Effects of phosphatidylserine-containing liposomes on odontogenic differentiation of human dental pulp cells

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Phosphatidylserine (PS) is known to enhance biomineralization due to the ability to accumulate calcium ions. In this study, the effects of PS on odontogenic differentiation and mineralization of human dental pulp cells (HDPCs) were investigated using phosphatidylserine-containing liposomes (PSLs). PSL was slightly cytotoxic at 125 µM in growth medium, and ALP activity was up-regulated in the PSL-treated HDPCs at non-cytotoxic concentrations. Mineralization was also enhanced by PSL, while mRNA expressions of DSPP and OCN genes were slightly attenuated. The mRNA expression of Runx2 was not altered by PSL. It is thus likely that PSL selectively affected odontogenic differentiation processes of HDPC. Finally, the interaction between PSL and HDPC was investigated by staining with annexin V-FITC in PSL-treated HDPC. It was found that PS was gradually incorporated into HDPC cytoplasm for several days. The results of this study suggest that PSL is able to stimulate dentin formation in dental pulps.

**Keywords**: Phosphatidylserine, Liposome, Human dental pulp cells, Mineralization

INTRODUCTION

Matrix vesicles (MVs), extracellular membrane-bound particles, are initiation sites of calcification in bone, predentin and cartilage\(^6\). In MVs, phosphatidylserine (PS) accumulates Ca\(^{2+}\) to produce calcium phosphate with annexin\(^2\). Due to its calcium ion binding property, PS has been used to enhance the osteoconductivity of bone grafts and dental implants in previous studies. Santin et al. showed that PS coating on (Ti) discs facilitated surface mineralization in simulated body fluids (SBF). PS rearranged into a three-dimensional matrix, a preparative transition for forming calcium phosphate mineral\(^3\), which increased in vitro mineralization in cell culture on Ti discs\(^4\). Calcium deposition at pericellular area of osteoblast-like cells was significantly enhanced on PS-coated Ti discs. Mineralization of the extracellular matrix (ECM) as well as the Ti surface showed potential of PS to improve osseointegration of dental implants. PS stimulated new bone formation at the surface of a Ti rod implanted in a rabbit femur\(^5\). The degree of osseointegration of dental implants. PS stimulated new bone formation at the surface of a Ti rod implanted in a rabbit femur\(^5\). The degree of osseointegration around the PS-coated Ti rod was higher than that of a hydroxyapatite-coated rod at 4 weeks after implantation. The effect of PS on new bone formation was also demonstrated in a study using a scaffold for bone tissue engineering\(^6\). A PS-Bioglass-collagen scaffold combined with rat mesenchymal stem cells (MSCs) accelerated new bone formation at a prepared rat femur defect. In the same study, mRNA expression of osteogenic genes in rat MSCs significantly increased on the PS-Bioglass-collagen scaffold compared to those on a corresponding scaffold without PS. The osteogenic effect of PS was also confirmed with human MSCs\(^7\). PS alone increased ALP activity and mineralization of human MSCs, and the extracellular signal-regulated kinase (ERK) pathway was involved in the osteogenic effects of PS.

A role for PS in forming mineral phase of dentin has been suggested by previous studies that observed MVs in dentin tissues. In an early study employing transmission electron microscopy, extracellular MVs were found in reparative dentin of rat molar pulp\(^8\). The MVs were also observed in diverse positions of human dentin including the proximal part of odontoblastic processes and intratubular spaces\(^9\). In cultured human dental pulp cells, MVs appeared at mineralized nodules, including hydroxyapatite\(^10\). The presence of MVs in dentin and dental pulp cell culture suggests a crucial role of PS in mineralizing dentin. Furthermore, considering the effects of PS on new bone formation, PS is expected to promote dentin-like structures in pulpal tissues under the clinical situation of vital pulp capping. However, no effects of PS on dentin formation or differentiation of dental pulp cells have been observed.

PS, having a long-chain fatty acid, is insoluble in water and can form micelles and liposomes due to its amphiphilic structure. PS in the outer leaflet of liposomes is expected to bind with calcium ions as it does in matrix vesicles. In this study, we investigated the effects of PS-containing liposomes (PSLs) on matrix mineralization of primary-cultured human dental pulp cells (HDPCs). Alkaline phosphatase (ALP) activity, an early biomarker of differentiation, and mRNA expression of odontogenic genes were also observed in PSL-treated primary HDPC.
MATERIALS AND METHODS

Preparation of PS-containing liposomes
To obtain PSL, PS, phosphatidylcholine (PC) and cholesterol (CH) at a 2:1:1 molar ratio were dissolved in chloroform-methanol (9:1 v/v). The solvent was evaporated under N₂ gas in a glass tube and further removed in a vacuum chamber for 1 h. Then the lipid film was hydrated in phosphate-buffered saline (PBS) (pH 7.4) and vigorously vortexed to obtain a 0.25 mM suspension of total lipid. The lipid suspension was filtered with a 0.1-µm membrane filter. The liposome size was verified by Nicomp 380 DLS (Particle Sizing Systems, Santa Barbara, CA, USA). The PS was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Other chemical reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise specified.

Cell culture
HDPC for primary culture was obtained from premolars that had been removed from patients for orthodontic purposes under guidelines approved by the Institutional Review Board of Seoul National University Dental Hospital (CR106015). The teeth were cut at the apex, and the dental pulp was removed from the pulp cavity and immersed in minimal essential medium (MEM) containing 20% fetal bovine serum (FBS) and antibiotic solution (100 U/mL of penicillin-G and 100 mg/mL of streptomycin). The pulp was minced into pieces and incubated at 37°C in a humidified atmosphere (5% CO₂/95% air) for 20 days with media exchanges at 3-day intervals. The explant-derived dental pulp cells were collected and subcultured to fourth passages in MEM containing 10% FBS for use in this study. Cell culture reagents and antibiotics were purchased from Gibco-BRL (Carlsbad, CA, USA).

Cell viability assay
The cell viability assay was performed with two different culture media (growth media [GM, MEM containing 10% FBS] and differentiation inducing media [DIM, MEM containing 10% FBS, 50 mg/mL L-ascorbic acid, 10⁻⁸ M dexamethasone and 2 mM β-glycerophosphate]). Fourth passage HDPC was grown in a 96-well plate at 37°C in a humidified atmosphere until confluent, and culture media was replaced with fresh media containing PSL. The final concentration of total lipids was between 12.5 and 250 µM. After the treatment with the liposomes for 1, 3 or 6 days, cell viability was evaluated by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] assay (Dojindo Laboratories, Kumamoto, Japan). The treated HDPC was incubated with 100 µL of culture medium containing 10% WST-8 and incubated for 1 h at 37°C. Optical density of the suspension was measured at 450 nm by using an automated plate reader (Sunrise, Tecan, Salzburg, Austria).

ALP activity assay
ALP activity was determined in HDPC that had been exposed to PSL for 3, 6 and 9 days in GM and DIM as described previously. In brief, cells in 96-well plates were incubated in a mixture of 140 µL of alkaline buffer solution and 10 µL of 1.5 M MgCl₂ containing 67 mM 4-nitrophenoxy phosphate (Fluka, Buchs, Switzerland) for 30 min at 37°C. The reaction was stopped by adding 0.5 M NaOH, and the optical density was measured at 405 nm. ALP activity was normalized to the amount of total protein. Cell protein was quantitated by using a bicinechonic acid (BCA) protein assay kit (iNtRon Biotechnology, Sungnam, Korea). During the treatment, liposomes were exchanged by replacing the culture medium containing fresh PSLs at every 3 days.

Gene expression assay
The effect of PSL on the expression of DSPP, OCN, and Runx2 mRNA was investigated. HDPC was exposed to 50 µM PSLs for 9 and 15 days in DIM to measure DSPP and OCN mRNA, and for 5 and 10 days to observe Runx2 expression. Total RNA was then extracted from the cells with an RNA extraction reagent (WelPrep Total RNA Isolation Reagent; Welgene, Daegu, Korea). cDNA was then prepared with a cDNA synthesis kit (Power cDNA Synthesis kit, iNtRon Biotechnology). Real-time PCR was performed in a 20-µL mixture containing 10 µL of SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan), 0.4 µL of ROX Reference Dye II (TaKaRa Bio), cDNA, and primers. The reaction was controlled with an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: DSPP, forward 5’-TGACTCAAAAGGAGAGAAGATGAT-3’, reverse 5’-ATTTACCTTTGCCACTGTCTGATTT-3’. OCN forward 5’-CTGACAGGATTGGCTGACC-3’, reverse 5’-CAAGGGGAAGAGGAAAGG-3’, Runx2, forward 5’-GAACACAAGTGGCCTGCA-3’, reverse 5’-ACTGCTTTCGCACCTTTAATGACTCT-3’; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5’-GTCGGAGTCAACGGATTTGG-3’, reverse 5’-GGTTGAATCATATTGGGAACATG-3’. The PCR conditions were 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 34 s. Gene expressions were evaluated by using the CT (threshold cycle) value and reported as the average expression ratio of the target gene to GAPDH to normalize expression.

Cell matrix mineralization
Alizarin red S staining was used to determine the extent of cell matrix mineralization. HDPC was exposed to PSL for 25 days in DIM. PSL treatment was divided into two groups: 1) exposure to PSL for only the first 6 days of cell culture and 2) exposure to PSL throughout the incubation period. Fresh PSL was supplied by exchanging the PSL-containing culture medium at 3-day intervals. To stain calcified nodules, cell cultures were fixed with 3.7% formaldehyde for 24 h, washed with PBS, and then stained with 20 mg/mL alizarin red S for 10 min. The amount of alizarin red S that bound to the cell matrix was quantified by extraction with 10% cetylpyridinium.
Annexin V staining of PS
PS was localized in PSL-treated HDPC by staining with annexin V-FITC and 4',6-diamidino-2-phenylindole (DAPI). Before staining, HDPC was grown in DIM on culture slides (BD Biosciences, San Jose, CA, USA) for 1, 3 and 6 days in the presence of PSL. The liposomes were replenished at day 3 for 6 days. The cell cultures were then washed with PBS and fixed with 3.7% paraformaldehyde for 20 min. The PS was stained with a FITC annexin V detection kit (BD Biosciences). Cell nuclei were stained with DAPI by UltraCruz Hard-set Mounting Medium (Santa Cruz, CA, USA). Fluorescent images were obtained under a laser scanning confocal microscope (Fluoview FV500, Olympus, Tokyo, Japan).

Statistical analysis
To determine the effect of PSL on cell viability and ALP activity in HDPC, statistical analysis was performed by using a paired t-test. One-way ANOVA test was used to determine the significance of the effects of PLS on the expression of DSPP and OCN mRNA, and mineralization. Values of \( p<0.05 \) were considered statistically significant.

RESULTS
Cell viability and ALP activity of PSL-treated HDPC
To observe the effects of PSL on the viability of HDPC, the pulp cells were incubated for 1, 3 or 6 days in the presence of PSL of which mean diameter was verified as 100 nm. The final PS concentrations estimated from the amount of PSLs in the culture medium ranged between 6.25 and 125 µM. PSL did not adversely affect the viability of HDPC at 1 and 3 days (data not shown). However, PSL treatment at the highest concentration (250 µM total lipids [125 µM PS]) for 6 days slightly reduced the viability of HDPC (Fig. 1A).

ALP activity was investigated in PSL-treated HDPC to observe the effects of PS on cell differentiation. As shown in Fig. 1B, ALP activity of untreated HDPC was enhanced in DIM compared to the levels in GM, which indicated an odontogenic potential of the HDPC used in this study. HDPC tended to have increased ALP activity upon exposure to PSL, and the increase became statistically significant at 25 and 50 µM PSL after 6 and 9 days. However, 250 µM PSL did not increase the ALP activity of HDPC.

Effects of PSL on expression of DSPP and OCN mRNA
To investigate the effect of PSL on expression of DSPP and OCN mRNA, HDPC was exposed to 50 µM PSL for 9 and 15 days. As shown in Fig. 2, there was no statistically significant difference in DSPP and OCN mRNA expression between PSL-treated and untreated HDPC at day 9. However, the mRNA expressions of both genes were slightly but statistically significantly attenuated by PSL for 15 days. The expression of Runx2, the early marker of differentiation, was not altered by PSL.

Effects of PSL on mineralization of HDPC
Mineralization of extracellular matrix was induced in HDPC that had been treated with PSL under differentiation-inducing conditions. To ensure the effect of PSL, two more HDPC (HDPC-P2, HDPC-P3) obtained from different patients were used for mineralization assessment. In untreated cells, HDPC and HDPC-P2 showed a similar degree of mineral deposition at day 25 of incubation, while HDPC-P1 rapidly developed cell matrix mineralization (Fig. 3A). HDPC-P2 produced microscopically-visible mineral deposition even at day 7 (data not shown). Due to the rapid cell matrix
mineralization, HDPC-P2 cells were stained at day 18 to avoid saturated matrix mineralization, while other primary cells were stained at day 25. Cells were exposed to PSL for either first 6 days or entire incubation period. As shown in Fig. 3B, continuous exposure to PSL during the entire culture period increased mineralization of all dental pulp cells. Mineral deposition did not increase statistically significantly in primary cells that had been treated for only 6 days.

In addition to its effects on matrix mineralization,
PSL also altered the appearance of deposited minerals in the well plates. As shown in Fig. 3A, in untreated cells, calcium precipitation was mostly localized to the center of the wells. The tendency to concentrate was most obvious in HDPC-P2 cells, in which mineralization occurred rapidly. When the cells were exposed to PSL, minerals were deposited evenly over the bottom of each well.

**PSL uptake in HDPC**

To observe the interactions between dental pulp cells and liposomal PS, PS was localized in PSL-treated HDPC cell by fluorescence microscopy with annexin-V-FITC. When HDPC was incubated with PSL for one day, small fluorescent liposomes were scattered over the culture surface. There were no signs of direct interactions between the cells and liposomes (Fig. 4A). After 3 days, the fluorescence was mostly localized at pulp cells, and amorphous fluorescent bodies were observed in cells. In addition, some cells were entirely fluorescent, as shown in Fig. 4B. Because the FITC fluorescence was brighter in the interiors of the cells than the edges, PS was likely localized throughout the pulp cells. At day 6, most cells became fluorescent, and high-intensity fluorescence was observed around vacuole-like structures in a few cells. Compared to the 3-day time point, fewer fluorescent particles appeared in the cells at day 6.

**DISCUSSION**

Odontogenic differentiation of dental pulp cells is important in the reparative dentinogenesis for forming a dentin bridge after pulp capping. A variety of osteogenic bioactive molecules, such as bone morphogenic proteins (BMPs), transforming growth factor beta (TGF-β) and bone sialoprotein have been found to induce reparative dentinogenesis. These results suggest that osteogenic molecules can enhance odontogenic differentiation of dental pulp cells. Previous studies have revealed the osteogenic activity of PS, leading us to speculate that it also has odontogenic activity. To investigate the effects of PS on human dental pulp cells, PS was provided as liposomes with PC and CH added to stabilize liposome structure. Liposomes containing PS, PC and CH have been frequently used in various studies for drug delivery, tissue repair, and molecular imaging. The calcium-binding activity of PS in PS-containing liposomes was identified previously.

When the cells were exposed to PSL, HDPC had a dose-dependent increase in ALP activity, within a range of concentrations, and the increase in ALP activity became statistically significant at 25 and 50 µM PSL. At the highest concentration, however, ALP activity was less than that of untreated cells, which was likely due to cytotoxic effects. Because the PSL increased the ALP activity of HDPC at 50 µM, we expected that PSL would also enhance the mRNA expression of odontogenic genes. However, PLS did not affect the expression of Runx2 mRNA. Furthermore, PSL slightly attenuated the expression of DSPP and OCN mRNA in HDPC. The change in ALP activity is an early event during odontogenic differentiation of dental pulp cells. Therefore, the inconsistency between ALP activity and expression of odontogenic genes. In contrast to the results of odontogenic gene expression, PSL enhanced matrix mineralization in HDPC. Although calcium deposition was not enhanced conspicuously, PSL certainly caused a statistically significant increase in mineralization. For mineralization assessment, we have employed three primary dental pulp cultures derived from different patients, which exhibited a higher amount of calcium deposition upon treatment with PSL. Thus PSLs definitely affected cell matrix mineralization of human dental pulp cells.

In addition to the degree of matrix mineralization, PSL also affected the configuration of mineral deposition in plastic culture wells; calcium accumulation on the culture surface was less concentrated at the center of the wells in treated groups compared to untreated. In untreated control, collagen lattice contraction of dental pulp cells is thought to induce concentrated calcium deposition. George et al. also reported significant...
contraction of the collagen matrix caused by dental pulp cells, demonstrating that the contraction could be an obstacle to pulp/dentine tissue engineering. The effects of PSL on contraction of HDPC cultures can be mediated by interactions between extracellular matrix (ECM) components and PSL, or by modulating cellular properties that affect the contraction. The localization of PS at HDPC in confocal fluorescence images provided a clue for the interaction of PSL with ECM or cells. PS staining with annexin V-FITC indicated that PS was concentrated in the inside of the cells as the exposure period increased. PSL endocytosis or fusion with the HDPC cytoplasmic membrane probably played a role in incorporating PS into cells. The predominant PS accumulation in cells suggests that PS affected the contraction of HDPC cultures after cellular uptake. In addition, the preferred localization of PS to the interior of HDPC, rather than at the ECM, after 6 days of treatment also suggests that PS is transported across the cell membranes to mineralize the ECM. PS in PSL may be transported into cells then exported to the ECM in a form capable of calcium deposition.

CONCLUSIONS

PSL enhanced ALP activity and extracellular-matrix mineralization of HDPC. However, PSL did not promote the expression of DSPP, OCN and Runx2 mRNA. PS of PSL was incorporated into HDPC during cell culture within several days, which indicated that the uptake of PS preceded ECM mineralization of PS. The results of present study suggest the usefulness of PS or PSL in the vital pulp capping via stimulation of dentin formation.

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