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# Phosphatidylserine-Containing Liposomes Inhibit the Differentiation of Osteoclasts and Trabecular Bone Loss

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**Liposomes containing phosphatidylserine (PS) are engulfed by phagocytes including macrophages, microglia, and dendritic cells. PS liposomes (PSLs) mimic the effects of apoptotic cells on these phagocytes to induce the secretion of anti-inflammatory molecules and to inhibit the maturation of dendritic cells. However, the effects of PSLs on osteoclasts, which are also differentiated from the common myeloid precursors, remain to be determined. This study investigated the effects of PSLs on the osteoclastogenesis. In the rat bone marrow culture system, osteoclast precursors phagocytosed PSLs to secrete TGF- $\beta$ 1 and PGE<sub>2</sub>, which in turn inhibited osteoclastogenesis through the downregulation of receptor activator for NF- $\kappa$ B ligand, receptor activator of NF- $\kappa$ B, ICAM-1, and CD44. Consistent with these in vitro observations, i.m. injection of PSLs significantly increased the plasma level of TGF- $\beta$ 1 and PGE<sub>2</sub> and decreased the expression of receptor activator for NF- $\kappa$ B ligand, receptor activator of NF- $\kappa$ B, and ICAM-1 in the skeletal tissues of ankle joints of rats with adjuvant arthritis (AA). A quantitative analysis using microcomputed tomography revealed that PSLs as well as TGF- $\beta$ 1 together with PGE<sub>2</sub> significantly inhibited AA-induced trabecular bone loss. These observations strongly suggest that PSLs generate TGF- $\beta$ 1 and PGE<sub>2</sub> release, leading to inhibit osteoclastogenesis and AA-induced trabecular bone loss. Because PS is a component of the cell membrane, PSLs therefore can be a potentially effective pharmacological intervention against abnormal bone loss, such as osteoporosis without deleterious side effects. *The Journal of Immunology*, 2010, 184: 3191–3201.**

Phagocytic cells in the living body play special roles depending on their location and physiological state. Mononuclear cells, including monocytes in the blood, macrophages in the spleen, microglia in the brain, and Kupffer cells in the liver, phagocytose apoptotic cells and necrotic cell debris in the tissues (1). Besides these professional phagocytes, recent reports have shown that non-professional phagocytes such as dendritic cells, the most professional APCs, can phagocytose apoptotic cells (2, 3). Furthermore, osteoclasts, the multinucleated bone cells, which generally remove mineralized matrix resulting in bone resorption, can also phagocytose apoptotic bone cells (4, 5).

Recognition and removal of apoptotic cells by phagocytes is mediated by changes in plasma membrane on dying cells. One of the most striking and consistent changes on the cell surface of

apoptotic cells is the exposure of phosphatidylserine (PS). PS is an anionic aminophospholipid restricted mostly to the inner leaflet of the plasma membrane in live cells (6). However, when cells undergo apoptosis, PS molecules are exposed on the cell surface (7, 8). Exposure of PS plays a central role in the recognition and phagocytosis of apoptotic cells by macrophages (9, 10). There is increasing evidence that PS-dependent phagocytosis of apoptotic cells transform macrophages to an anti-inflammatory phenotype (11–13). On the other hand, phagocytosis of apoptotic cells inhibits the maturation of dendritic cells and their secretion of proinflammatory cytokines (2, 14). PS liposomes (PSLs) can mimic the effects of apoptotic cells on macrophages and microglia to induce the secretion of anti-inflammatory mediators including TGF- $\beta$ 1 and PGE<sub>2</sub> (15, 16). Moreover, PSLs inhibit the maturation of dendritic cells and enhance their secretion of anti-inflammatory cytokines (17).

Osteoclasts are differentiated from the common myeloid precursors with macrophages and dendritic cells depending the kinds and amount of factors in the bone marrow (BM) microenvironment (18, 19). However, it is not known how PSLs affect the osteoclastogenesis. It is well known that receptor activator for NF- $\kappa$ B ligand (RANKL)/receptor activator of NF- $\kappa$ B (RANK) signal plays essential roles in differentiation and the fusion of the precursors into mature osteoclasts (20–23). In addition, the RANKL/RANK pathways are primarily involved in trabecular bone loss in rats with adjuvant arthritis (AA) (24). On the other hand, ICAM-1 and CD44 are known as the important adhesion molecules for the fusion of osteoclast precursor (OP) cells in osteoclastogenesis (25–27). This study attempted to elucidate the effects of PSLs on osteoclastogenesis in rat BM culture systems and bone loss following the AA, a widely used bone destruction model. The present study highlights the novel role of PSLs that inhibit both osteoclastogenesis and AA-induced bone loss through the secretion of TGF- $\beta$ 1 and PGE<sub>2</sub>.

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Abbreviations used in this paper: 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; AA, adjuvant arthritis; BM, bone marrow; CLSM, confocal laser scanning microscopy; CTR, calcitonin receptor; htROSCM, ROS17/2.8 cell-conditioned medium;  $\mu$ CT, microcomputed tomographic; MNC, multinucleated cell; NBD, 4-nitrobenz-2-oxa-1,3-diazole; OP, osteoclast precursor; OPG, osteoprotegerin; PC, phosphatidylcholine; PCL, phosphatidylcholine liposome; PS, phosphatidylserine; PSL, phosphatidylserine liposome; RANK, receptor activator of NF- $\kappa$ B; RANKL, receptor activator for NF- $\kappa$ B ligand; TGF- $\beta$ R2, TGF- $\beta$  type II receptor; TRAP, tartrate-resistant acid phosphatase.

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## Materials and Methods

### Reagents

$\alpha$ -MEM was from Life Technologies BRL (Grand Island, NY).  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> [ $1\alpha,25(\text{OH})_2\text{D}_3$ ] and SQ22536 were from BIOMOL (Plymouth Meeting, PA). FBS was from BioWhittaker (Walkersville, MD). Soluble RANKL was from Peprotec Science (London, U.K.). TGF- $\beta$ 1 was from AbD Serotec (Kidlington, U.K.) PGE<sub>2</sub> was from Cayman Chemical (Ann Arbor, MI). PS, phosphatidylcholine (PC), 4-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled PS, and NBD-labeled PC were from Avanti Polar Lipids (Alabaster, AL). Rabbit polyclonal anti-TGF- $\beta$  type II receptor (TGF- $\beta$ RII), anti-RANKL, anti-RANK, anti-osteoprotegerin (OPG), mouse monoclonal anti-ICAM-1, anti-CD44, goat polyclonal anti-calcitonin receptor (CTR), and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-ED1 was from BMA (Augst, Switzerland), mouse anti-actin was from Abcam (Tokyo, Japan). Texas Red-conjugated phalloidin was from Molecular Probes (Eugene, OR). Heat-killed *Mycobacterium butyricum* and mineral oil were from Difco (Detroit, MI).

### Liposome

PSLs consisted of PC and PS at a molar ratio of 7:3; PC only, PSLs, and PC liposomes (PCLs) were prepared as described previously (16). In some experiments, NBD-labeled PS and PC were used for preparing NBD-labeled PSLs and PCLs, respectively.

### Animals

Female Lewis rats (4 to 5 wk old) were from Kyudo (Fukuoka, Japan) and cared for according to the manual of Animal Care and Use Committee, Kyushu University, Fukuoka, Japan.

### Rat cell cultures for forming OP cells and osteoclast-like multinucleated cells

Two rat osteoclast-like multinucleated cell (MNC)-generating culture systems, BM and OP cell cultures, were used in the current study as previously described (28, 29). For BM cell culture, whole BM cells were flushed out from tibia and femurs of rats and cultured in 24-multiwell culture plates ( $1 \times 10^6$  cells/well) in  $\alpha$ -MEM with 15% FBS,  $10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ , and 10% (v/v) ROS17/2.8 cell-conditioned medium (htROSCM) for 96 h. For OP cell cultures, osteoblasts and stromal cells were depleted from BM cell culture using a Sephadex G10 column (Amersham Biosciences, Uppsala, Sweden), and then remaining cells were cultured in 24-multiwell culture plates ( $1 \times 10^6$  cells/well) in  $\alpha$ -MEM with 15% FBS,  $10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ , and 10% (v/v) htROSCM for 96 h. In this culture condition, only mononuclear tartrate-resistant acid phosphatase (TRAP)-positive cells were formed as OP cells (28). OP cells were cultured in 24-multiwell culture plates ( $1 \times 10^6$  cells/well) for 96 h in  $\alpha$ -MEM with 15% FBS,  $10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ , and 10% (v/v) htROSCM and soluble RANKL (20 ng/ml) for 96 h. Various concentrations of PSLs, PCLs, and chemical components were added to the BM and OP cell cultures. At day 4 (96 h) of the cultures, cells were fixed by 4% paraformaldehyde in PBS for 30 min and stained with a TRAP kit (Sigma-Aldrich, St. Louis, MO), the general marker of osteoclast. TRAP-positive cells with  $>3$  nuclei were counted as osteoclast-like MNCs (mature osteoclasts).

### Western blotting

PSL-treated or untreated BM and OP cell extracts were prepared with cell lysis buffer (16), and Western blots were performed with the following Abs: rabbit polyclonal anti-RANKL (1:500 dilution), anti-RANK (1:500 dilution), anti-OPG (1:500 dilution), or anti-actin (1:2000 dilution). Blots were final-detected by an ECL detection system (ECL kit, Amersham Bioscience, Uppsala, Sweden) and quantified with image analyzer FLA3000 (Fuji Photo Film, Tokyo, Japan). Values were normalized to actin.

### Flow cytometry analysis

OP cells were treated with PSLs (100  $\mu\text{M}$ ), TGF- $\beta$ 1 (500 ng/ml), or PGE<sub>2</sub> (500 ng/ml). After removal by cell dissociation buffer enzyme-free PBS-based GIBCO (Invitrogen, Carlsbad, CA) at each time point, the cells ( $5 \times 10^5$ ) were incubated with mouse monoclonal ICAM-1 (1:200) or anti-CD44 (1:200) Abs for 1 h on ice, with normal mouse IgG as negative control. After washes by PBS with 2% FBS, the cells were incubated for 30 min on ice with donkey anti-mouse FITC-labeled IgG. After washes by PBS with 2% FBS, immunofluorescence was analyzed using a FACScan analyzer (BD Biosciences, San Jose, CA).

### Systemic PSL treatment for AA rats and sample preparation

Female Lewis rats (5 wk old,  $n = 120$ ) were used for in vivo experiments. The animals ( $n = 93$ ) were injected intradermally with CFA (1.5 mg heat-killed *M. butyricum* well suspended in 0.15 ml mineral oil) at the base of the tail to induce AA as described previously (30, 31). Rats were injected with mineral oil only as sham rats ( $n = 27$ ). We injected i.m. with PSLs (5 mg/kg/day; PSL-treated AA rats,  $n = 27$ ) or PCLs (5 mg/kg/day; PCL-treated AA rats,  $n = 27$ ) in the hind limbs from days 14–28 after CFA injection. At this time point, acute synovial inflammation of ankle joints was confirmed without bone destruction occurring (31, 32). The AA rats were injected i.m. with PBS ( $n = 27$ ). Rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) at days 2, 7, and 14 after PSL or PCL treatment. After obtaining the blood samples, animals were perfused intracardially with 2% paraformaldehyde fixative in PBS. The ankle joints were removed and immersed in the same fixative for 12 h at 4°C. Some samples were for microcomputed tomographic ( $\mu\text{CT}$ ) analyses ( $n = 6$ ); other samples were decalcified in 10% EDTA for another 3 wk. After being immersed in 30% sucrose in PBS for 2 d, samples were embedded in an optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). Serial coronal frozen sections (10  $\mu\text{m}$ ) were prepared for histological and immunohistochemistry as described previously (31). For real-time PCR analyses, the rats ( $n = 3$  for each time point) were perfused intracardially with PBS, and the ankle joints were removed and stored at  $-80^\circ\text{C}$ . In other groups, we injected rats i.m. with TGF- $\beta$ 1 (25  $\mu\text{g}/\text{kg}/\text{day}$ ) and PGE<sub>2</sub> (2.5  $\mu\text{g}/\text{kg}/\text{day}$ ) from days 14–28 after CFA injection at same time course of PSL treatment on AA rats ( $n = 6$ ). We also injected rats i.m. with anti-TGF- $\beta$ 1 Ab (100  $\mu\text{g}/\text{kg}/\text{day}$ ) and SQ22536 (125  $\mu\text{g}/\text{kg}/\text{day}$ ) from day 12, and then treatment with PSLs from days 14–28 after CFA injection ( $n = 6$ ). Rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and perfused intracardially with 2% paraformaldehyde fixative in PBS after 14 d of the treatments. The ankle joints were removed and immersed in the same fixative for  $\mu\text{CT}$  analyses.

### $\mu\text{CT}$ analysis

The ankle joints of sham and the AA rats at day 14 after the treatments of each group ( $n = 6$ ) were examined using ScanXmate-E090S40 in vivo (Comscantecno, Kanazawa, Japan). Briefly, rat paws were scanned, and the bones including tibia, talus, and calcaneus in the ankle joints were examined by three-dimensional surface rendering with a common threshold, optimized using histomorphometric techniques (GEMS MicroView, Toronto, Canada). Because the trabecular bone loss within the tibia and talus in the AA rats was too severe to quantify, calcanei were used for bone qualitative analysis. The parameters of trabecular bone in each group were analyzed using TRI/3D-BON software (Ratoc System Engineering, Tokyo, Japan).

### ELISA

Plasma samples from rats and the supernatants of cultured cells were measured using TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) and PGE<sub>2</sub> (Amersham Bioscience) ELISA kits. The assay followed the protocol provided by the manufacturer. The absorbency at 450 nm was performed by a microplate reader (Bio-Rad, Hercules, CA).

### Immunohistochemistry

The sections of ankle joints from sham and the AA rats were blocked with 10% normal donkey serum and incubated with monoclonal anti-ICAM-1 IgG (1:100) overnight at 4°C. After being incubated with biotinylated anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at 24°C, the sections were incubated with peroxidase-conjugated streptavidin (1:300, Dako Japan, Kyoto, Japan) for 30 min at 24°C. The peroxidase was developed using 3,3'-diaminobenzidine (DAB substrate kit, Vector Laboratories, Burlingame, CA); Mayer's hematoxylin was used as counterstain.

### Immunofluorescence

NBD-labeled PSLs (100  $\mu\text{M}$ ) or NBD-labeled PCLs (100  $\mu\text{M}$ ) were applied to cultured OP cells in chamber slides (Nalge Nunc International, Rochester, NY), and the cells were obtained from 24 to 96 h. In the in vivo experiments, after 6 h of systemic treatment with NBD-labeled PSLs (5 mg/kg) or NBD-labeled PCLs (5 mg/kg) in the AA rats, BM cells and peripheral blood cells were obtained and incubated in chamber slides ( $2 \times 10^5$  cells/well) at 37°C for another 24 h. The cells were fixed with 2% paraformaldehyde for 30 min at 24°C. The cultured cells and the cells from BM and peripheral blood of the AA rats were incubated with mouse anti-ED1 (1:400) or goat anti-CTR (1:200) overnight at 4°C. After washing with PBS, the slides were incubated with rhodamine-conjugated donkey anti-mouse or goat IgG for 1 h at 24°C. In some experiments, culture cells were incubated with Texas Red-conjugated phalloidin for visualizing the margins of OP cells. In the separated

experiments, the ankle joint sections were incubated with anti-goat CTR Ab mixed with anti-mouse ICAM-1 overnight at 4°C. After being incubated with a mixture of FITC-conjugated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-goat IgG for 1 h at 24°C, the slides were mounted in antifading medium Vectashield (Vector Laboratories) and examined by a confocal laser scanning microscope LSM510MET (Carl Zeiss, Jena, Germany).

#### Real-time RT-PCR

Skeletal tissues of ankle joints of rats were obtained after removal of the soft tissues. RNA from the skeletal tissues was extracted with RNeasy Lipid Tissue Midi Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA was reversely transcribed to cDNA using PrimeScript RT reagent Kit (Takara, Shiga, Japan). The real-time RT-PCR was performed using SYBR Premix Ex Taq (Takara). The thermal cycling was holding at 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 60°C for 30 s. After amplifying the PCR reaction, melting curve analysis was performed from 55°C to 95°C (0.5°C/s). GAPDH was used as an internal control. Primer sequences were as follows; RANKL: 5'-TCGGGTTCCCATAAAGTCAG-3' and 5'-CTGAAGCAAATGTTGGCGTA-3'; RANK: 5'-TCGGGTTCCCATAAAGTCAG-3' and 5'-CTGAAGCAAATGTTGGCGTA-3'; OPG: 5'-ACACACCAACTGCAGCTCAC-3' and 5'-TGTCACAGAACACTCAGC-3'; and GAPDH: 5'-AAGTACCCATTGAACCGG-3' and 5'-ATCACAATGCCAGTGGTACG-3'. The standard curve method was used for quantification of gene expression, and target genes/GAPDH were compared between the AA rats and sham rats and between the PSL- or PC liposome-treated AA rats and the untreated AA rats.

#### Statistical analysis

Data are expressed as means  $\pm$  SD. The significant differences between groups were determined with the Student *t* test.

## Results

### Phagocytosed PSLs by OP inhibits rat osteoclastogenesis

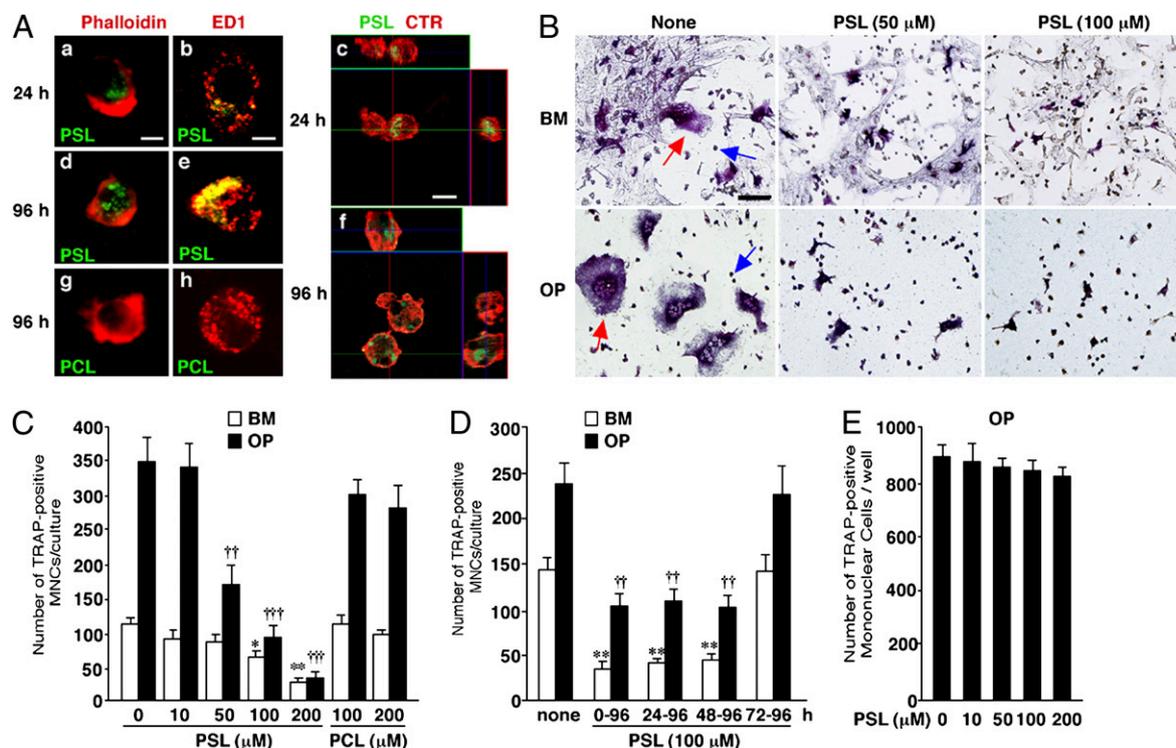
The present study used two rat osteoclast-generating culture systems: BM and OP cell cultures. BM cell cultures contain the whole BM cells including stromal cells and osteoblasts, whereas OP cell cultures contain only mononuclear OP cells (29). To assess the effects of PSLs on osteoclastogenesis, it was necessary to determine whether PSLs could be phagocytosed by OP cells. To visualize the margin of the cells, OP cells were stained by Texas red-conjugated phalloidin, which binds to F-actin. At 24 h after treatment, large granular aggregates of NBD-labeled PSLs apparently localized in the cytoplasm of OP cells, for which cell marginal F-actin was visualized by Texas red-conjugated phalloidin (Fig. 1*Aa*). PSLs were also detected in the cytoplasm up to 96 h after treatment (Fig. 1*Ad*). The merged confocal laser scanning microscopic (CLSM) images show that NBD-labeled PSLs (green) corresponded well with the immunoreactivity for ED1 (red), a lysosomal membrane Ag of rat monocytes (33) (Fig. 1*Ab, e*), thus suggesting that PSLs were transported into the endosome/lysosome of OP cells after engulfment (16). To further elucidate a phagocytosis of PSLs by OP cells, CLSM was used to construct orthogonal cross sectional images of cultured OP cells that were immunohistochemically stained by an anti-CTR Ab, a marker for OP cells. CLSM images of a plane view with two orthogonal cross-sectional views of CTR-labeled cultured OP cells clearly demonstrated that PSLs were engulfed by OP cells (Fig. 1*Ac, f*). However, no granular aggregates of the NBD-labeled PCLs were found in OP cells posttreatment (Fig. 1*Ag, h*).

Next, the effects of PSLs on rat osteoclastogenesis were examined using both OP and BM cell cultures. TRAP-positive cells with >3 nuclei are counted as osteoclast-like MNCs. On the other hand, TRAP-positive mononuclear cells are considered OP cells. In the control condition of both BM and OP cell cultures, the TRAP-positive MNCs were not detected until 48 h, and the number of TRAP-positive MNCs peaked at 96 h (Fig. 1*B*, indicated by red arrows), whereas some cells still remained as TRAP-positive

mononuclear cells (Fig. 1*B*, blue arrows). Fig. 1*B* also showed TRAP-negative cells consisting mainly of osteoblasts and stromal cells in BM cell cultures. The effect of PSLs on rat osteoclastogenesis was thus examined in both BM and OP cell cultures at 96 h. PSLs significantly decreased the mean number of TRAP-positive MNCs in a dose-dependent manner in both cell cultures at 96 h (Fig. 1*B, 1C*). These results suggest that the primary target of PSLs is OP cells to inhibit osteoclastogenesis. As previously noted, the minimal concentration of PSLs for a significant inhibition of TRAP-positive MNC formation in OP cell cultures was lower than that in BM cell cultures. However, PCLs did not affect the mean number of TRAP-positive MNCs in either type of cultured cells at 96 h (Fig. 1*C*). Next, the stage when PSLs inhibited TRAP-positive MNC formation was determined. As shown in Fig. 1*D*, the number of TRAP-positive MNCs significantly decreased when application of PSLs (100  $\mu$ M) was started at an early stage (from 0–48 h) but not at the later stage (from 72 h) in both types of cultured cells. However, PSLs did not inhibit the mean number of TRAP-positive mononuclear cells (OP cells) at 96 h (Fig. 1*E*). These observations strongly suggest that PSLs inhibit rat osteoclastogenesis from the precursors by acting on OP cells directly without affecting the precursor formation.

### PSLs downregulated the expression of RANKL, RANK, ICAM-1, and CD44 in cultured cells

To elucidate the mechanism underlying the inhibitory effects of PSLs on rat osteoclastogenesis, the effects of PSLs on the expression levels of RANKL, RANK, and OPG, which play essential roles in osteoclastogenesis, were examined. The expression of RANKL and OPG was examined in BM cell cultures, because the treatment of soluble RANKL (20 ng/ml) is required for TRAP-positive MNC formation in OP cell cultures. The protein amount of RANKL in BM cells increased from 48 h to 72 h during the 96-h culture period. PSLs significantly decreased the mean level of RANKL from 48 to 72 h (Fig. 2*A, 2B*), but not that of OPG (Fig. 2*A, 2C*). On the other hand, the protein amount of RANK in OP cell cultures increased from 48 h during the culture period. PSLs significantly decreased the mean level of RANK from 48 h in OP cell cultures (Fig. 2*A, 2D*). Furthermore, PGE<sub>2</sub> significantly decreased the mean level of RANKL in BM cell cultures, but did not affect that of either OPG in BM cell cultures or RANK in OP cell cultures. In contrast, TGF- $\beta$ 1 significantly decreased the mean level of RANK in OP cells from 48 to 72 h, but did not affect that of either RANKL or OPG in BM cells (Fig. 2*A–D*). On the other hand, PCLs did not affect the mean level of RANKL and OPG in BM cell cultures or that of RANK in OP cell cultures (Fig. 2*A–D*). Next, the effects of PSLs, PCLs, TGF- $\beta$ 1, and PGE<sub>2</sub> on the expression of both ICAM-1 and CD44 by cultured OP cells were further examined using flow cytometry, because ICAM-1 and CD44 are involved in the fusion of the precursors into mature osteoclasts (25–27). As shown in Fig. 3*A, 3C*, ICAM-1 was expressed in OP cells during the culture period. PSLs significantly decreased the number of ICAM-1-positive OP cell cultures to 53.4% and 75.8% of the control level at 48 and 72 h, respectively. In addition, both TGF- $\beta$ 1 and PGE<sub>2</sub> also significantly decreased the number of ICAM-1-positive cells to 77.3% and 78.2% of the control level at 48 h, respectively. As shown in Fig. 3*B, 3D*, CD44 was also expressed in OP cell cultures during the culture times. PSLs significantly decreased the number of CD44-positive OP cells to 79.5% of the control level at 48 h. Furthermore, TGF- $\beta$ 1 but not PGE<sub>2</sub> significantly decreased the number of CD44-positive cells to 86.2% of the control level at 48 h. It was also noted that PSLs, TGF- $\beta$ 1, and PGE<sub>2</sub> did not affect the expression level of either ICAM-1 or CD44 in OP cell cultures at 72 h posttreatment. On the other hand, PCLs did not affect the expression of both ICAM-1 and CD44 in OP cell cultures (Fig. 2*C, 2D*). These observations strongly suggest that



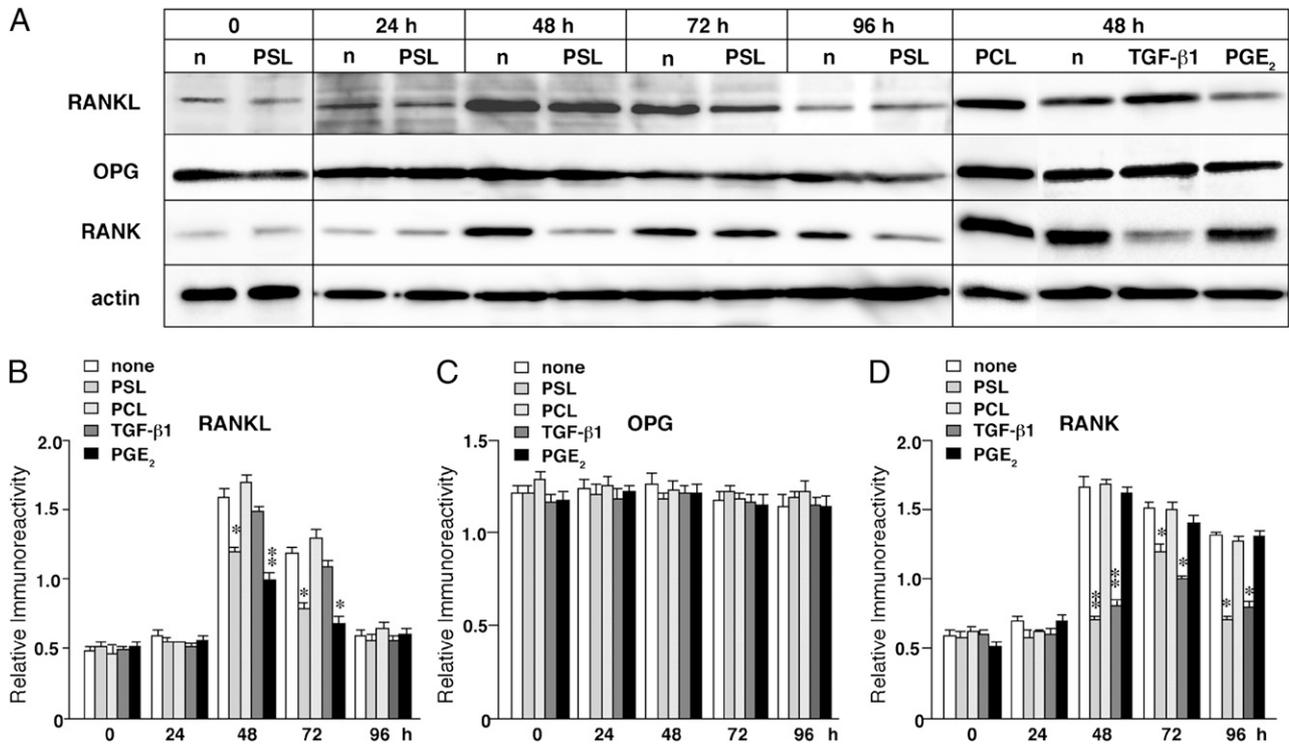
**FIGURE 1.** Inhibitory effect of PSLs on the formation of TRAP-positive multinuclear cells in BM and OP cell cultures. *A*, NBD-leveled PSL (green) was phagocytosed by OP cells and stained with phalloidin (red) at 24 h (*a*) and 96 h (*d*) posttreatment in cultured OP cells. NBD-leveled PSL colocalized with immunoreactivity for ED1 (red) at 24 h (*b*) and 96 h (*e*) posttreatment in cultured OP cells. CLSM images of plane view with two orthogonal cross-sectional views obtained by a  $\times 63$  oil immersion objective clearly showed that PSLs were engulfed by OP cells, which labeled with anti-CTR Ab at 24 h (*c*) and 96 h (*f*) posttreatment. No phagocytosis of NBD-leveled PCL by OP cells stained by phalloidin (*g*) or anti-ED1 Ab (*h*) was observed even at 96 h posttreatment (scale bar, 10  $\mu\text{m}$  in *a* and *b* and 2  $\mu\text{m}$  in *c*). *B* and *C*, The dose dependency of the inhibitory effect of PSL on the rat osteoclastogenesis in BM and OP cell cultures at 96 h. TRAP-positive MNCs and mononuclear cells are indicated by red and blue arrows, respectively (scale bar, 50  $\mu\text{m}$ ) (*B*). PSLs significantly inhibited the formation of TRAP-positive MNCs in a dose-dependent manner in both BM and OP cell cultures; PCLs did not affect the formation of TRAP-positive MNCs (*C*). *D*, The time dependency effect of PSL on the osteoclastogenesis at 96 h. It was noted that a PSL (100  $\mu\text{M}$ ) significantly inhibited osteoclastogenesis in both rat culture cells when applied at an early stage (from 24–48 h) but not at the later stage (from 72 h). Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisks indicate significant differences between the PSL-treated and untreated BM cell cultures (\* $p < 0.05$ ; \*\* $p < 0.01$ ; Student *t* test). Daggers indicate significant differences between the PSL-treated and untreated OP cell cultures ( $^{\dagger\dagger}p < 0.01$ ;  $^{\dagger\dagger\dagger}p < 0.001$ ; Student *t* test). *E*, PSL failed to inhibit the formation of TRAP-positive mononuclear OP cells at 96 h. Each column and bar represents the mean  $\pm$  SD of three experiments.

PSLs, TGF- $\beta$ 1, and PGE<sub>2</sub> decreased the expression levels of RANKL, RANK, ICAM-1, and CD44 that are involved in the differentiation and fusion of OP cells.

#### *PSLs increase the release of TGF- $\beta$ 1 and PGE<sub>2</sub> and thereby inhibit rat osteoclastogenesis*

Next, the effects of PSLs on secretion of TGF- $\beta$ 1 and PGE<sub>2</sub> from both BM and OP cells were examined, because PSLs can induce the production of these molecules after they are phagocytosed by macrophages and microglia (11, 16). TGF- $\beta$ 1 release in both culture cells significantly increased from 24 h after PSL treatment in comparison with that in the untreated cells (Fig. 4*A*), and the increased level of TGF- $\beta$ 1 lasted up to 96 h (data not shown). The release of PGE<sub>2</sub> reached the maximal level at 24 h posttreatment with PSLs, lasted up to 48 h, the early period of the culture, and then returned rapidly to the control level thereafter (Fig. 4*B*). However, no increase of TGF- $\beta$ 1 and PGE<sub>2</sub> was found in either BM or OP cell culture posttreatment with PCLs (Fig. 4*A*, 4*B*). These observations indicate that OP cells induce the secretion of both TGF- $\beta$ 1 and PGE<sub>2</sub> following phagocytosis of PSLs. The roles of TGF- $\beta$ 1 and PGE<sub>2</sub> in osteoclastogenesis are still matters of controversy, depending on the cell types, species, and concentrations of these molecules. It has been reported that TGF- $\beta$ 1 enhances osteoclastogenesis even at low concentrations (<100 pg/ml), whereas it inhibits it at a higher dose

(e.g., 2 ng/ml) (34, 35). Although PGE<sub>2</sub> is generally believed to stimulate osteoclastogenesis in murine cultured cells at a relatively high concentration (36–38), PGE<sub>2</sub> has also been shown to inhibit osteoclastogenesis in human and rat culture systems (39–42). Therefore, the effects of TGF- $\beta$ 1 and PGE<sub>2</sub> on rat osteoclastogenesis were examined. As shown in Fig. 4*C*, TGF- $\beta$ 1 significantly decreased the mean number of TRAP-positive MNCs at the concentration of 500 pg/ml but not 50 pg/ml. The inhibitory effect of TGF- $\beta$ 1 (500 pg/ml) was completely reversed by rabbit anti-TGF- $\beta$ 1R2 Ab (5  $\mu\text{g}/\text{ml}$ ), but not control rabbit IgG (Fig. 4*C*). Furthermore, PGE<sub>2</sub> also significantly inhibited the formation of TRAP-positive MNCs at the concentration of 500 pg/ml but not 50 pg/ml. The inhibitory effect of PGE<sub>2</sub> was completely antagonized by SQ22536 (10  $\mu\text{M}$ ), a specific inhibitor of adenylate cyclase. It is noted that combined treatment with TGF- $\beta$ 1 (50 pg/ml) and PGE<sub>2</sub> (50 pg/ml) significantly inhibited the formation of TRAP-positive MNCs. Moreover, the inhibitory effect of PSLs (100  $\mu\text{M}$ ) on the formation of mature osteoclasts was reversed by either rabbit anti-TGF- $\beta$ 1 Ab (5  $\mu\text{g}/\text{ml}$ ) or SQ22536 (Fig. 4*D*). However, control rabbit IgG did not affect the inhibitory effects of PSLs on the formation of TRAP-positive MNCs (Fig. 4*D*). PSL-induced secretion of either TGF- $\beta$ 1 or PGE<sub>2</sub> in the culture medium did not reach the concentration to inhibit osteoclastogenesis. Therefore, it is reasonable to consider that PSL-induced the inhibition of osteoclastogenesis was mediated by presumable synergistic effect of TGF- $\beta$ 1 and PGE<sub>2</sub>.



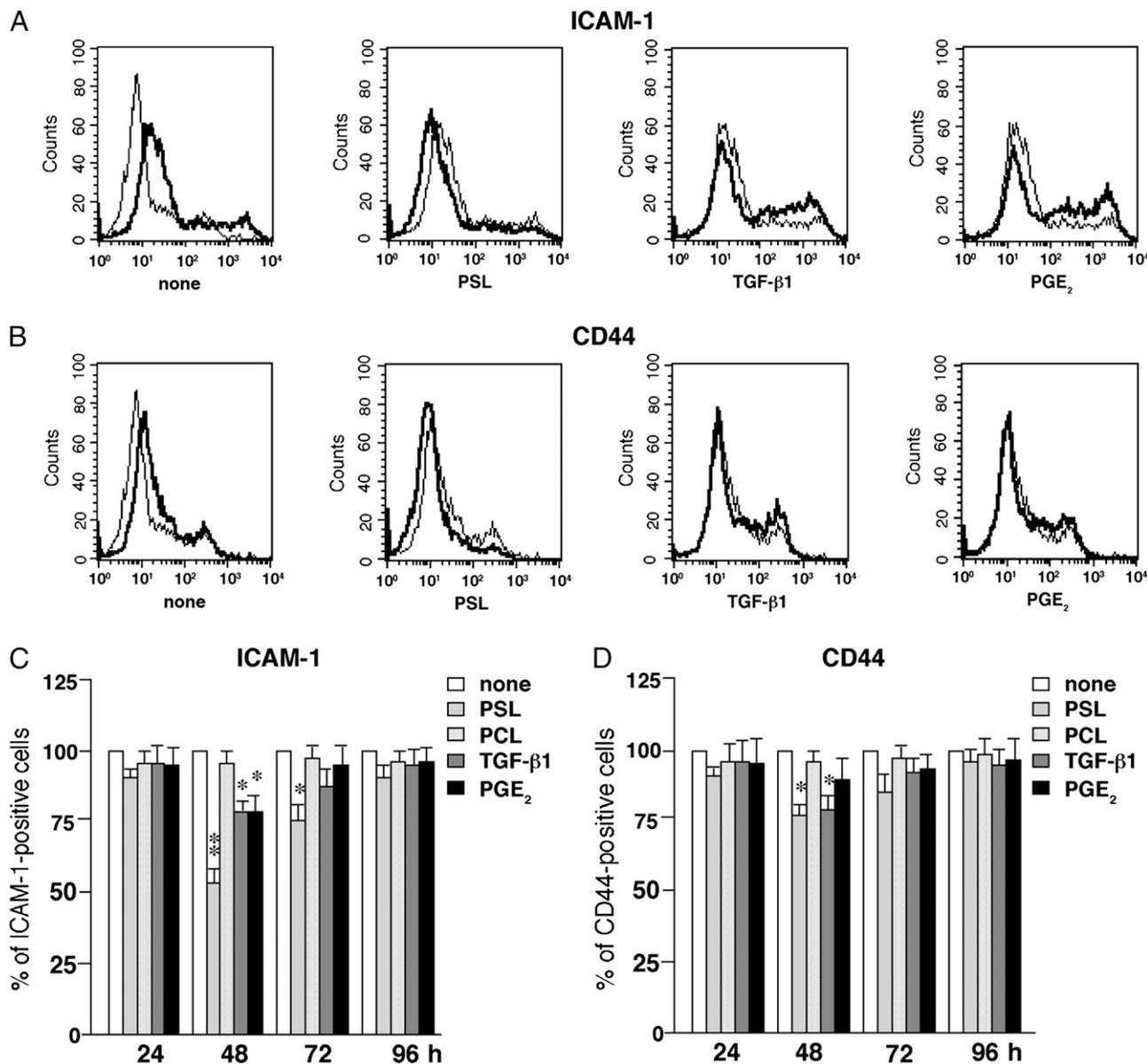
**FIGURE 2.** Effects of PSLs, TGF- $\beta$ 1, and PGE<sub>2</sub> on the protein expression of RANKL, RANK, and OPG in the rat culture cells. *A*, Immunoblot analyses of RANKL, OPG, and RANK posttreatment with PSLs, PCLs, TGF- $\beta$ 1, and PGE<sub>2</sub>. *B–D*, The time course changes of RANKL and OPG in BM cell cultures and RANK in OP cell cultures. The mean protein levels of RANKL (*B*), OPG (*C*), and RANK (*D*) posttreatment with PSL, PCL, TGF- $\beta$ 1, and PGE<sub>2</sub>. The mean relative immunoreactivity for RANKL, OPG, and RANK in untreated and PSL-treated BM or OP cell cultures was determined by comparison with that for actin. Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisks indicate significant differences among the PSL-, TGF- $\beta$ 1-, and PGE<sub>2</sub>-treated and untreated BM or OP cell cultures at each time points (\**p* < 0.05; \*\**p* < 0.01; Student *t* test).

*Systemic treatment with PSLs increases the plasma level of TGF- $\beta$ 1 and PGE<sub>2</sub> and markedly suppresses trabecular bone loss in the AA rats*

To confirm the inhibitory effect of PSLs on osteoclast formation in vivo, the effects of PSLs on an osteoclastic-induced trabecular bone loss model, the AA rats, was investigated. Systemic treatment with PSLs was initiated 14 d after CFA injection, when the peak of paw inflammation was established. However, the number of osteoclasts increases with bone destruction after the peak of inflammation in the ankle joints of the AA rats (30, 31). At 24 h after i.m. treatment, CLSM images of a plane view with two orthogonal cross-sectional views clearly demonstrated that PSLs were engulfed by CTR-labeled OP cells in the tibia marrow (Fig. 5*Aa*) of the AA rats. In the peripheral blood of the AA rats, CLSM images of a plane view with two orthogonal cross-sectional views also clearly demonstrated that PSLs localized in ED1-positive lysosomes of mononuclear cells (Fig. 5*Ab*). The paw volume increase of AA rats occurred in two stages, an acute inflammation stage from days 10–21 after CFA injection and the bone/joints destruction stage thereafter (31, 32). As shown in Fig. 5*B*, systemic treatment with PSLs or treatment with TGF- $\beta$ 1 and PGE<sub>2</sub> together significantly reduced the paw volume of AA rats during the bone/joints destruction stages. However, treatment with PCLs did not reduce the paw volume of AA rats. Furthermore, treatment with anti-TGF- $\beta$ 1 Ab and SQ22536 together completely reversed the inhibitory effects of PS on the paw volume of AA rats. At day 2 posttreatment with PSLs, the mean plasma levels of TGF- $\beta$ 1 and PGE<sub>2</sub> in the AA rats significant increased in comparison with those in the PBS- and PCL-treated AA rats, and the significant high plasma level of both TGF- $\beta$ 1 and PGE<sub>2</sub> in the PSL-treated AA rats lasted up to 7 d after PSL treatment (Fig. 5*C*). The mRNA

expression levels of RANKL and RANK significantly increased in the skeletal tissues of the AA rats from 21 d after CFA injection. Treatment with PSLs for 7 d significantly decreased the mean expression levels of both RANKL and RANK in the AA rats; however, treatment with PCLs did not decrease the expression level of either RANKL or RANK in the AA rats (Fig. 5*D*). On the other hand, treatment with PSLs for 7 d did not affect the mean expression levels of OPG in the AA rats (Fig. 5*D*). Furthermore, the number of ICAM-1-positive mononuclear cells, which were identified as CTR-positive OP cells, increased in the BM cavities of the AA rats from 21 d after CFA injection. Treatment with PSLs for 7 d markedly decreased the number of ICAM-1-positive OP cells in the AA rats (Fig. 5*E*). These observations obtained in the AA rats were closely consistent with those obtained in the cultured cells. Therefore, a possible decrease in osteoclast formation was examined in the tibia BM cavity of the AA rats. Consistent with previous observations (32), the number of TRAP-positive MNCs in the BM cavity of the distal epiphysis of tibia rapidly increased from 21 d, and numerous TRAP-positive MNCs were found at 28 d after CFA injection. Systemic treatment with PSLs significantly decreased the mean number of TRAP-positive MNCs in the tibia BM cavity of the AA rats; however, PCLs did not decrease the mean number of TRAP-positive MNCs (Fig. 5*F*).

To further prove the essential roles of TGF- $\beta$ 1 and PGE<sub>2</sub> in PSL-induced inhibitory effects on the trabecular bone loss in AA rats, we finally quantified the trabecular bone network of the bones in the ankle joints at 14 d posttreatment with PSLs or TGF- $\beta$ 1 and PGE<sub>2</sub> together on AA rats (28 d after CFA injection) using  $\mu$ CT analysis. The  $\mu$ CT analysis was conducted in the calcaneus, because trabecular bone losses within the tibia and talus in the AA rats were too severe to quantify. In comparison with the sham rats



**FIGURE 3.** Effects of PSLs, TGF- $\beta$ 1, and PGE<sub>2</sub> on the expression of ICAM-1 and CD44 in OP cell cultures. *A* and *B*, Effects of PSL, TGF- $\beta$ 1, and PGE<sub>2</sub> on the expression of ICAM-1 (*A*) and CD44 (*B*) in OP cells at 48 h posttreatment. In untreated OP cell cultures, the thick lines represent the ICAM-1 or CD44 expression, and the thin lines represent the controls using mouse IgG. In treated OP cell cultures, thin lines represent untreated cells and thick lines represent treatment with PSL, PGE<sub>2</sub>, or TGF- $\beta$ 1, respectively. The time course changes in the mean normalized percentage of ICAM-1-positive cells (*C*) and CD44-positive (*D*) OP cells posttreatment with PSLs, PCLs, TGF- $\beta$ 1, and PGE<sub>2</sub>. Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisks indicate significant differences between the PSL-, TGF- $\beta$ 1-, or PGE<sub>2</sub>-treated and untreated OP cell cultures (\* $p$  < 0.05; \*\* $p$  < 0.01; Student *t* test).

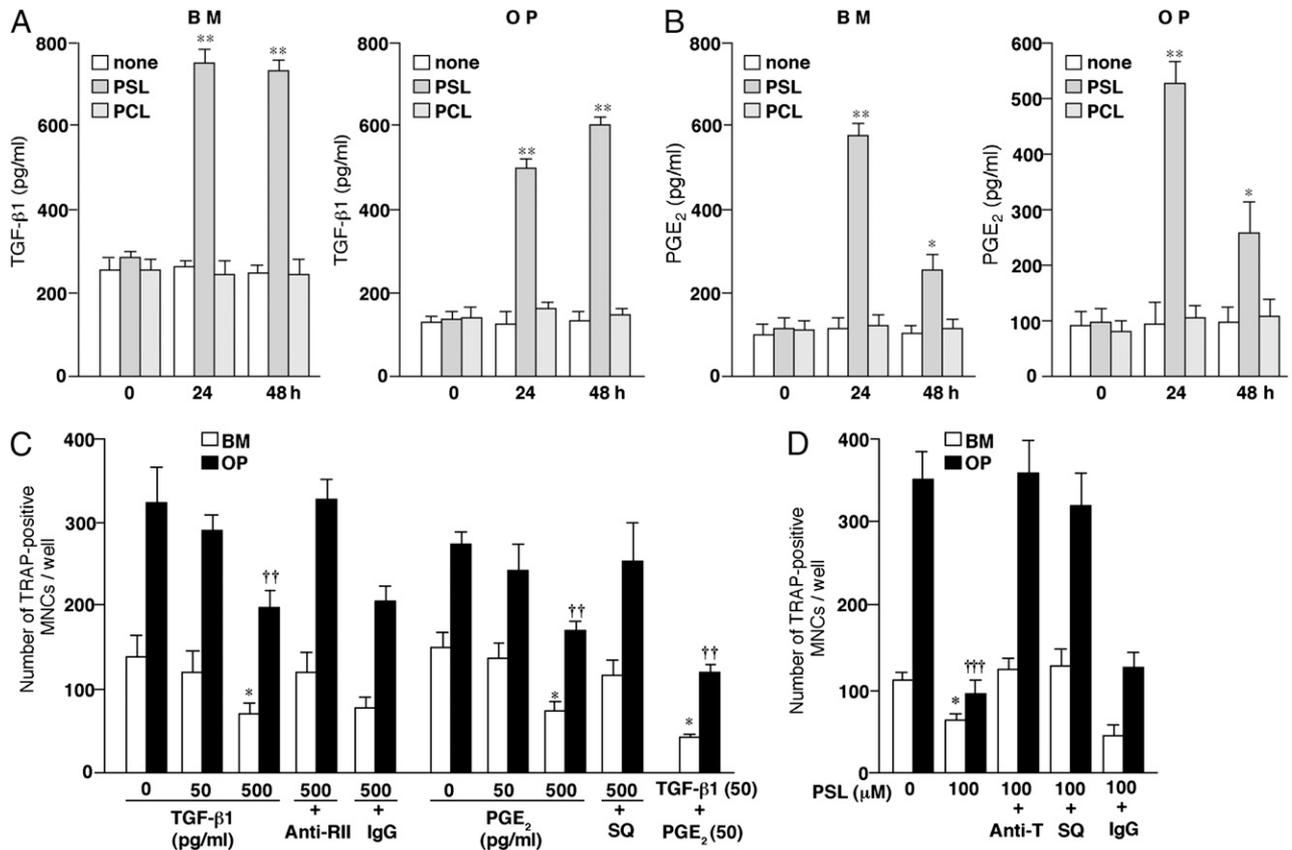
(Fig. 6A), a severe trabecular bone loss was observed in the AA rats (Fig. 6B). The AA-induced trabecular bone loss was markedly inhibited by treatment with PSLs (Fig. 6C) as well as treatment with TGF- $\beta$ 1 and PGE<sub>2</sub> together (Fig. 6D). However, the AA-induced trabecular bone loss was not inhibited by treatment with PCLs (Fig. 6E). Furthermore, the AA-induced trabecular bone loss was not inhibited by treatment with PSLs and simultaneous treatment with anti-TGF- $\beta$ 1 Ab and SQ22536 (Fig. 6F). In analysis of the parameters of trabecular bone shown in Fig. 6G–I, PSLs, as well as TGF- $\beta$ 1 and PGE<sub>2</sub> together, significantly inhibited the AA-induced decrease of trabecular bone volume per unit of metaphysis (BV/TV, Fig. 6G), trabecular thickness (Tb/Th, Fig. 6H), and trabecular numbers (Tb/N, Fig. 6I). On the other hand, PCLs showed no effect on any parameters in AA-induced trabecular bone loss. Moreover, TGF- $\beta$ 1 Ab and SQ22536 sig-

nificantly blocked the inhibitory effects of PSLs on AA-induced trabecular bone loss.

## Discussion

The major finding of this study is that PSLs inhibit osteoclastogenesis and AA-induced trabecular bone loss in rats. This is the first report to clarify the effects of PSLs on osteoclasts. However, the effects of PSLs on macrophages and dendritic cells, which are differentiated from the common myeloid precursors with osteoclasts, have been described elsewhere (18, 19). The current findings are consistent with a recent observation that PSLs inhibit the maturation of myeloid dendritic cells (17).

TGF- $\beta$ 1 and PGE<sub>2</sub> secreted from PSL-engulfed OP cells were thought to be closely associated with the inhibitory effects of PSLs on osteoclastogenesis and AA-induced bone loss for two reasons.

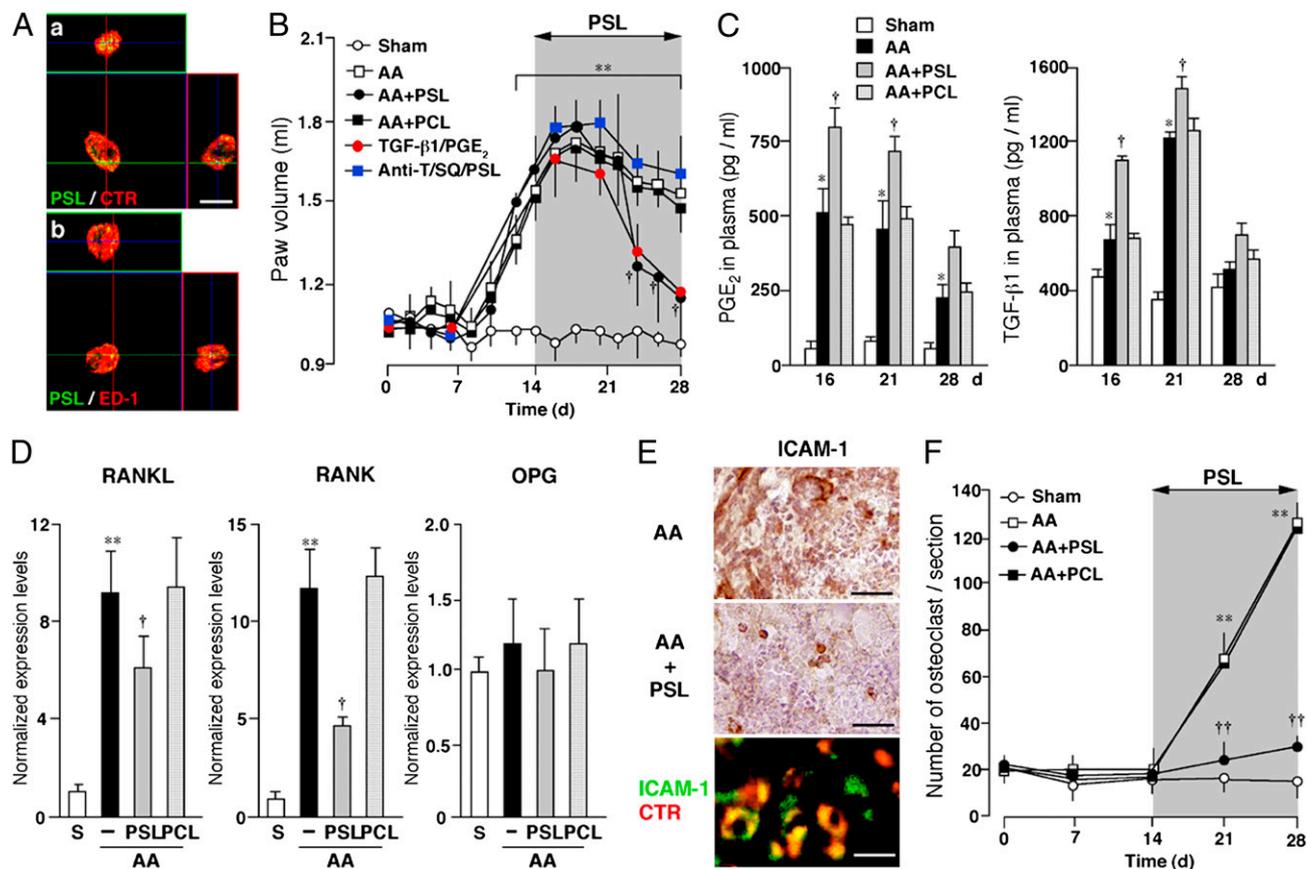


**FIGURE 4.** Involvement of TGF- $\beta$ 1 and PGE<sub>2</sub> in PSL-induced inhibition of osteoclastogenesis in rat BM and OP cell cultures. Release of TGF- $\beta$ 1 (A) and PGE<sub>2</sub> (B) from the cultured cells following treatment with PSLs and PCLs. Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisks indicate significant differences between the PSL-treated and untreated BM cell cultures (\* $p$  < 0.05; \*\* $p$  < 0.01; Student  $t$  test). Daggers indicate significant differences between the PSL-treated and untreated OP cell cultures ( $\dagger p$  < 0.05;  $\dagger\dagger p$  < 0.01; Student  $t$  test). C, The number of TRAP-positive MNCs was decreased in a dose-dependent posttreatment with TGF- $\beta$ 1 or PGE<sub>2</sub>. TGF- $\beta$ 1- and PGE<sub>2</sub>-induced inhibition was reversed by anti-TGF- $\beta$ RII Ab (Anti-RII) and SQ22536 (SQ), a specific inhibitor for adenylate cyclase, respectively, but was not reversed by control rabbit IgG. The mean number of TRAP-positive MNCs was not affected by either TGF- $\beta$ 1 or PGE<sub>2</sub> alone at the concentration of 50 pg/ml, but was significantly suppressed by their combined treatment. Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisks indicate a significant difference between the PSL-treated and untreated BM cells (\* $p$  < 0.05; Student  $t$  test). Daggers indicate a significant difference between the PSL-treated and untreated OP cells ( $\dagger p$  < 0.01; Student  $t$  test). D, The inhibitory effect of PSL (100  $\mu$ M) on TRAP-positive MNC formation was reversed by either anti-TGF- $\beta$ 1 (Anti-T) or SQ22536 (SQ), but was not reversed by control rabbit IgG. Each column and bar represents the mean  $\pm$  SD of three experiments. Asterisk indicates a significant difference between the PSL-treated and untreated BM cell cultures (\* $p$  < 0.05; Student  $t$  test). Daggers indicate a significant difference between the PSL-treated and untreated OP cell cultures ( $\dagger\dagger p$  < 0.001; Student  $t$  test).

First, addition of either TGF- $\beta$ 1 or PGE<sub>2</sub> alone significantly inhibited osteoclastogenesis (the formation of TRAP-positive MNCs) in both rat culture systems at the concentration of 500 pg/ml. Second, the inhibitory effect of PSLs on osteoclastogenesis was significantly reversed by an Ab against TGF- $\beta$ RII, the receptor for the signaling of response to TGF- $\beta$ 1 (43). The effect of PSLs on osteoclastogenesis was also significantly antagonized by SQ22536, an adenylate cyclase inhibitor, thus suggesting the involvement of EP2 and/or EP4 receptors that are coupled to adenylate cyclase (44). The substantial concentration of TGF- $\beta$ 1, which alone could suppress the osteoclastogenesis in BM and OP cell cultures, was estimated to be 800 pg/ml and 650 pg/ml, respectively, because of the relatively high basal level of TGF- $\beta$ 1 in the culture medium (i.e., 300 pg/ml in BM cells, 150 pg/ml in OP cells). On the other hand, the substantial concentration of PGE<sub>2</sub>, which alone could suppress osteoclastogenesis, was estimated to be 650 pg/ml and 580 pg/ml in BM and OP cell cultures, respectively, because of relatively high basal level of PGE<sub>2</sub> in the culture medium (i.e., 150 pg/ml in BM and 80 pg/ml OP cells). PSL-induced secretion of either TGF- $\beta$ 1 or PGE<sub>2</sub> in the culture medium did not reach the concentration to inhibit osteoclastogenesis in BM and OP cell cultures. Either TGF- $\beta$ 1 or PGE<sub>2</sub>

alone at the concentration of 50 pg/ml had no effect on osteoclastogenesis; however, their combined treatment significantly suppressed it. Therefore, it is reasonable to consider that PSLs inhibited the osteoclastogenesis by a synergistic effect of TGF- $\beta$ 1 and PGE<sub>2</sub>. This can explain why anti-TGF- $\beta$ 1 Ab alone or SQ22536 alone can suppress the PSL-induced inhibitory effect on osteoclastogenesis. The effects of TGF- $\beta$ 1 and PGE<sub>2</sub> on bone metabolism are still matters of controversy. TGF- $\beta$ 1 enhances osteoclastogenesis even at low concentrations (<100 pg/ml), whereas it inhibits it at a high dose (e.g., 2 ng/ml) (34, 35). Although PGE<sub>2</sub> is generally believed to stimulate osteoclastogenesis in murine-cultured cells at a relatively high concentration (36–38), it also inhibits osteoclastogenesis in human and rat culture systems (39–44). Therefore, it is conceivable that the effects of PGE<sub>2</sub> depend on experimental conditions, including the cell types, species, and concentrations. Additional experiments are necessary to elucidate the effect of PSLs on human osteoclast formation.

PSLs could be phagocytosed by OP cells, thus inhibiting osteoclastogenesis. These results indicate that PSLs affect the destiny of OP cells by generating TGF- $\beta$ 1 and PGE<sub>2</sub>, which may act in an autocrine manner. On the other hand, PSLs also inhibit

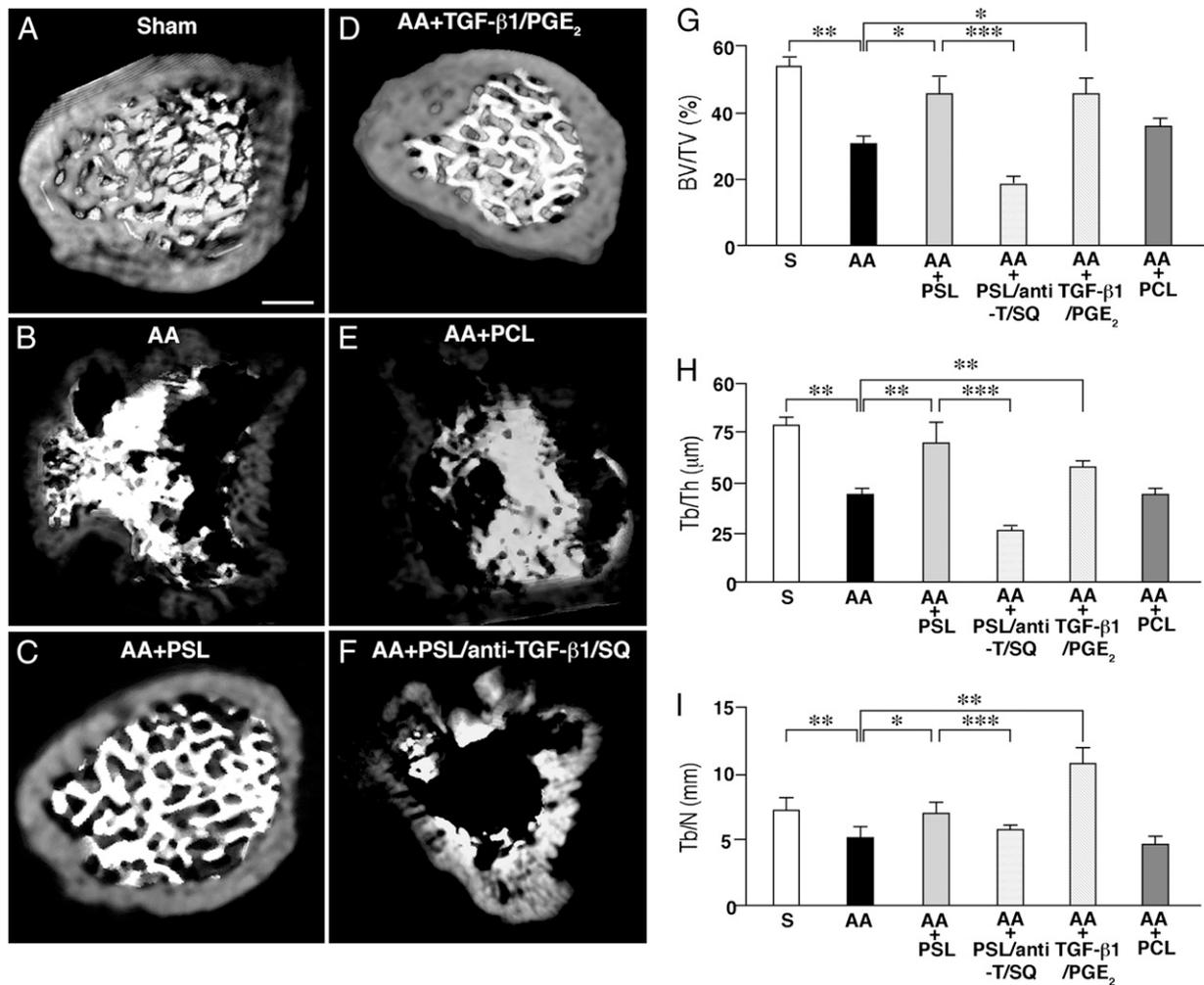


**FIGURE 5.** Effects of systemic treatment with PSLs on osteoclast formation in the AA rats. *A*, CLSM images of plane view with two orthogonal cross-sectional views obtained by a  $\times 63$  oil immersion objective clearly showed that NBD-PSL (green) were engulfed by CTR-positive OP cells (red) in the tibia BM of AA rats (*a*). In the peripheral blood of the AA rats (*b*), NBD-PSL (green) localized in ED1-positive mononuclear cells (red) (scale bar,  $10\ \mu\text{m}$ ). *B*, Effects of PSLs and PCLs on the mean paw volume after CFA injection. *C*, The time course levels of TGF- $\beta$ 1 and PGE $_2$  in the plasma of the AA rats after systemic treatment with PSLs and PCLs. *D*, Effects of PSLs and PCLs on the mean mRNA levels of RANKL, RANK, and OPG in skeletal tissues of the AA rats after 7 d treatment. *E*, The immunoreactivity for ICAM-1 in the tibia BM cavities of AA or PSL-treated AA rats (scale bar,  $50\ \mu\text{m}$ ), and a CLSM image shows that the immunoreactivity for ICAM-1 corresponded with that of CTR in the AA rats (scale bar,  $20\ \mu\text{m}$ ). *F*, The effects of PSLs and PCLs on the mean number of TRAP-positive osteoclasts in the tibia BM cavity in the AA rats. Each symbol and bar represents the mean  $\pm$  SD from six rats in each group. Asterisks indicate significant differences between the sham rats and untreated AA rats (\* $p < 0.05$ ; \*\* $p < 0.01$ ; Student *t* test). Daggers indicate a significant difference between the PSL-treated AA rats and untreated AA rats ( $\dagger p < 0.05$ ;  $\ddagger p < 0.01$ ; Student *t* test).

osteoclastogenesis from BM cell cultures. This suggests that PSLs also affect the functions of osteoblasts and stromal cells in a paracrine manner because they have receptors for both TGF- $\beta$ 1 and PGE $_2$  (45, 46). PSLs activate p44/p42 ERK but not p38 MAPK in primary cultured rat microglia (16) and macrophages (15). In addition, the PSL-induced PGE $_2$  production is mediated by cyclooxygenase-1 and its functionally coupled upregulated terminal PGE synthases, especially microsomal PGE synthase 2 (16). Furthermore, bafilomycin A $_1$ , a specific inhibitor of vacuolar-type H $^+$ -ATPase, almost completely inhibited PSL-induced PGE $_2$  generation, thus suggesting the involvement of lysosomal proteolytic functions in the PGE $_2$  generation following phagocytosis of PSLs. It is reasonable to consider that PSLs also activate such unique intracellular pathways in OP cells to generate TGF- $\beta$ 1 and PGE $_2$ . Further studies to clarify the intracellular signaling pathways activated postphagocytosis of PSLs are now in progress.

The downregulation of both RANKL and RANK may be a potential mechanism for the inhibitory effect of PSLs on rat osteoclastogenesis, because the RANKL/RANK pathway plays essential roles in differentiation and the fusion of the precursors into mature osteoclasts (20–22). The disruption of either the RANKL or RANK gene causes severe osteopetrosis in mice, with very few osteoclasts in the skeletal tissues (21, 23). In addition, the RANKL/RANK

pathways are primarily involved in trabecular bone loss in the AA rats (24). In the current study, PSLs significantly decreased the protein amounts of RANKL in BM cells and RANK in the rat BM cultures. Furthermore, TGF- $\beta$ 1 inhibited the RANK expression in OP cells as reported previously (47–49), and PGE $_2$  suppressed the RANKL expression in BM cell cultures in our rat culture system, thus suggesting that PSLs affect the proximal signaling of osteoclastogenesis. PSLs inhibited the formation of mature osteoclasts but not that of OP cells, suggesting that PSLs may consequently inhibit cell-to-cell contact and/or cell fusion during osteoclastogenesis. These observations prompted us to further examine the effect of PSLs on the expression level of ICAM-1 and CD44, which are the important adhesion molecules for the fusion of OP cells during differentiation (25–27). Consistent with published reports (26, 27, 50), ICAM-1 and CD44 are expressed on the OP cells. Furthermore, PSLs decreased ICAM-1 and CD44 expression on OPs at the early stage of osteoclastogenesis in rat BM cell cultures. Therefore, another potential mechanism underlying the inhibitory effect of PSLs on osteoclastogenesis is the inhibition of OP cell fusion through the reduction of ICAM-1 and CD44 expression. Moreover, TGF- $\beta$ 1 and PGE $_2$  reduced ICAM-1 and CD44 expression, respectively, in OP cell cultures during the early stage of osteoclastogenesis. These are in line with the previous findings that



**FIGURE 6.**  $\mu$ CT analyses of the effect of PSLs on the AA-induced trabecular bone loss.  $\mu$ CT images of calcaneus in the ankle joints of sham rats (A), untreated AA rats (B), the PSL-treated AA rats (C), the TGF- $\beta$ 1- and PGE<sub>2</sub>-treated AA rats (D), the PCL-treated AA rats (E), and the anti-TGF $\beta$ 1 and SQ22536 and PSL-treated AA rats (F) at day 14 after the treatments. The mean volume of trabecular bone per unit of metaphysis (BV/TV, G), the mean of trabecular thickness (Tb/Th, H) and the mean of trabecular numbers (Tb/N, I) in sham rats (S), untreated AA rats (AA), PSL-treated (AA+PSL), PCL-treated (AA+PCL), TGF- $\beta$ 1 with PGE<sub>2</sub>-treated (AA+TGF- $\beta$ 1/PGE<sub>2</sub>), anti-TGF- $\beta$ 1 with SQ22536 and PSL-treated (AA+PSL/anti-T/SQ) AA rats. Each column and bar represents the mean  $\pm$  SD from six rats at each group. Asterisks indicate significant differences between the values (\* $p$  < 0.05; \*\* $p$  < 0.01; Student  $t$  test). Daggers indicate a significant difference between the PSL-treated AA rats and untreated AA rats ( $\dagger p$  < 0.05;  $\dagger\dagger p$  < 0.01; Student  $t$  test).

both TGF- $\beta$ 1 and PGE<sub>2</sub> inhibit ICAM-1 expression in monocytes (51–54). Taken together, these observations strongly suggest that TGF- $\beta$ 1 and PGE<sub>2</sub> are major causative factors for PSL-induced inhibition of rat osteoclastogenesis through reduction of the expression of RANK, RANKL, ICAM-1, and CD44 at the early stage of osteoclastogenesis.

In parallel with the *in vitro* experiments, the effects of PSLs were further examined on the bone destruction associated with AA, which is widely used in research of inflammation and bone destruction (55, 56). We have noted previously that the inflammation of the synovial tissues peaks at 14 to 21 d after CFA injection, and the trabecular bone loss in the bone cavities occurs thereafter (31, 32). To focus on the effects of PSLs on AA-induced trabecular bone loss, systemic treatment with PSLs was started at 14 d after CFA injection, when the inflammation is established without bone destruction in the ankle joints. The following sequential events were observed after systemic treatment of PSLs in the AA rats: 1) phagocytosis of PSLs by OP cells in BM and monocytes in blood; 2) a significant increase in the plasma level of both TGF- $\beta$ 1 and PGE<sub>2</sub>; 3) a significant decrease in AA-induced elevated expression of RANKL, RANK, and ICAM-1 in the skeletal tissues with the decrease in TRAP-positive osteoclast formation; and 4) a significant

inhibition of the AA-induced trabecular bone loss. Furthermore, systemic treatment with TGF- $\beta$ 1 and PGE<sub>2</sub> together significantly reduced the AA-induced trabecular bone loss, and with anti-TGF- $\beta$ 1 Ab and SQ22536, completely reversed the inhibitory effects of PSL and the AA-induced trabecular bone loss. These *in vivo* findings further support the hypothesis that PSLs inhibit osteoclastogenesis by generating TGF- $\beta$ 1 and PGE<sub>2</sub>. The deficit of osteoclastogenesis may further lead to the inhibition of AA-induced bone loss. Moreover, in addition to the regulation of osteoclastic bone resorption, TGF- $\beta$ 1 and PGE<sub>2</sub> are also potent stimulators of osteoblastic bone formation. In the present study, we confirmed that systemic treatment with TGF- $\beta$ 1 and PGE<sub>2</sub> markedly improved the AA-induced decrease of trabecular bone network, especially in trabecular numbers. Those results were in accordance with the previous reports, which showed that local s.c. injection of either TGF- $\beta$ 1 or PGE<sub>2</sub> increases the activity of osteoblasts to cause the bone formation (57, 58). Therefore, increased TGF- $\beta$ 1 and PGE<sub>2</sub> levels at the early stage posttreatment with PSLs contribute not only to the prevention of trabecular bone loss, but also to the facilitation of trabecular bone formation in the AA rats.

In conclusion, the current findings strongly suggest that PSLs potentially inhibit rat osteoclastogenesis and AA-induced trabecular

bone loss in rats by generating TGF- $\beta$ 1 and PGE<sub>2</sub>. Because PS is a component of mammalian cell membranes, PSLs can be used as potential pharmacological interventions against abnormal bone loss such as osteoporosis without any deleterious side effects.

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## Disclosures

The authors have no financial conflicts of interest.

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