metabolite, $24R,25(OH)_2D_3$, in a surface-dependent manner, possibly by a similar pathway that exists in resting zone chondrocytes and that it enhanced osteoblast differentiation.

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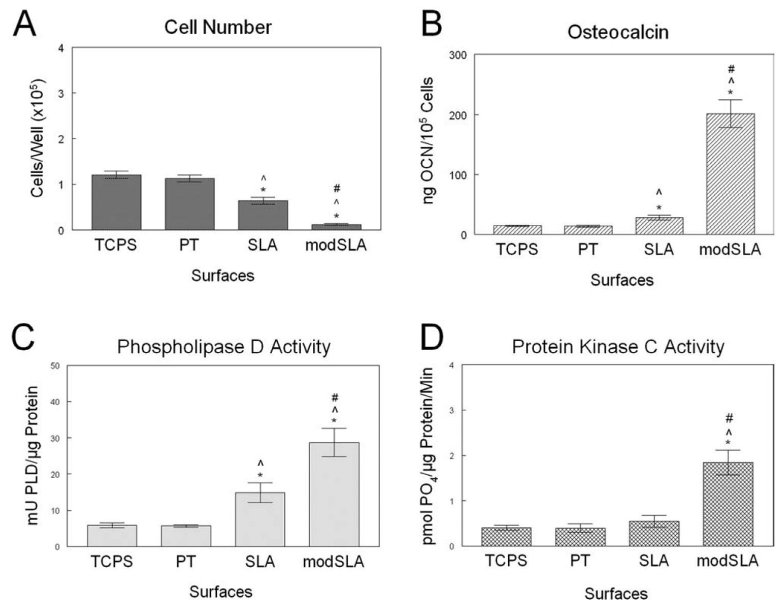

Figure 1.

Effect of substrate on specific activity of phospholipase D (PLD) and protein kinase C (PKC) in confluent cultures of MG63 cells. (A) Cell number; (B) osteocalcin (OCN) content of the conditioned media; (C) PLD activity, where 1 unit (U) of PLD will liberate 1.0 µ*M* of choline from L-α-phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C; and (D) PKC activity in MG63 cells cultured on tissue culture polystyrene (TCPS), pretreatment Ti (PT), grit blasted/acid etched Ti (SLA), and hydrophilic SLA (modSLA) surfaces. *TCPS versus Ti surfaces $p < 0.05$; ^PT versus SLA and modSLA surfaces $p < 0.05$; $^{\#}$SLA versus modSLA $p < 0.05$.

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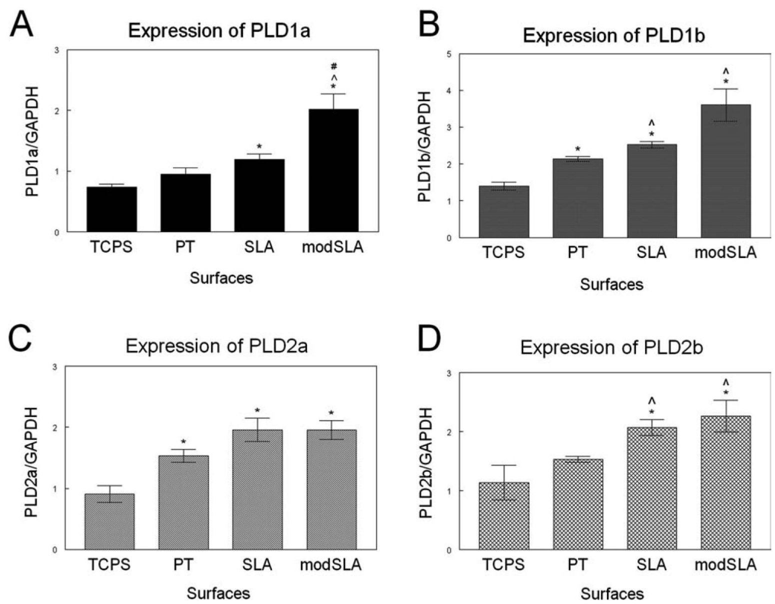

Figure 2.

Effect of substrate on expression of PLD isoforms in confluent cultures of MG63 cells. (A) PLD1a, (B) PLD1b, (C) PLD2a, and (D) PLD2b. *TCPS versus Ti surfaces, $p < 0.05$; ^PT versus SLA and modSLA, $p < 0.05$; #SLA versus modSLA, $p < 0.05$.

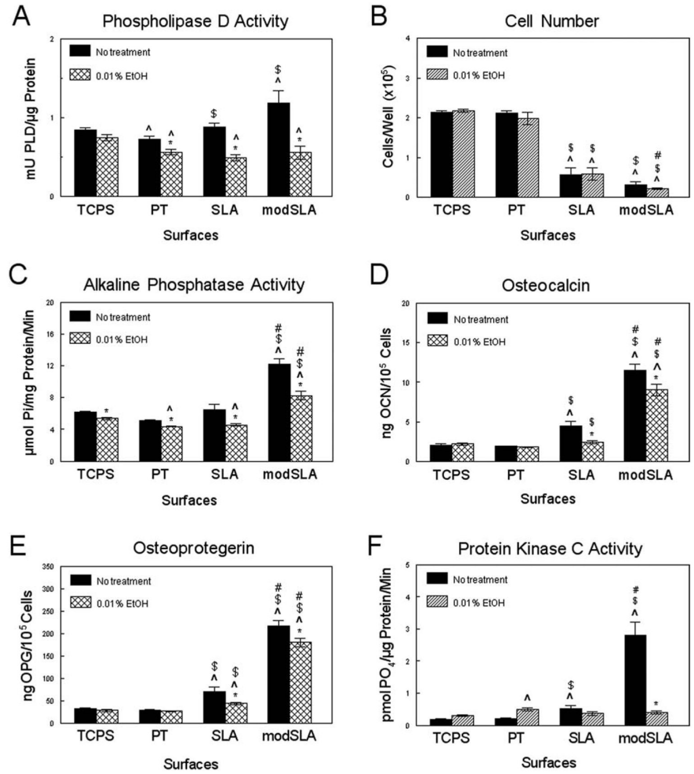

Figure 3.

Effect of PLD inhibition on MG63 response to substrate properties. Confluent cultures of MG63 cells were treated for 24 h with 0.01% ethanol (EtOH). (A) PLD activity, where 1 U of PLD will liberate 1.0 μ*M* of choline from L-α-phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C, (B) cell number, (C) alkaline phosphatase activity, (D) amount of osteocalcin (OCN), (E) amount of osteoprotegerin (OPG), and (F) PKC activity in MG63 cells cultured on TCPS, PT, SLA, and modSLA Ti surfaces. *No treatment versus treatment $p < 0.05$; ^TCPS versus Ti surfaces $p < 0.05$; $^{\$}$PT versus SLA and modSLA, $p < 0.05$; $^{\#}$SLA versus modSLA $p < 0.05$.

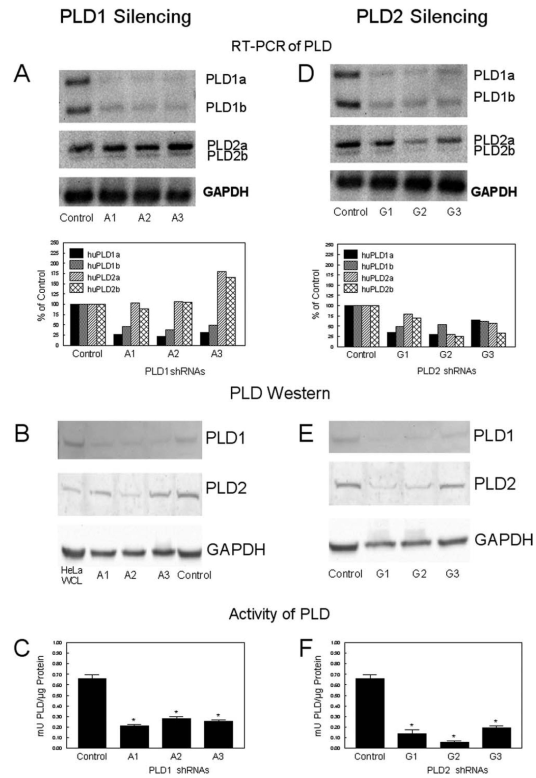

Figure 4.

Characterization of PLD knockdown clones. (A C) MG63 cells were transfected with PLD1 shRNAs; (D F) MG63 cells were transfected with PLD2 shRNAs. Agarose gels of the RT-PCR products PLD1a, 1b, 2a, 2b, and GAPDH in cells transfected with PLD1-shRNAs (A) and PLD2-shRNAs (D). Gels were scanned and relative densities determined with respect to GAPDH for each clone (A1, A2, A3 for PLD1-shRNA and G1, G2, G3 for PLD2-shRNAs) (% wild type control). Western blots of whole cell lysates (WCL) of MG63 cells transfected with PLD1-shRNA (B) and PLD2-shRNA (E). HeLa WCLs were used as a positive control for PLD1 and PLD2. PLD specific activity in cell lysates of the PLD1 (C) and PLD2 (F) silenced cells. Data are mean ± SEM; Each variable was tested in six independent cultures. Data are from a single representative experiment. *Control versus PLD shRNA, $p < 0.05$.

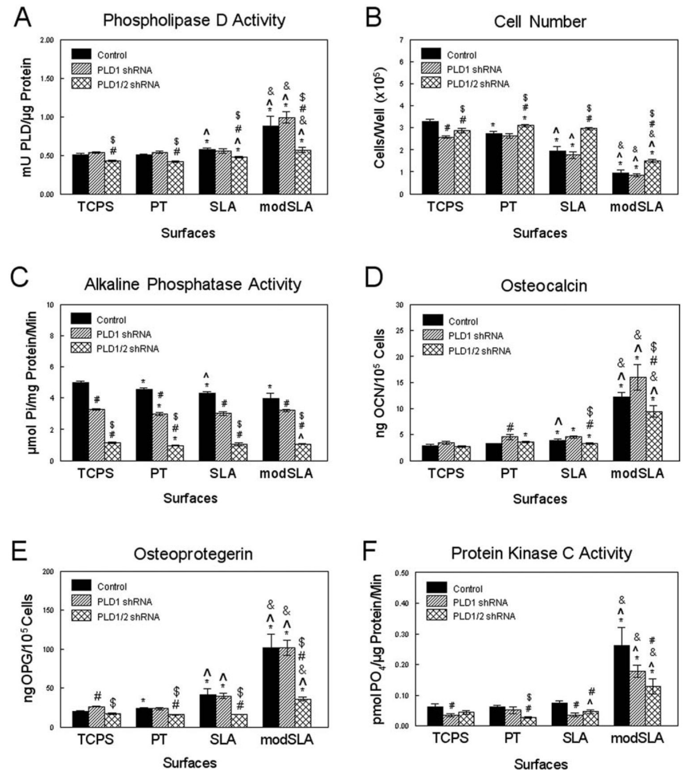

Figure 5.

Effect of PLD silencing on MG63 cell response to substrate microstructure and surface energy. (A) PLD activity, where 1 U of PLD will liberate 1.0 µ*M* of choline from L-α-phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C, (B) cell number, (C) alkaline phosphatase activity, (D) amount of OCN, (E) amount of OPG, and (F) PKC activity in control MG63 cells, PLD1 shRNA silenced cells, and PLD1/2 shRNA silenced cells cultured on TCPS, PT, SLA, and modSLA Ti surfaces. *TCP versus surfaces $p < 0.05$; ^PT versus surfaces $p < 0.05$; &SLA versus modSLA $p < 0.05$; #Control versus PLD1 or PLD1/2 shRNA $p < 0.05$; $PLD1 shRNA versus PLD1/2 shRNA $p < 0.05$.

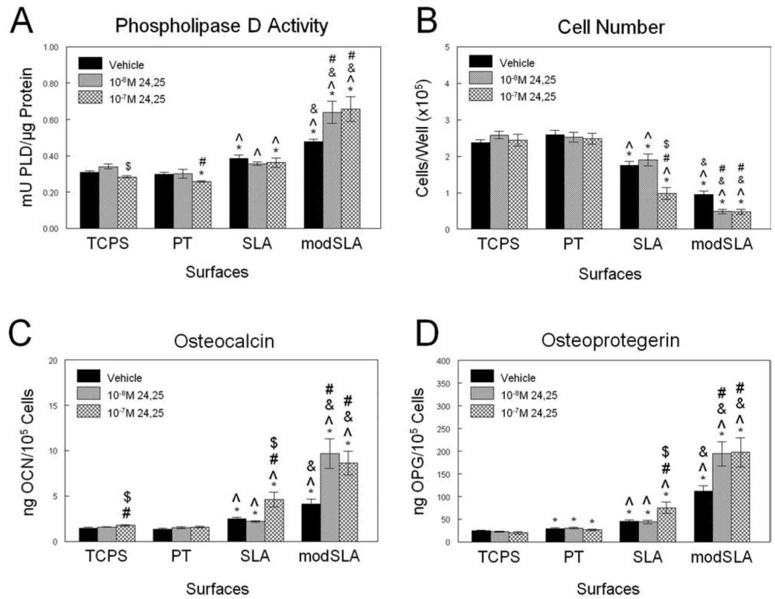

Figure 6.

Effect of 24R,25$(OH)_2D_3$ on MG63 cell response to surface microstructure and surface energy. (A) PLD activity, where 1 U of PLD will liberate 1.0 µ*M* of choline from L-α-phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C, (B) cell number, (C) amount of OCN, and (D) amount of OPG from MG63 cells cultured on TCPS, PT, SLA, and modSLA Ti surfaces after treatment with vehicle, 10^{-8} *M*, and 10^{-7} *M* 24, 25 for 24 h. *TCP versus surfaces $p < 0.05$; ^PT versus surfaces $p < 0.05$; $^{\&}$SLA versus modSLA $p < 0.05$; $^{\#}$Vehicle versus 24, 25 treatment $p < 0.05$; $^{\$}10^{-8}$ *M* 24, 25 versus 10^{-7} *M* 24, 25 $p < 0.05$.

TABLE I

Real-Time PCR PLD Primer Sequences

	PLD1a	PLD1b	PLD2a	PLD2b
Sense primer sequence (5′ → 3′)	GAA AGT TCT CCA AAT TTA GTC	GCA CCT CCA ATA CCG GGT	AAT GAC CGG AGC TTG CTG	AAT GAC CGG AGC TTG CTG
Antisense primer sequence (5′ → 3′)	GGA TTA AAT TGT GAT GAC TTC	AGT TGA ACC CAG TCT TTG AAG	GCA GAC TCA AGG CAA ACC TG	AGA ATC ACA CTG CCC GCC

TABLE II

PLD1 and PLD2 shRNA Hairpin Sequences

ID	PLD Isoform	Sequence
A1	PLD1	CCG GGC AAG TTA AGA GGA AAT TCA ACT CGA GTT GAA TTT CCT CTT AAC TTG CTT TTT
A2	PLD1	CCG GGC ATT CTC GTA TCC AAC CCA ACT CGA GTT GGG TTG GAT ACG AGA ATG CTT TTT
A3	PLD1	CCG GCC ACT AGA AGA CAC ACG TTT ACT CGA GTA AAC GTG TGT CTT CTA GTG GTT TTT
G1	PLD2	CCG GCC GAA AGA TAT ACC AGC GGA TCT CGA GAT CCG CTG GTA TAT CTT TCG GTT TTT G
G2	PLD2	CCG GCC TCT CTC ACA ACC AAT TCT TCT CGA GAA GAA TTG GTT GTG AGA GAG GTT TTT G
G3	PLD2	CCG GCG ATG AGA TTG TGG ACA GAA TCT CGA GAT TCT GTC CAC AAT CTC ATC GTT TTT G