Abstract: Phosphatidylserine (PS)—normally present on the inner leaflet of the plasma membrane—translocates to the outer leaflet at an early stage of apoptosis. PS-containing liposomes (PSLs) can mimic the effect of apoptotic cells in inducing the secretion of prostaglandin E₂ from phagocytes and inhibiting the maturation of dendritic cells and osteoclast precursors. The present study attempted to evaluate the effect of calcium phosphate (in the form of hydroxyapatite [HAP]) in the presence or absence of PSLs for repair of rat calvarial bone defects. The defects, each 5 mm in diameter, were created in the calvaria parietal bone of 8-week-old Wistar rats and subjected to one of the following treatments: no augmentation (Sham), HAP alone, or a mixture of HAP and PSL (HAP+PSL). Micro-computed tomography data showed that the HAP+PSL complexes promoted greater bone regeneration in comparison with either the Sham procedure or HAP alone at 4 and 8 weeks after implantation. The regeneration of calvarial bone defects induced by PSLs was mediated partly through upregulation of the osteogenic marker Alkaline Phosphatase, Type I collagen, osteocalcin, Runx2, and Osterix mRNAs. These data are the first to show that PSLs can influence bone regeneration by regulating osteoblast differentiation.

Keywords: PS liposome; hydroxyapatite; rat calvarial bone defects.

Introduction
Bone grafting is a surgical procedure used widely to replace alveolar bone that has been lost as a result of severe periodontitis. Alveolar regeneration using bone grafting is a complex process involving the coordinated migration, proliferation and differentiation of various cell types. To achieve effective bone regeneration in large bone defects, the pathological inflammatory condition that caused them must be brought under control (1). Phosphatidylserine (PS) is an acidic phospholipid related to phosphatidylcholine (PC), and is usually located on the inner leaflet of the plasma membrane. Since the discovery that PS is a key initial trigger for phagocytosis in macrophages, various studies have focused on the mechanism by which the production of inflammatory mediators can be suppressed via the PS-PS receptor (2). PS-containing liposomes (PSLs) are engineered nanoparticles that can mimic the effects of apoptotic cells in inducing the
secretion of anti-inflammatory molecules, including transforming growth factor-beta (TGF-β), by phagocytes. TGF-β is thought to contribute to the process of structural healing downstream of cell surface molecules recognizing PS (2). In addition to their anti-inflammatory and phagocytic roles, PSLs are also known to inhibit osteoclastogenesis, which may help to prevent bone loss under pathophysiological conditions (3). Administration of PSLs in an adjuvant arthritic rat model was reported to induce anti-inflammatory responses, resulting in inhibition of inflammatory bone loss (3). The purpose of the present study was to evaluate the effect of PSLs on bone regeneration in experimentally created bone defects.

Materials and Methods

Liposomes

The PSLs, comprising PC and PS at a molar ratio of 7:3, were prepared as described previously (1,3,4). In brief, dried lipid films containing various phospholipids were suspended in PBS and sonicated (Tomy UD-200, Tokyo, Japan) for 10 min on ice.

Animals

Sixty-seven 8-week-old male Wistar rats weighing 250-300 g were used. They were obtained from Kyudo Corporation (Tosu, Japan) and kept under a standard light-dark schedule and relative humidity. A stock diet and tap water were available ad libitum and food and water were available ad libitum. The experimental rats were anesthetized with 2% isoflurane (Abbott Laboratories, Abbott Park, IL, USA) in air, the gas mixture being applied at a constant flow rate of 1.0 L/min using an anesthesia gas machine (Anesthesia Machine SF-B01; MR Technology, Inc., Tsukuba, Japan). A skin incision was made aseptically along the temporal line bilaterally to the middle of the forehead. An incision was then made through the peristeum, and a flap was gently retracted to expose the calvarial bone. A full-thickness standardized trephine defect, 5 mm in diameter, was created in the parietal bone under continuous irrigation with saline buffer. The calvarial bone defects were then subjected to exposure of inflammatory bone loss (3). The purpose of the present study was to evaluate the effect of PSLs on bone regeneration in experimentally created bone defects.

Bone regeneration evaluation

Bone regeneration was evaluated using an in vivo micro-computed tomography (Micro-CT) system (Skyscan-1176 Micro-CT; Bruker, Kontich, Belgium) at 50 kVp and 500 μA. Rats under anesthesia as described above were scanned immediately after treatment and at 2, 4, and 8 weeks later. Each image data set had a scan size of approximately 35 µm. As described previously (5,6), the percentage of newly formed bone in each calvarial defect (New-Bone%) was calculated as the area of newly formed bone / area of the defect originally created by trephination and filled with HAP only or HAP plus PSL at week 0. For quantitative analysis of the Micro-CT images, the pixels within the healing defect were counted digitally using WinROOF image analysis software (Mitani Corp., Tokyo, Japan).

Tissue preparation and histological analysis

At 1, 2, 4, and 8 weeks after the defects had been created, the rats were anesthetized by intraperitoneal administration of sodium pentobarbital, then fixed by aortic perfusion using a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The calvariae were resected and kept in the same fixative overnight at 4°C. The fixed specimens were then decalcified in autoclaved 10% EDTA in 0.01 M PBS, pH 7.4, for 4-6 weeks at 4°C. After dehydration through a graded series of ethanol solutions, the tissues were embedded in paraffin. Serial sections 5 µm thick were cut, and selected sections were processed for staining with hematoxylin-eosin (H-E), Masson’s trichrome (MT), and tartrate-resistant acid phosphatase (TRAP) (TRAP staining kit, Wako, Tokyo, Japan).

RNA extraction

The rats were killed by an overdose of sodium pentobarbital on day 0, and at 2, 4, and 8 weeks after the start of the experimental treatment. Using an 8-mm-diameter trephine bur, tissues were extracted from a defined area that included newly formed bone within the original 5-mm-diameter bone defect. The extracted tissues were homogenized mechanically, and the total RNA was isolated with TRizol reagent (Life Technologies, Tokyo, Japan). RNA samples were collected from 4-5 rats in each experimental group at each time point. cDNA synthesized using 1-μg aliquots of the total RNA extracts was treated in parallel in the presence or absence of reverse transcriptase (ReverTra Ace, Toyobo, Tokyo, Japan).

Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad, Tokyo, Japan) and a MiniOpticon System (Bio-Rad) in accor-
dance with the manufacturer’s protocols. Gene-specific primer sets were designed using Mac Vector (Ceres Bioscience, Saitama, Japan) to span exon-exon junctions to minimize the possibility of amplifying genomic DNA and also to obtain PCR products of 50-250 bases (Table 1), which were based on each gene-coding DNA sequence (cds) recorded in the Nucleotide Database (National Center for Biotechnology Information, USA).

The genes of interest for osteogenesis examined were: alkaline phosphatase (ALP); collagen type I (Col1); osteocalcin (OCN); Runt-related transcription factor 2 (RUNX2); and Osterix (OSX). The PCR conditions used were a 30 s initial enzyme activation step followed by 40 cycles of 15 s at 95°C and 30 s at 60°C.

Gene expression values were expressed as the ratios of differences between the CT values for each gene of interest and GAPDH (DCt = Ct interest - Ct GAPDH). By using a D(DCt) method, \( \{D(DCt)\} = DCt_{\text{initial}} - DCt_{\text{treatment}} \), the fold change \( \{2^{-D(DCt)}\} \) was calculated for each gene.

**Statistical analysis**

The results are given as mean ± standard deviation (SD). The group results were compared by one-way analysis of variance (ANOVA) and Scheffe’s multiple comparison test. Differences at \( P < 0.05 \) were considered to be significant.

**Results**

**Expression of genes involved in the bone defect healing process**

To investigate whether HAP+PSL implantation was able to induce new bone formation in bone defects via the expression of osteogenic genes, the gene expression levels of ALP (Fig. 1a), Type I collagen (Fig. 1b), Osteocalcin (Fig. 1c), Runx2 (Fig. 1d), and Osterix

**Table 1** Oligonucleotide primers used for reverse transcription-polymerase chain reaction. ALP, alkaline phosphatase, GAPDH, glyceraldehyde-3-phosphate dehydrogenase

<table>
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<th>Forward primer (5’–3’):</th>
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<td>CACTGACTGACTGACTGACTG</td>
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**Fig. 1** Gene expression profiles of osteogenic differentiation markers in calvarial bone defects treated with implants of HAP alone (HAP) or HAP with PSL (HAP+PSL). Gene expression was measured by qRT-PCR analysis for alkaline phosphatase (ALP) (a), Type I collagen (b), Osteocalcin (c), Runx2 (d), and Osterix (e). The expression of each gene was normalized to GAPDH and expressed as mean ± SD for 3-5 independent experiments. Each gene expression profile is shown as the fold-change relative to that at week 0 (where week 0 = 1). Significant differences are denoted by: *\( (P < 0.05 \) vs. Sham at the same time point); and **\( (P < 0.01 \) vs. Sham at the same time point).
(Fig. 1e) were analyzed using qRT-PCR at each time point after each treatment (Sham, HAP, and HAP+PSL implantation). Following HAP implantation alone (Fig. 1, HAP), all genes exhibited significantly increased expression at 2 weeks after the procedure compared with the sham operation (Fig. 1, Sham), although in the case of Runx2, this increase was only small (Fig. 1d). At 2 weeks after HAP+PSL implantation (Fig. 1, HAP+PSL), the expression of all genes was dramatically increased in comparison with both the sham operation (Fig. 1, Sham) and the HAP alone implantation (Fig. 1, HAP). Furthermore, the gene expression of ALP (Fig. 1a), Type I collagen (Fig. 1b), and Osteocalcin (Fig. 1c) was increased to a significantly greater degree by HAP+PSL than by HAP alone throughout the observation period from 2 to 8 weeks after the implantation procedure. These results indicated that HAP+PSL promoted the expression of osteogenic markers in regenerated tissue within the bone defect area from 2 weeks after implantation.

**Micro-CT analysis**

Because the induction of osteogenic marker gene expression at 2 weeks was dramatically and significantly different for HAP alone and for HAP+PSL, additional morphological analysis was performed using Micro-CT for each set of calvarial bone defects at 2 and 8 weeks. By 8 weeks postoperatively, some new bone formation was observable in the marginal zone of the initial bone defect in the sham group (Fig. 2A, c).

In rats that had received HAP implantation alone, Micro-CT images demonstrated small peninsulas in the implanted area at 2 weeks (Fig. 2A, b) and by 8 weeks these areas displayed enhanced bone regeneration (Fig. 2A, f). By contrast, bone defects that had been treated with HAP+PSL implants exhibited enhanced bone regeneration even at 2 weeks (Fig. 2A, h) and almost complete healing at 8 weeks (Fig. 2A, i).

To analyze the Micro-CT images quantitatively, the pixels representing the healing defect were counted digitally using a computer application. The proportion of new bone formation in the defect was calculated relative to the area of the defect originally created by trephination and filled with HAP only or HAP plus PSL at week 0. For 4 and 8 weeks, the proportion of new bone formation was significantly greater in the HAP+PSL group than in the HAP alone group at 4 and 8 weeks, and there was an increased tendency for the same outcome at 2 weeks, although the degree was not significant. At 4 and 8 weeks, the mean percentages of new bone in the HAP group were 19.21 ± 4.99% and 40.60 ± 2.16%, whereas those in the HAP+PSL group were 41.76 ± 3.57% and 53.1 ± 2.76%, respectively. The differences in the mean percentages of new bone between the HAP and HAP+PSL

![Fig. 2](image-url) Calvarial healing assessed by Micro-CT observed in a vertical direction (a-i). The treatments of the calvarial defects included no treatment (Sham, a-c), implantation of HAP alone (d-f), and implantation of HAP+PSL (g-i). Micro-CT analysis revealed slight healing in the Sham group (a-c), and slightly more enhanced healing in the HAP group. In contrast, bone defects treated with HAP+PSL implants (g-i) were healed to a greater degree than those treated with HAP alone (d-f) at 8 weeks. Dotted circles (a, c, f, and i) indicate the initial bone defect at week 0 (a). Quantitative Micro-CT analysis. The extent of healing in the defects was expressed by calculating the fraction of the total defect that was filled with new bone under each condition. The percentage of newly formed bone in the defect was calculated relative to the area of the defect originally created by trephination and filled with HAP only or HAP plus PSL at week 0. For 4 and 8 weeks, there was significantly more new bone formation in the HAP+PSL group than in the HAP alone group (HAP). n = 4-6 per group. *: P < 0.01 for HAP vs. HAP+PSL; #: P < 0.01 for 2 weeks vs. 8 weeks.
These results demonstrated that HAP+PSL complexes promoted the formation of calcified areas more potently than HAP alone in experimental rat calvaria bone defects at 4 weeks after implantation.

**Histological findings**

Next, histological evidence of bone healing in the calvarial defects was investigated and compared with the Micro-CT findings. For this purpose, H-E and MT staining of serial sections obtained at 8 weeks was performed. Although the defects were not completely filled with new
bone after any of the treatments, there was appreciable new bone formation around the implanted HAP particles (stained red by MT; Figs. 3e, f, h, i, arrow) in both the HAP alone and HAP+PSL groups. Interestingly, in the HAP+PSL group, this newly formed bone-like tissue was also observed in marginal areas of the bone defects on the dura mater side (Fig. 3i, double arrowhead). This tissue had likely formed independently of the growing bone mass (Fig. 3e, f, h, i, arrow) connected to the initial margin of the defect (Fig. 3a-f, arrowhead).

Since it has been reported that PSL inhibits osteoclastogenesis in vivo (3), TRAP staining of the bone defects was performed in each experimental group at 4 weeks. This revealed several TRAP-positive cells, indicating the presence of osteoclasts in both the Sham (Fig. 4d) and HAP alone (Fig. 4e) groups, although very few were evident in the HAP+PSL implantation group (Fig. 4f).

Discussion
Bone grafting is a surgical procedure commonly used for healing large bone defects, HAP often being the material of choice for alloplastic grafts. To achieve better outcomes, mixing of HAP with other components such as polylactic acid (PLA), which neutralizes alkaline HA upon its degradation, has been investigated as a method for generating novel organic/inorganic hybrid materials (7,8). Other studies have focused on the combination of HAP with growth factors such as BMP, which is a potent inducer of osteogenesis (9-14). However, both techniques have adverse effects. It has been reported that the acid produced by PLA causes local inflammation (15), and that implantation of BMP-2 combined with other materials to promote clinical bone regeneration in humans can cause bone resorption or even increase the risk of de novo malignancy if higher doses of BMP-2 are applied in cases of major trauma or rheumatoid arthritis (16). These findings indicate that the pathological inflammation induced by implantation of HAP and other materials must be brought under control. It has been reported that engineered PSLs possess anti-inflammatory properties and can inhibit osteoclastogenesis (3), and for this reason the effect of a combination of HAP and PSLs for enhancement of bone regeneration in experimental bone defects was investigated in the present study.

The present quantitative analysis of morphological bone formation using Micro-CT showed that the proportion of new bone formation in the control group was increased at 4 and 8 weeks in comparison with the initial bone defect (Fig. 2B). To date, many variants of the rat calvarial bone defect model have been applied for studies of biomaterial implantation (6,17,18). Quantitative Micro-CT analysis has demonstrated an increase in bone mineral content from 4 weeks to 8 weeks after creation of bone defects 4 mm in diameter (17). Conversely, a similar Micro-CT study reported no evidence of new bone formation in 8-mm rat calvarial defects at 3 months (6). In the rat calvarial bone defect model, there appears to be a critical defect size window of 3.8-8.8 mm for spontaneous complete bone repair after 36 weeks (19,20). The defects used in the present study (5 mm) were within this critical size window for new bone formation; clear spontaneous bone formation was observed in the control group at 4 and 8 weeks (Fig. 2B), and this was significantly increased in the HAP+PSL group (Fig. 2B). Implantation of these HAP+PSL complexes therefore appears to promote more efficient spontaneous bone formation than implantation of HAP alone at later postoperative stages.

After 8 weeks in the present study, TRAP-positive cells were absent from bone defects in which PSL+HAP had been implanted, despite being present in the Sham and HAP groups (data not shown). It has been reported that systemic administration of PSLs in an experimentally induced adjuvant arthritis rat model suppressed trabecular bone loss by decreasing the number of osteoclasts (1). The same study yielded in vitro evidence that PSL inhibits the formation of TRAP-positive multinuclear cells among osteclast precursor cells and also in bone marrow cells derived from rat long bones, and that the mechanism underpinning this activity involves RANK-RANKL signaling (1). These findings suggest that local administration of PSLs, when mixed with HA, might suppress osteoclast formation in experimental membranous bone defects like those in the calvaria model. In the present study, enhanced bone formation was observed at the margins of defects containing implants of HAP+PSL complex, especially on the dura mater side. The origin of the osteoblasts that were stimulated to promote bone formation in the calvarial defects was unclear. Bone marrow (21), periosteum (22,23), or dura mater (24,25) may provide osteoprogenitor cells that differentiate into osteoblasts upon stimulation with PSL (Fig. 3i). Systemic administration of PSLs significantly decreases the expression of IL-1β mRNA and the number of IL-1β-positive cells in rat ankle joints at the acute stage of adjuvant arthritis (3), as well as causing in vitro suppression of IL-1β production in lipopolysaccharide (LPS)-stimulated macrophages (3). These previous findings suggest that local administration of PSL may inhibit both osteoclastogenesis and the production of pro-inflammatory factors. These actions would facilitate the role of HAP in increasing total bone mass and spontaneous bone formation at the margins of the initial bone defect.
The present q-PCR data suggested that the significant increase of bone mass in defects treated with HAP+PSL implants may have been due to the promotion of osteoblastic differentiation within the defects. The expression of RUNX2, ALP, OPN, and OCN was upregulated at 2 weeks after implantation of the PSL+HAP complex (Fig. 1), even though the degree of new bone formation at this time point was similar to that induced by HAP alone (Fig. 2). This increase in osteogenic gene expression is consistent with findings, in which PSLs have been shown to enhance the expression of ALP and OCN in rat primary osteoblasts (25). Since short-term administration of prostaglandin E2 (PGE$_2$) stimulates osteogenesis (25) and PSLs increase PGE$_2$ production in whole bone marrow cells and osteoclast precursors (1), it is reasonable to conclude that PSL enhancement of osteogenic gene expression may involve the PGE$_2$ pathway.

Many of the drugs currently used to treat bone resorption act either by inhibiting the differentiation of osteoclasts or by activating osteoblasts. PSLs inhibit osteoclast formation and also appear to induce osteoblast differentiation, thus exerting a bimodal positive action on the temporal and spatial processes underlying bone remodeling. This is the major finding of the present study, in which PSLs promoted bone regeneration in experimental calvarial bone defects partly through upregulation of osteogenic marker genes. This appears to be the first report to have addressed the effect of PSLs on bone regeneration through osteoblast differentiation in vivo. In conclusion, the present results suggest that local treatment with PSLs induces the repair of calvarial bone defects by increasing the activity of osteogenic markers. As PS is a component of the cell membrane, PSLs may be a potentially useful approach for pharmacological intervention against periodontal inflammatory bone loss without any apparent deleterious side effects. However, further studies are required to clarify the mechanism by which PSLs induce osteoblast cell differentiation and to test whether their combination with other osteo-inducing agents exerts similar positive effects on osteoinduction.

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Conflict of Interest
The authors have no conflicts of interest to disclose.

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