Osteoblast response and calcium deposition on phospholipid modified surfaces

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In this study, the effect of calcium phosphate complexed phospholipid (Ca–PL–PO_4) coatings on solid surfaces on the *in vitro* calcium (Ca) deposition and on the osteoblast responses was evaluated. Commercially available phospholipids were converted to their Ca–PL–PO_4, and were coated on glass Petri dishes. The coated dishes were immersed in the simulated body fluid for up to 14 days under sterilized conditions at 37 °C, and the amount of calcium (Ca) deposited was quantified. Similarly, by measuring the alkaline phosphatase specific activity, the differentiation of osteoblast precursor cells were evaluated after seeding the cells on Ca–PL–PO_4 coated cell culture plastics. It was observed that all Ca–PL–PO_4 enhanced Ca deposition on coated surfaces. The, polar head group of phospholipids in coated surfaces was observed to have an influence on the Ca deposition as well as the osteoblast differentiation. Among the phospholipids evaluated, phosphatidylserine (Ca–PS–PO_4) exhibited the strongest calcium deposition and more enhanced alkaline phosphatase specific activity. It was therefore concluded from this study that Ca–PS–PO_4 surface modification may be an alternative method for enhancing bone–implant interactions.

Introduction

Bone response to implant surfaces has been reported to be dependent on the chemical and physical properties of the implant surfaces, thereby affecting their success [1]. It was reported that dental and orthopedic implants coated with hydroxyapatite (HA) enhanced osteoconduction [2–5]. The superior performance of HA-coated implants is attributed to more rapid Osseo-integration and the development of increased interfacial strength that results from the early skeletal attachment and increased bone contact with the implant surface [6–9]. Extensive *in vivo* research indicates that HA-coated implants are biocompatible and may perform better than non-coated Ti implants. However, over the past 10 years, the clinical use of implants coated with HA using plasma spray technology has initiated substantial controversy in the field of implantology, mainly because of variation in coating thickness between vendors, variation in bond strength at the coating–metal interface and variation in structural and chemical properties of HA depending on its source, and non-uniformity in coating density [10–12]. In addition, there are no accepted standards for plasma spraying HA on implants [13–15], and as a result of variable coating quality, many conflicting animal and clinical observations have been reported. In addition to alteration in HA-coating properties during plasma spraying, clinical failures of plasma sprayed HA-coated implants have been observed to occur due to premature degradation or delamination at the HA–metal interface, indicating a weak bond at the coating–substrate interface compared to bone–HA interface [16–24]. However, these problems do not reflect shortcomings inherent in the rationale for HA coating, but rather in the plasma spray and other technologies currently used to apply the coatings.

Recently, an alternative implant surface modification using phospholipids coatings have been suggested. It has been reported that various kind of calcium deposition processes involve the use of phospholipids [25–31]. The role of phospholipids has also been suggested in the initiation of calcium phosphate deposition in cartilage [32, 33], bone [34], healing fracture callus [35], and certain calcifying bacteria [27]. It has also been reported that a complex between calcium-inorganic phosphate and the phospholipid was essential for inducing the deposition of calcium phosphate [36]. Therefore, it was postulated that a non-living surface (such as that of orthopedic and dental implants) coated with the aforesaid calcium–phospholipid–phosphate (Ca–PL–PO_4) complexes should be able to attract hydroxyapatite. Studies have reported that the phospholipid coated biomaterials were fairly biocompatible [37], Though numerous chemical, structural and biological characteristics of phospholipids are well defined; cellular responses to

phospholipids-coated surfaces have yet to be evaluated. As such, the effect of phospholipid coatings on solid surfaces on the *in vitro* calcium deposition and osteoblast responses was evaluated in this paper.

Materials and Methods

Confirmation of phospholipid stability

In order to ensure the stability of phospholipids used after UV sterilization, three samples were prepared by dissolving 1 mg of phosphatidylcholine in 200 µL of phased chloroform. One of the samples was stored at 4 °C to serve as control while the other two samples were transferred to glass Petri dishes. The solvent was allowed to evaporate at room temperature, and the residue was UV sterilized for 96 h. The sterilized samples were then dissolved in 200 µL phased chloroform. All sterilized and control samples were spotted on a $5 \times 20\,cm^2$ silica gel TLC plate, and the spots were air dried at room temperature. A chromatogram was developed by keeping the TLC plate in a TLC chamber containing elution solvent (chloroform, methanol and water in the ratio of 65 : 35 : 5). To visualize all fractions, the chromatogram was then sprayed with sulphuric acid in methanol (50 : 50) followed by hot air charring.

Synthesis and coating of Ca–PL–PO_4

Phospholipids (Fig. 1) [phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI)] were purchased from Sigma Chemical Company, St. Louis, MO, and were converted to their respective Ca–PL–PO_4 complex by the method of Boskey and Posner [36]. Briefly, 2.5 mg of phospholipid was taken in 5 mL of a solution containing 1 mM $CaCl_2$ and 1 mM $(NH_4)_2HPO_4$ in 0.05 M Tris buffer (pH 7.45) and the mixture was sonicated in ice for 1 min. The dispersion was agitated at 37 °C for 24 h. The solution was phased by the addition of mixture of chloroform and methanol (2 : 1, V/V, 15 mL) followed by centrifugation at 2000 rpm for 15 min at 5 °C. The lower phase, thus obtained, was washed three times with 10^{-5} N HCl to remove the unchanged phospholipid. The Ca–PL–PO_4, recovered in the lower phase was finally used for coatings on glass Petri dishes and tissue culture plates.

Coating on tissue culture plate was performed by mixing Ca–PL–PO_4 in chloroform and methanol (2 : 1, V/V, 1 mL) and pouring the mixture on to glass Petri dishes and tissue culture plates (98 mm diameter). Coated surface was then covered and left at room temperature under UV light (253.7 nm) for 48 h. Complete evaporation of solvent occurred after 48 h and surfaces were rinsed with deionized water. Uncoated glass dishes and tissue culture plates were used as control. All coated and uncoated surfaces were sterilized under UV light (253.7 nm) for 48 h prior to experiment.

Immersion study

Coated and control glass Petri dishes (98 mm long and 10 mm wide) were immersed in the simulated body fluid (SBF; prepared as aqueous solution of 93.9 mM NaCl, 1.24 mM K_2HPO_4, 0.66 mM KH_2PO_4, 0.94 mM $MgCl_2$, 1.48 mM $CaCl_2$, 18 mM $KHCO_3$ at pH 7.4 and at 37 °C. After 0, 7 and 14 days immersion, triplicate samples were removed and calcium concentrations were measured.

Flame atomic absorption was used to measure calcium concentration. The blank was prepared by adding 90.0 mL ddH_2O to 10.0 mL 1% $LaCl_3$, and 20% HNO_3. Samples were 10 times diluted with 0.1% $LaCl_3$ and 2.0% HNO_3. The blank and samples were measured at 422.7 nm using a Perkin Elmer 3030 Atomic Absorption Spectrophotometer with a Perkin-Elmer Intensitron calcium lamp with a slit of 0.7 and a current of 10 ampere. Differences in calcium concentration were statistically compared using ANOVA, with the Student Newman–Keuls procedure as *post hoc* test for the evaluation of significant differences at $P < 0.05$.

Cell culture

Coated and uncoated tissue culture plates were used in this study. The cell culture study was conducted using the

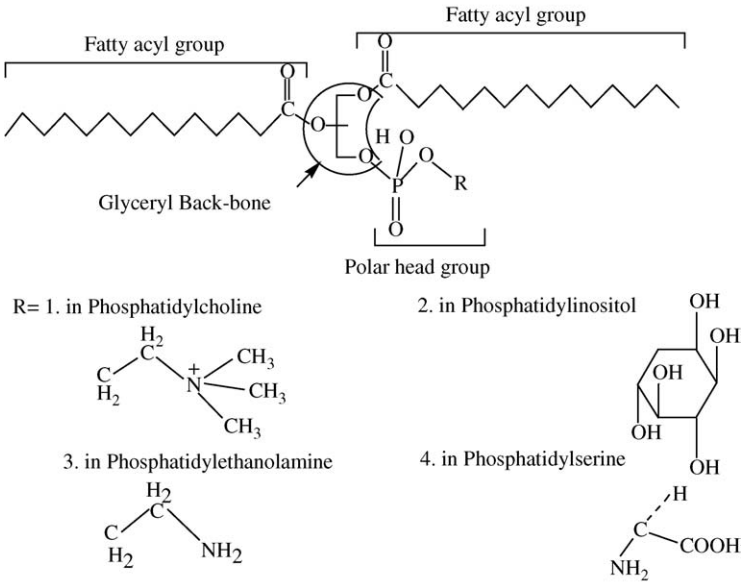

Figure 1 Common natural phospholipids.

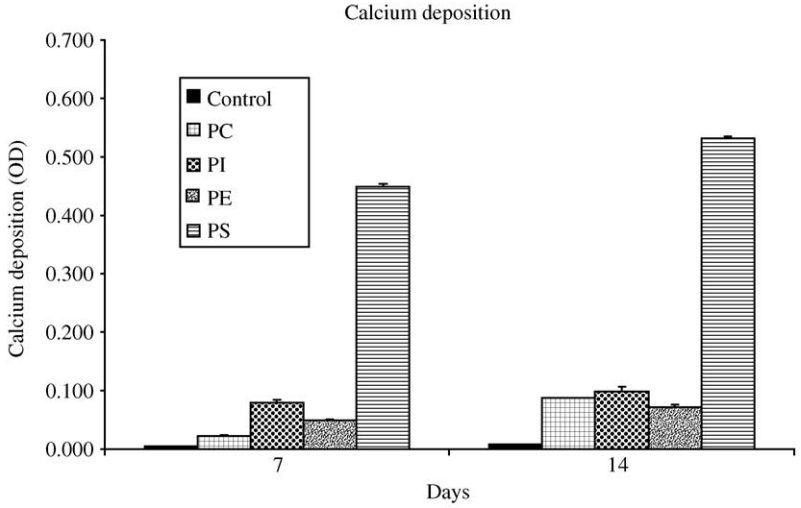

Figure 2 Induction of calcium deposition on surface modified glass Petri dishes immersed in SBF for seven and 14 days.

American Type Culture Collection (Manassas, VA, USA) CRL-1486 human embryonic palatal mesenchyme cells (HEPM), an osteoblast precursor cell line, over an 8-day period. Surfaces were seeded with 15 000 cells/mL in Alpha modified Eagle's medium (MEM) containing 7% fetal bovine serum, 1% antibiotic–antimycotic solution, and 50 μg/mL ascorbic acid. The cell suspension was placed (1 mL/per well) in a 24-well-plate containing either the control or the coated surfaces. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. At confluence, the media was removed and replaced with MEM containing 7% fetal bovine serum, 1% antibiotic–antimycotic solution, 50 μg/mL ascorbic acid, and 4 mM β-glycero-phosphate. The culture medium was changed every two days with MEM media containing 7% fetal bovine serum, 1% anti-biotic-antimycotic solution, 50 μg/mL ascorbic acid, and 4 mM β-glycerophosphate. On days 0, 3, and 6, media were decanted and surfaces were washed twice with a phosphate buffered solution. The cells were then lysed using 0.2% Triton-X-100 solution (Fisher Scientific, Houston, USA) and stored at -20 °C until assayed.

Protein production assay

Triplicate samples from each group were analyzed for total protein synthesis. Protein synthesis was performed using the Pierce BCA protein assay (Pierce, IL). The cell layer suspension (30 μL) was added to 200 μL of working reagent (sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartrate in 0.1 M NaOH, and 4% copper sulfate). The samples were incubated for 30 min at 37 °C and read using a micro-plate reader at 600 nm. The absorbance for the cell layer suspension was correlated to a standard protein curve and differences in protein synthesis were statistically compared using the ANOVA test ($P < 0.05$), with differences analyzed using the Student Newman–Keuls *post hoc* test.

Alkaline phosphatase (ALP) specific activity assay

Triplicate samples from each treatment were used for measuring the ALP specific activity. The cell layer suspension (50 μL) was added to 50 μL of working reagent (1.5 M 2-amino-2 methyl-1-propanol, 20 mM *p*-nitrophenol phosphate, and 1 mM magnesium chloride). The samples were then incubated for 3 h at 37 °C. After 3 h incubation, the reaction was stopped with the addition of 100 μL of 1 N NaOH and read using a Titertek Multiscan Plus MK II microplate reader (MTX Labsystems, Inc, VA, USA) at 410 nm. The absorbance for the cell layer suspension was correlated to a standard ALP activity curve prepared using *p*-nitrophenol stock standard. Alkaline phosphatase specific activity was calculated by normalizing ALP activity with protein production. Differences in ALP specific activity were statistically compared using the ANOVA test ($P < 0.05$), with differences analyzed using the Student Newman–Keuls *post hoc* test.

Results and discussion

Prior to calcium deposition and cell culture studies, the stability of phospholipids after UV sterilization was evaluated. The UV exposed samples were analyzed by thin layer chromatographic study as compared to unexposed (control) sample. The chromatogram revealed that UV exposed samples developed into single spot, testifying to the purity and homogeneity of samples. Moreover, the UV exposed samples had the same RF value as that of the control sample and co-eluted in the superimposed chromatographic analysis. Thus, the UV exposed samples had the same chemical identity as that of control and the phospholipids were stable to mandatory exercise for UV sterilization of implant products. This result conforms to a few previous observations describing the stability of lecithin and related phospholipids to UV exposure [38, 39].

Mineral deposition after immersion of Ca–PL–PO_4 coated surfaces in SBF was evaluated in this study by measuring the amount of deposited calcium. As shown in Fig. 2, all Ca–PL–PO_4 coatings induced significantly higher calcium deposition after immersion in SBF as compared to control surface. It was suggested that the variation in polar head group of phospholipid part of Ca–PL–PO_4 imparted a pronounced effect on Ca

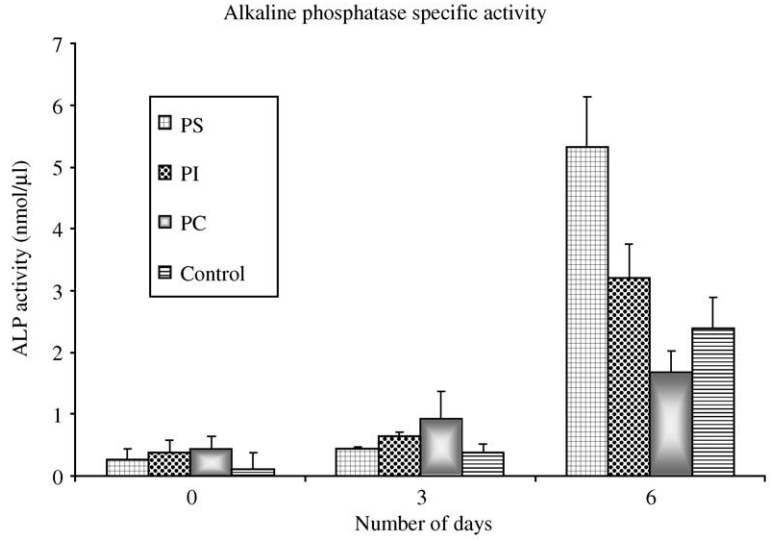

Figure 3 Alkaline phosphatase specific activity expressed by differentiating HEPM cells (osteoblast progenitors) cultured on Ca–PL–PO_4 coated tissue culture plates (98 mm diameter). Uncoated tissue culture plates were used as control.

deposition. It was observed that all phospholipid complex coatings induced enhanced deposition of calcium during the first seven days of immersion in SBF, as compared to control (uncoated) Ti samples. Among four phospholipids used, the complex of PC induced statistically the poorest calcium deposition, whereas complex of PS induced statistically the best calcium deposition during the first seven days of immersion. After 14 days of immersion also, it was observed that all phospholipids complex coated surfaces induced far better deposition of calcium, as compared to the uncoated control Ti surface. Once again, among the phospholipids coated surfaces, the one coated with calcium phosphate complex of phosphatidylserine (Ca–PS–PO_4) exhibited the strongest calcium deposition. But after 14 days immersion in SBF, the poorest calcium deposition among coated surface was exhibited by the calcium phosphate complex of phosphatidylethanolamine (Ca–PE–PO_4) and therefore it was not included in the following cell culture study.

The alkaline phosphatase specific activity is widely recognized as a biochemical marker for the osteoblast phenotype [40], their proliferation [41] and may be considered an important factor in bone mineralization [42], and as such has been associated with increased osteoblastic activity. Up to day three of culture, the data of alkaline phosphatase specific activity on different coated surfaces relative to each other and to the uncoated control surface, were statistically not significant (Fig. 3). However, by day six of culture, all phospholipids coated surfaces induced a significantly greater alkaline phosphatase specific activity as compared to uncoated control surface. In comparing all phospholipids coatings with each other, a significantly higher alkaline phosphatase specific activity was observed on PS coated surfaces after the six day incubation compared to the other phospholipids and control surfaces. The surface coated with Ca–PS–PO_4 registered a 122% increment in the specific alkaline phosphatase activity compared to control uncoated surface after six days, indicating a significantly enhanced expression of osteoblast phenotype by osteoblast precursor cell line [43] on this surface.

Conclusions

From this study, it was observed that the surfaces coated with calcium phosphate complex of phosphatidylserine exhibited the highest amount of calcium deposition. In addition, osteoblast differentiation, as indicated by alkaline phosphatase specific activity, was enhanced when the precursor cells were seeded on surfaces coated with the complex of phosphatidylserine. It was therefore concluded that Ca–PS–PO_4 surface modification may be an alternative method for enhancing bone–implant interactions.

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