Role of Prostaglandin E in Receptor Activator of Nuclear Factor-κB Ligand (RANKL) Expression in Osteoblasts Induced by Cell Adhesion to Bone Marrow B-lymphocytes

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Estrogen deficiency caused by ovariectomy (OVX) induces bone loss and increased B-lymphopoiesis in bone marrow. In OVX mice, the production of prostaglandin E (PGE) and the expression of receptor activator of nuclear factor-κB ligand (RANKL) were elevated in osteoblasts, and the cell adhesion of B cells induced RANKL expression in osteoblasts. However, the roles of PGE in RANKL expression and bone resorption are not clear. To examine the relationship between B-lymphopoiesis and PGE production by osteoblasts, B cells were purified from bone marrow, fixed, and co-cultured with mouse osteoblasts. Most of the fixed B cells adhered to cell surface of osteoblasts, and the cell-cell interaction markedly elevated the expression of cyclooxygenase (COX)-2 and membrane-bound PGE synthase (mPGES)-1 mRNAs, and PGE_2 production in osteoblasts. Adding B cells also induced the expression of RANKL mRNA in osteoblasts, and the RANKL expression was suppressed by indomethacin, COX-2 inhibitor (NS398) and selective antagonist for PGE receptor EP4, suggesting that PGE production and EP4 signals are involved in RANKL-dependent bone resorption induced by cell-cell contact between B cells and osteoblasts. Therefore, the increased B-lymphopoiesis and PGE production by osteoblasts may contribute to the pathogenesis of osteoporosis due to estrogen deficiency.

Key words —— prostaglandin E, bone marrow, B-lymphocyte, bone resorption, osteoporosis

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INTRODUCTION

Postmenopausal osteoporosis is characterized by decreased bone mass due to the elevation of bone resorption. OVX animals have been used to understand the mechanism of bone loss due to estrogen deficiency. We have reported that OVX mice exhibited the marked stimulation of bone marrow B-lymphopoiesis, resulting in an accumulation of B cells in mouse bone marrow, and that the treatment of estrogen recovered both bone mass and B-lymphopoiesis to normal levels in OVX mice.[1] In addition, the increased B-lymphopoiesis by the administration of interleukin-7 (IL-7) resulted in marked bone loss caused by stimulated bone resorption in mice with intact ovarian function.[2] Bone-resorbing cytokines such as IL-1, IL-6 and tumor necrosis factor α may have been reported to a possible factor(s) in the pathogenesis of osteoporosis, but the mechanism of bone loss in OVX mice is still controversial.[3–5]

As a pivotal factor in osteoclast differentiation, previous studies have identified the receptor activator of nuclear factor-κB ligand (RANKL), which is expressed on the cell surface of osteoblasts in response to bone-resorbing factors.[6–9] Osteoclast precursors possess RANK, a receptor for RANKL, and can differentiate into mature osteoclasts via a mechanism of RANK-RANKL recognition.

Prostaglandin E_2 (PGE_2) is a typical bone-resorbing factor involved in bone loss associated with inflammation. We have reported that bone-resorbing cytokines such as IL-1 and IL-6 are known to induce PGE_2 production by osteoblasts, and the produced PGE_2 stimulates adenylate cyclase to accumulate cellular cAMP in osteoblasts, which induces the expression of RANKL for osteoclast differentiation through its receptor sub-

type EP4.[10,11] PGE synthesis is regulated by three metabolic steps; the release of arachidonic acid from the membranous phospholipids by phospholipase A_2 (PLA_2), the conversion of arachidonic acid to PGH_2 by cyclooxygenase (COX) and the synthesis of PGE_2 by PGE synthase (PGES).[12–14] We have reported that the purpose of cytosolic PLA_2 ($cPLA_2$) expression in osteoblasts is to release arachidonic acid following PGE_2 production, and that the expression of membrane-bound PGE synthase (mPGES)-1 is markedly induced by inflammatory stimuli, and functionally coupled with COX-2.[15,16] The coordinate induction of COX-2 and mPGES-1 is essential for PGE_2 production by osteoblasts.

We previously reported that both PGE_2 production and RANKL expression was elevated in osteoblasts and bone marrow in OVX mice.[17,18] However, the roles of PGE in RANKL expression and bone resorption are not clear. In the present study, we show that PGE_2 produced by cell-to-cell interaction between B-lymphocytes and osteoblasts markedly induce RANKL expression in osteoblasts, suggesting a possible role of B-lymphopoiesis and PGE_2 in bone loss due to estrogen deficiency.

MATERIALS AND METHODS

Animals and Reagents —— DdY mice, day 2, 6-weeks and 8-weeks of age, were obtained from Japan SLC (Shizuoka, Japan). Eight-week-old mice were either sham-operated or OVX. All procedures were performed in accordance with institutional guideline for animal research. Indomethacin and NS398 (an inhibitor of COX-2) were purchased from Calbiochem (San Diego, CA, U.S.A.). EP4 antagonist (ONO-AE2-227) was provided by Ono Pharmaceutical Co., Ltd (Osaka, Japan).

Radiographic Analysis of the Femur —— The bone mineral density (BMD) of the femurs collected from sham-operated or OVX mice were measured by dual X-ray absorptiometry (model DCS-600R; Aloka, Tokyo, Japan).[2] The bone mineral content (BMC) of the mouse femur was closely correlated with the ash weight. The BMD was calculated by dividing the BMC of the measured area by the area. The scanned area was dividing equally into three regions comprising the proximal, middle and distal femur to assess regional differences.

Flow Cytometric Analysis —— Bone marrow cells (2×10^6) were obtained from sham-operated or OVX mice 2 week after surgery, and incubated with fluorescein isothiocyanate (FITC)-conjugated B220 (RA3-6B2; Pharmingen, San Diego, CA, U.S.A.), as described previously.[1] These cells were washed with phosphate-buffered saline (PBS) (−) containing 1% bovine serum albumin (BSA) and analyzed on a flow cytometer (FACScalibur, Becton Dickinson, San Jose, CA, U.S.A.).

Isolation of Bone Marrow B-lymphocytes —— Bone marrow cells were prepared from the tibiae and femora of 6-week-old mice, centrifuged and resuspended in 2 ml of ammonium chloride-Tris buffer to lyse red blood cells. The cell suspension was washed, and isolated from bone marrow with a magnetic cell-sorting (MACS) system using magnetic micro-beads coated with anti-mouse CD45R/B220 (Pharmingen).[18] The isolated B cells were more than 98% positive to B220 in flow cytometric analysis.

Co-culture of Mouse Osteoblasts and B-lymphocytes —— Primary osteoblasts were isolated from 2-day-old mouse calvariae after five routine sequential digestions with 0.1% collagenase (Wako Pure Chemical Industries (Osaka, Japan)) and 0.2% dispase (Godo Shusei, Tokyo, Japan). Osteoblasts were cultured in α-modified minimum essential medium (αMEM) supplemented with 10% fetal calf serum (FCS) at 37°C under 5% CO_2 in air. Isolated B-lymphocytes were fixed with 4% paraformaldehyde, washed three times with PBS (−) and added to the cell layer of mouse osteoblasts, and cultured until 24 hr.

RT-PCR Analysis —— Total RNA was extracted from mouse osteoblasts using the acid guanidium-phenol-chloroform method. cDNA was synthesized from 5 μg of total RNA by reverse transcriptase (Superscript II Preamplification System, Invitrogen, Carlsbad, CA, U.S.A.) and amplified via PCR. The primers in PCR for mouse COX-1, COX-2, mPGES-1, RANKL, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as reported previously.[15,16] The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. The signals were densitometrically quantified using an National Institutes of Health (NIH)-image analyzer.

Measurement of PGE_2 Content —— The concentrations of PGE_2 in the cultured medium were determined using an enzyme immunoassay (EIA; Amersham Biosciences). The antibody had the following cross-reactivity when calculated by the bound/free ratio: PGE_2, 100%; PGE_1, 70%; 6-keto-$PGF_1\alpha$,

5.4%; $PGF_2\alpha$, 4.3%; and PGD_2, 1.0%.

Statistical Analysis —— The data are expressed as means ± standard error of the mean (SEM). The significance of differences was analyzed using Student's *t*-test.

RESULTS

Bone Loss and Increased B-lymphopoiesis in OVX Mice

Figure 1 shows the influence of OVX on femoral bone mass and fluorescence activated cell sorter (FACS) analysis of bone marrow B cells. OVX mice showed a significant decrease in femoral BMD, and an increased number of bone marrow B cells 2–4 weeks after surgery (Fig. 1). The expression of B220 in the B-cell lineage is developmentally regulated, and immature pre-B cells was weakly stained with B220 antibody, whereas mature B cells showed the intense staining. In FACS analysis, we detected two peaks of B cells, peak I (pre-B cells) and peak II (mature B cells), in sham-operated mice. In OVX mice, the percentage of B cells was elevated, and major peak of B cells was peak I, indicating that estrogen loss induces the accumulation of immature B cells in bone marrow (Fig. 1B). Levels of PGE_2 in bone marrow supernatant in OVX mice were higher than those in sham mice (sham: 2.4 ± 1.1 ng/ml; OVX: 5.0 ± 0.8 ng/ml), suggesting a possible role of PGE produced by bone and bone marrow in bone resorption due to estrogen deficiency.

Expression of COX-2 and mPGES-1 mRNAs and PGE_2 Production in Osteoblasts by the Contact with Fixed B-lymphocytes

We have reported that B-lymphocytes induces the expression of RANKL in osteoblasts via cell adhesion.[18] Then, we examined the possible role of B cells in PGE_2 production by osteoblasts. B cells were purified from mouse bone marrow using B220-conjugated magnetic microbeads, and fixed with paraformaldehyde to avoid PGE_2 production by B cells. Most fixed-B cells attached to osteoblast surfaces 24 hr after the addition. The expression of COX-2 and mPGES-1 mRNAs was markedly induced in osteoblasts after the addition of fixed B cells, and the level of PGE_2 was elevated in the conditioned medium of the co-cultures of osteoblasts and fixed-B cells (Fig. 2). In contrast, the expression of COX-1 mRNA was suppressed by adding fixed B cells. The expression of COX-2 and mPGES-1 mRNAs could not be elevated in osteoblasts in the separate co-cultures (data not shown), indicating that cell-to-cell contact with B cells is essential for induction of PGE_2 production by osteoblasts.

Relationship between PGE_2 Production and RANKL Expression in Osteoblasts

To examine the relationship between PGE_2 production and RANKL expression in osteoblasts after cell-to-cell contact with B cells, indomethacin, NS398 (an inhibitor of COX-2) and EP4 antagonist were added to the co-cultures of osteoblasts and fixed-B cells. As shown in Fig. 3, the cell-to-cell contact with B cells significantly induced the expression of RANKL mRNA in osteoblasts, and these inhibitors significantly suppressed the expression of RANKL mRNA in osteoblasts 24 hr after the contact with fixed-B cells. Therefore, PGE_2 production and EP4-mediated signals may be involved in the mechanism of RANKL expression in osteoblasts.

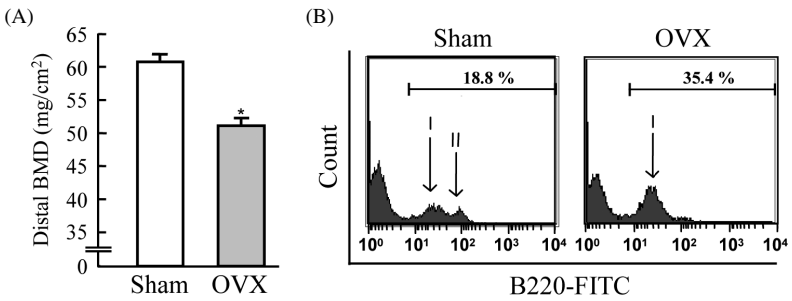

Fig. 1. Femoral Distal BMD and Bone Marrow B-lymphopoiesis in Sham-operated and OVX mice. Eight-week-old Mice were Sham-operated or OVX

(A) Femur was collected 4 weeks after operation, and BMD at distal femur was measured. Significantly different from sham, $^*p < 0.001$, Data are expressed as means±SEM of 5–6 mice. (B) B220-positive cells in bone marrow was analyzed by FACS, and the percentage of B220 positive cells was calculated in sham-operated and OVX mice 2 weeks after operation. In sham mice, B220-positive cells were separated into two subpopulations, peak I (immature B cells) and peak II (mature B cells).

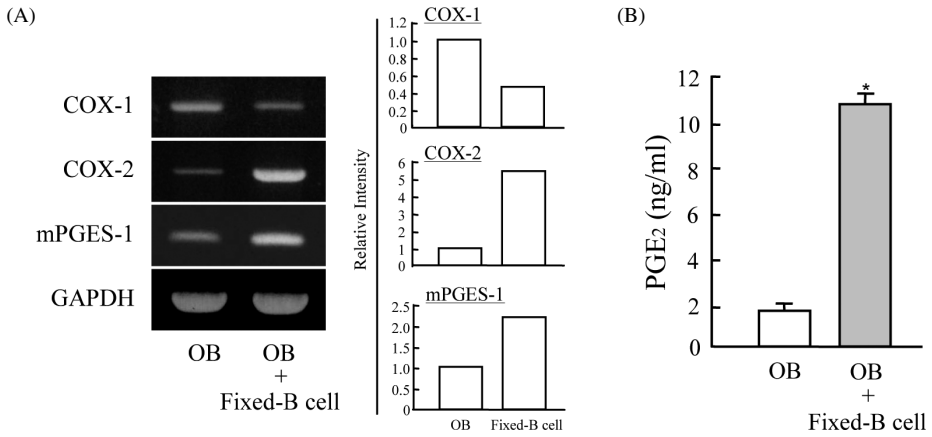

Fig. 2. The Expression of COX-2 and mPGES-1 mRNAs in Osteoblasts by Cell-to-cell Contact with Bone Marrow B Cells

B cells, 98% positive to B220, were collected from mouse bone marrow as described in Materials and Methods, and fixed with 4% paraformaldehyde. The fixed-B cells (2×10^6) were added to the layer of mouse osteoblasts (1×10^5) and co-cultured for 24 hr. (A) The expression of COX-1, COX-2 and mPGES-1 mRNAs in osteoblasts was analyzed by RT-PCR. (B) The level of PGE_2 in the conditioned medium was measured. Significantly different from osteoblast. $^{*}p < 0.001$. Data are expressed as means±SEM of 3 wells.

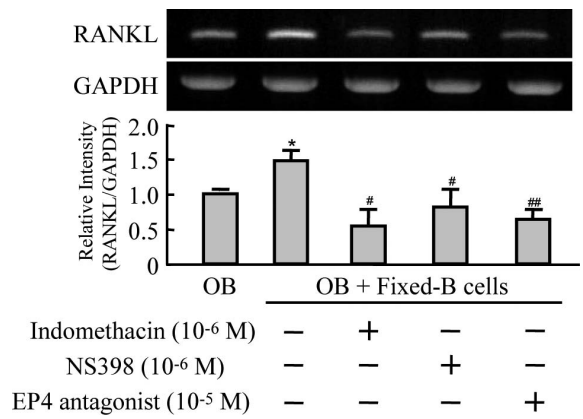

Fig. 3. Effects of Indomethacin, NS398 and EP4 Antagonist on the Expression of RANKL mRNA in Osteoblasts after the Contact with Fixed-B Cells

Fixed-B cells (2×10^6) were added to the layer of osteoblasts (1×10^5) in 24 well plate, and cultured for 24 hr with or without indomethacin (10^{-6} M), NS398 (10^{-6} M) and EP4 antagonist (10^{-5} M) to extract total RNA. The expression of RANKL mRNA was analyzed by RT-PCR, and relative intensity was calculated. Data are expressed as means±SEM of three experiments. A significant difference between the two groups is indicated, $^{*}p < 0.05$ *vs.* control osteoblasts (OB); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* OB+fixed-B cells.

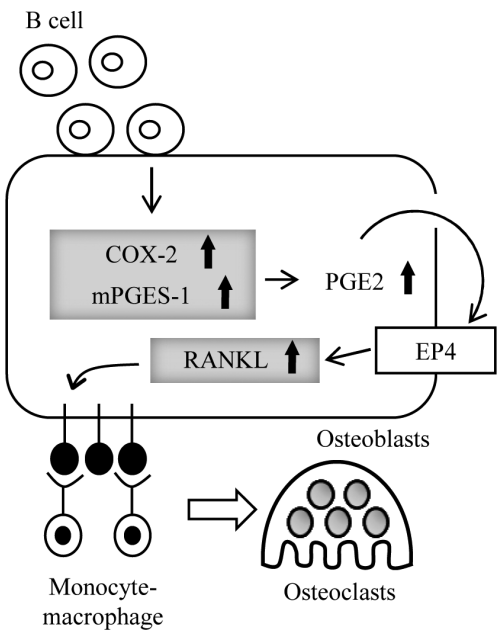

Fig. 4. Model of the Relationship between PGE_2 Production and RANKL Expression in Osteoblasts Induced by Cell-to-cell Contact with B-lymphocytes

Contact of B cells with osteoblasts induces the expression of COX-2 and mPGES-1 mRNAs in osteoblasts to produce PGE_2, and binds to EP4 to induce the expression of RANKL in osteoblasts.

DISCUSSION

PGE_2 is a typical inducer of bone resorption associated with inflammation. We have reported that PGE_2 binds to the EP4 receptor, one of the PGE receptor subtypes EP1–EP4, and induces RANKL expression to stimulate bone resorption, using agonist and antagonist of EPs and respective EP-knockout mice.[11] In the present study, the cell-to-cell contact with fixed-B cells markedly induced the expression of COX-2 and mPGES-1 and PGE_2 production in osteoblasts. The cell-to-cell contact with B cells also induced RANKL expression in osteoblasts, and the RANKL expression was completely suppressed by adding inhibitors of PGE_2 production and EP4 antagonist. Therefore, PGE_2 produced by osteoblasts after the cell-to-cell contact with B cells binds to EP4, and induces RANKL expression via

EP4-mediated signals (Fig. 4).

Using mPGES-1 null mice, we have reported that mPGES-1 is essential for PGE synthesis in osteoblasts treated with bone resorbing cytokine, and RANKL-dependent osteoclast formation could not be induced by IL-1 in mPGES-1 null mice.[16] It is well known that the induction of COX-2 is essential for PGE_2 synthesis induced by various stimuli. Therefore, the coordinate induction of COX-2 and mPGES-1 is critical for PGE_2 production by osteoblasts treated with various stimuli. Previous studies have shown that the mouse COX-2 gene promoter possesses functional regulatory elements for NFκB and AP-1.[19] In contrast, the mouse mPGES-1 gene promoter possesses AP-1, but not NFκB.[20] In the present study, the cell-to-cell contact with B cells induced the expression of COX-2 and mPGES-1 in osteoblasts, but the expression of COX-1 was suppressed by the contact with B cells. Osteoblasts are known to express various integrins such as α1, αv, and β1, and adhesion signals are involved in the activation of several kinases and NFκB.[21] Recently, cell adhesion has been shown to regulate intracellular key mediators including LFA-1, VLA-4, Focal adhesion kinase (FAK), MAPKK/ERK kinase (MEK), Rho kinase, and NFκB in the field of cancer.[22] We have reported that osteoblasts produce PGE_2 after the cell-to-cell contact with cancer cells by the induction of COX-2, and that the PGE_2 production is involved in the mechanism of osteolysis due to bone metastasis.[23] Further studies are needed to define a possible adhesion molecule(s) involved in the cell-to-cell contact between B cells and osteoblasts to induce COX-2 and mPGES-1 genes. In addition, the mechanism of regulation of COX-1 and COX-2 after the cell-to-cell contact is the question to define in the future study.

It is well known that RANKL is a pivotal factor in osteoclast differentiation, and that bone loss due to estrogen deficiency is caused by increased osteoclastic bone resorption. Ikeda *et al.* have reported that the expression of RANKL mRNA was detected in femoral primary spongiosa in OVX rats but not in sham-operated rats.[24] Recently, Li *et al.* have shown that both B cells and T cells are critical for bone mass *in vivo*, using B cell knockout mice and T-cell-deficient nude mice.[25] We have detected the increased expression of RANKL mRNA in bone and bone marrow in OVX mice.[18] In the present study, we showed that RANKL expression in osteoblasts could be induced by PGE_2 after cell adhesion to bone marrow B cells. Therefore, the interaction between osteoblasts and bone marrow B-lymphocytes may contribute to the pathogenesis of bone loss due to estrogen deficiency.

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